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Original article

Synthesis, cytotoxicity and antiplasmodial activity of novel *ent*-kaurane derivatives

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A B S T R A C T

This paper reports on the syntheses and spectrometric characterisation of eleven novel *ent*-kaurane diterpenoids, including a complete set of ^{1}H, ^{13}C NMR and crystallographic data for two novel *ent*-kaurane diepoxides. Moreover, the antineoplastic cytotoxicity for kaurenoic acid and the majority of *ent*-kaurane derivatives were assessed *in vitro* against a panel of fourteen cancer cell lines, of which allylic alcohols were shown to be the most active compounds. The good *in vitro* antimalarial activity and the higher selectivity index values observed for some *ent*-kaurane epoxides against the chloroquine-resistant W2 clone of *Plasmodium falciparum* indicate that this class of natural products may provide new hits for the development of antimalarial drugs.

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1. Introduction

Malaria is among the leading causes of death from a single infectious agent in the developing world. An estimated 3.3 billion people were at risk of malaria in 2010, of which 2.2 billion were at low risk. The 1.2 billion at high risk were living in the WHO African (47%) and South East Asian Regions (37%). Malaria episodes and deaths in 2010 were estimated to be 216 million and 655,000, respectively. The cases and deaths were mostly in the WHO African region. Globally, malaria deaths rates were still high, approximately 86%, among children. In Brazil, 2% of the population (4,480,000) is at high risk for malaria. This at-risk population is represented mostly by people living in the Brazilian Amazonia region. Among the four etiological agents causing malaria in humans, *Plasmodium falciparum* is the most virulent and is responsible for nearly one million deaths per year. However, in Brazil, *Plasmodium vivax* is the major species [1]. As the burden of this disease is worsening mainly because of the increasing resistance of *P. falciparum* to the widely available antimalarial drugs [2], together with the lack of highly effective vaccines and inadequate control of mosquito vectors, malaria continues to be a global challenge. Thus, there is an urgent need for new, more affordable and accessible antimalarial agents with original modes of action [3,4]. Within this context, new antimalarial lead compounds may emerge from tropical plant sources, as natural products have been playing a dominant role in the discovery of leads in the development of drugs to treat human diseases [3,5].

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Kaurane diterpenes have been identified from numerous medicinal plants that have been used for the treatment of inflammation, cancer, zoonosis and infection, as well as other disorders and ailments [6–10]. Kaurenoic, *iso*-kaurenoic and grandiflorenic acids [*ent*-kaur-16-en-19-oic (**1**), *ent*-kaur-15-en-19-oic (**2**) and *ent*-kaur-9:11,16-dien-19-oic (**3**) acids, respectively] have been abundantly found in *Wedelia paludosa* D.C. (Asteraceae) [8,9,11]. Kaurenoic (**1**) and grandiflorenic (**3**) acids have been shown to be trypanocides [8,12], cytotoxic [10,13] and potent stimulators of uterine contraction [6], whereas *iso*-kaurenoic acid (**2**) has been found to display potent antifungal activity against the soil-borne plant pathogenic fungi *Pythium ultimum* and *Rhizoctonia solani* [11].

Given the wide array of biological activities already described for *ent*-kaurane diterpenes [7,14], along with the promising antimalarial activities observed for three zoapatlin diterpene lactones structurally related to a rearranged kaurane skeleton [15,16], it was assumed that kaurenoic acid (**1**) and its related *ent*-kaurane diterpenoids and derivatives might also possess promising antimalarial properties, even though the literature only reports a weak antiplasmodial activity for kaurenoic acid via the parasite lactate dehydrogenase assay (pLDH) [17].

This work describes the *in vitro* antiplasmodial activity of kaurenoic (**1**) and grandiflorenic (**3**) acids, both isolated from *W. paludosa* D.C. Furthermore, hydroxyl and other oxygen substituents in the skeletons of kaurane diterpenoids may improve their biological effects [7,18]. In addition, the presence of epoxide groups is known to increase the capacity of molecules to inhibit proteases by covalent reactions [19]. Accordingly, this paper also reports the synthesis and characterisation of a number of oxidised/epoxidised *ent*-kaurane derivatives obtained from the naturally occurring *ent*-kaurenes of *W. paludosa* D.C. and describes the results of *in vitro* evaluation of the antineoplastic and antimalarial activities for most of these compounds.

2. Results and discussion

2.1. Chemistry

The naturally occurring diterpenes kaurenoic (**1**), *iso*-kaurenoic (**2**) and grandiflorenic (**3**) acids, obtained from *W. paludosa* [8,9,11], were used as the starting materials for the chemical transformations presented in this paper. The three acids are difficult to separate by standard chromatographic techniques, although small amounts of **1** and **3** can be isolated [8,11]. The transformations performed in this work included the syntheses of 11 novel *ent*-kaurane derivatives (**8**, **9**, **10a**, **10b**, **13a**, **13b**, **14**, **15**, **21**, **22** and **23**) and four known compounds (**7**, **11**, **12** and **24**) whose structures are depicted in Scheme 1. Physical and spectrometric data for alcohol **11** are described here for the first time, although its crystallographic data have been published before by our group [18]. The syntheses and complete spectrometric characterisation of substances **7**, **12**, **16**, **17**, **18**, **19** and **20** are available in the literature [12,20,21].

The first aim of the study was to synthesise *ent*-kaurane epoxides starting from the naturally occurring kaurenes. A mixture of kaurenoic (**1**), *iso*-kaurenoic (**2**) and grandiflorenic (**3**) acids, obtained previously from *W. paludosa* D.C. in a 14:8:17 ratio [11], were esterified with an ethereal solution of diazomethane to afford a mixture of esters **4**, **5** and **6** in the same ratio. Then, the mixture of esters was epoxidised [22], affording the known expected methyl *ent*-kauran-16β,17-epoxy-19-oate (**7**), identified by comparison with literature data [20,21], and two novel *ent*-kaurane diepoxides, methyl *ent*-kauran-9β:11,16β:17-diepoxy-19-oate (**8**) and methyl *ent*-kauran-9β:11,16α:17-diepoxy-19-oate (**9**).

Diepoxides **8** and **9**, obtained as colourless crystals, were shown to be isomers via presentation of very similar IR, ^{13}C and ^{1}H NMR spectra, in addition to presenting the same molecular formula $C_{21}H_{32}O_4$ by HRFABMS, but with slightly different specific rotation values [**8**, −9.5° ($CHCl_3$, $c = 0.9$); **9**, −7.2° ($CHCl_3$, $c = 0.5$)] and very distinct melting points (**8**, 168–170 °C; **9**, 96–99 °C). The structure of both compounds was determined by means of bidimensional spectroscopic experiments (COSY, ROESY, HMBC and HMQC). The correlations observed allowed the complete assignment of the NMR data (Table 1) and indicated that both derivatives possess the same kaurane skeleton and the same atomic connectivities, the difference being due to the relative configuration of their epoxide groups at C-9/C-11 and/or C-16/C-17 positions. Fortunately, compounds **8** and **9** were obtained as colourless crystals after recrystallisation in hexane and their structures were confirmed unequivocally by X-Ray crystallography. The x-ray diffraction analysis of a single crystal of **8** and **9** (Fig. 1) proved the *ent*-β orientation of the epoxide group at C-9/C-11 for both compounds, whereas the epoxide group at C-16/C-17 was oriented at the *ent*-β configuration on compound **8** and at the *ent*-α configuration on compound **9**. The three epoxides (7, 8 and 9) were the results of the total epoxidation of the two major components **4** and **6** of the starting mixture.

When grandiflorenic acid (**3**) alone was treated with MCPBA, only the exocyclic double bond was epoxidised, as deduced from the ^{1}H and ^{13}C NMR data of the reaction product. Column chromatography over silica gel afforded the two novel epoxides **10a** and **10b** in a 1:10 ratio. Comparison of their NMR data with that of **8** and **9** allowed the assignment of the *ent*-16α configuration for the minor isomer **10a** and *ent*-16β for **10b,** which is in agreement with those expected from the stereoselective epoxidation of the C-16/C-17 double bond taking place at the less hindered face (*exo* side).

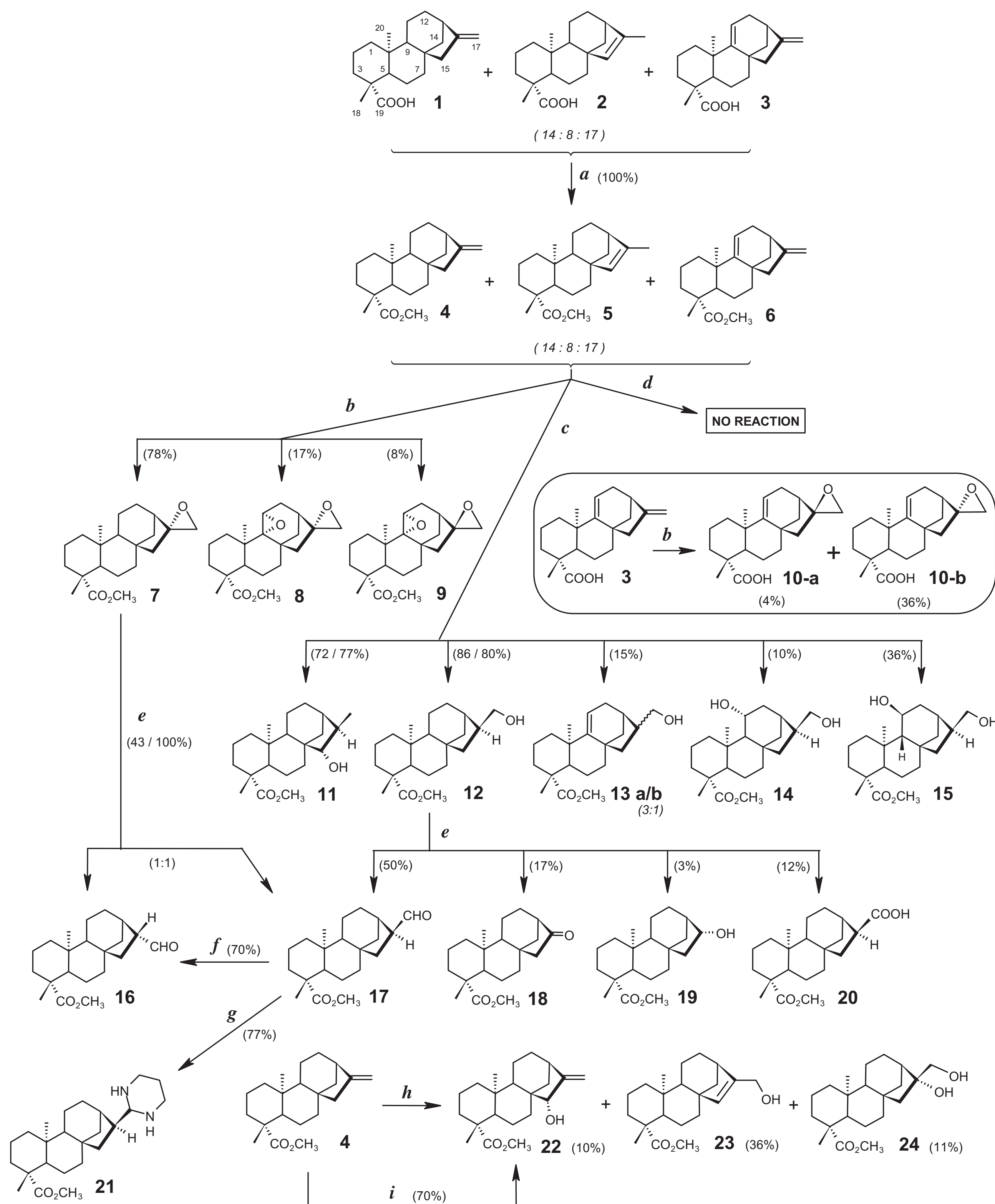
To obtain new derivatives with hydroxyl groups at different positions of the kaurenoic skeleton, a mixture of **4**, **5** and **6** was submitted to different oxidation reactions, such as hydroboration-oxidation [23], allylic oxidation [24,25] by SeO_2 and photo-oxidation with Bengal Rose [26]. The first approach was to employ 9-borabicyclo[3.3.1]nonane (9-BBN) as a hydroboration agent to favour the synthesis of less hindered *ent*-kaurane alcohols, taking into account that the selectivity obtained with 9-BBN is generally higher than that previously realised with diborane or disiamylborane [27]. Unfortunately, no reaction was observed when 9-BBN was used. When the mixture of esters was treated with diborane generated *in situ,* the alcohols **11**–**15** were obtained, which were separated by column chromatography (CC). After 1 h of the hydroboration reaction, alcohols **11**, **12** and **13a**/**b** were obtained, and partial hydroxylation of ester **6** was observed. However, when the reaction time was extended to 2 h, complete hydroboration of all the double bonds was observed, and the isolated products were **11**, **12**, **14** and **15**.

Alcohol **11** came from the hydroboration of ester **5**. The absolute configuration of C-15 and C-16 for compound **11** was deduced from their spectroscopic data and confirmed unequivocally by X-Ray crystallography [18].

Alcohol **12** was identical to the compound obtained by hydroboration [12] of **4**, and it was transformed into the more oxidised derivatives **17**–**20** as previously described [20]. Also the new derivative **21** was obtained from the reaction of aldehyde **17** with propylenediamine.

Derivatives **13**, **14** and **15** came from ester **6**. The ^{1}H and ^{13}C NMR data for alcohol **13** indicated that the double bond at C-9/C-11 was present in the molecule and it was a 3:1 mixture of the two isomers that could not be separated by chromatographic techniques. However, the comparison of the C-16/C-17 NMR data of **13a** and **13b** with the C-16/C-17 NMR data available for related *ent*-kaurane alcohols [28] allowed us to attribute the *ent*-16α configuration for **13a** and *ent*-16β for **13b**.

Compounds **14** and **15** were also shown to be isomers via the presentation of the same molecular formula $C_{21}H_{34}O_4$ but very distinct melting points (**14**, 159–162 °C; **15**, 88–91 °C). No olefinic



Scheme 1. Synthesis of the *ent*-kaurane diterpenoids **4**–**24**. Reagents and conditions: (a) CH_2N_2, Et_2O, 4 h; (b) MCPBA, $NaHCO_3$, CH_2Cl_2, 30 min.; (c) $NaBH_4$, $BF_3.Et_2O$, THF, 1 or 2 h; NaOH, H_2O_2, 50 °C, 1 or 2vh; (d) 9-BBN, THF, 3 h; H_2O_2, NaOH, 50 °C, 1 h; (e) Ref. [20]; (f) HCl, AcOH, 80 °C, 48 h; (g) $H_2N(CH_2)_3NH_2$, CH_2Cl_2, $MgSO_4$, 6 h; (h) Bengal Rose, *i*-PrOH, *hν*, 16 days; $NaBH_4$, MeOH; and (i) SeO_2, *t*-BuOOH, CH_2Cl_2, 45 min.

carbon/hydrogen signals were found on their $^{13}C/^{1}H$ NMR spectra, indicating the hydroxylation of both double bonds. The deshielded C-9 signals observed for **14** (δ 59.4) and **15** (δ 65.4) confirmed the expected anti-Markovnikov hydroxylation on the C-9/C-11 double bond of **3**. *Ent*-16α configuration was assigned to both *ent*-kaurane derivatives on the basis of comparison of ^{13}C NMR data with literature data [28]. NOE experiments confirmed the *ent*-11β-hydroxy group in **14** and the *ent*-11α-hydroxy group in **15**.

Table 1
Complete assignments of ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for the *ent*-kaurane diepoxides **8** and **9** ($CDCl_3$).

Position	**8**		**9**	
	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
1	39.5, CH_2	1.46, m 1.36, dd (4.2; 12.0)	39.5, CH_2	1.91, m 1.25, m
2	19.0, CH_2	1.93, dd (4.4; 10.6) 1.71, d (4.1)	19.1, CH_2	1.87, m 1.49, m
3	37.9, CH_2	2.23, d (13.4) 1.03, dt (4.2; 13.4)	37.8, CH_2	2.21, d (12.0) 1.01, dt (4.2; 13.2)
4	44.3, C		44.2, C	
5	48.7, CH	1.76, dd (4.4; 12.2)	48.7, CH	1.77, dd (3.9; 12.3)
6	19.8, CH_2	1.41, d (5.2) 1.46, m	19.8, CH_2	1.49, m 1.43, m
7	38.9, CH_2	1.56, m 1.91, dd (2.4; 11.5)	39.2, CH_2	1.91, m 1.49, m
8	43.4, C		42.3, C	
9	69.9, C		69.4, C	
10	38.1, C		38.1, C	
11	53.7, CH	3.07, dd (0.9; 4.0)	54.2, CH	3.10, dd (1.0; 4.0)
12	29.1, CH_2	2.04, dd (6.0; 15.4) 1.66, m	26.8, CH_2	2.00, m 1.84, dd (5.5; 15.1)
13	38.9, CH	1.46, m	36.3, CH	1.69, bt (9.0)
14	31.8, CH_2	1.95, dd (4.4; 10.6) 1.65, m	32.1, CH_2	1.95, m 1.88, m
15	45.6, CH_2	2.50, dd (2.4; 14.5) 1.75, d (14.7)	46.2, CH_2	2.25, dd (3.5; 14.4) 1.95, dd (4.1; 14.4)
16	69.0, C		66.1, C	
17	50.6, CH_2	2.84, d (4.5) 2.79, d (4.5)	54.8, CH_2	2.83, d (5.3) 2.78, d (5.3)
18	28.6, CH_3	1.21, s	28.6, CH_3	1.19, s
19	177.8, C		177.9, C	
20	15.0, CH_3	0.68, s	14.8, CH_3	0.66, s
1'	51.3, CH_3	3.64, s	51.2, CH_3	3.63, s

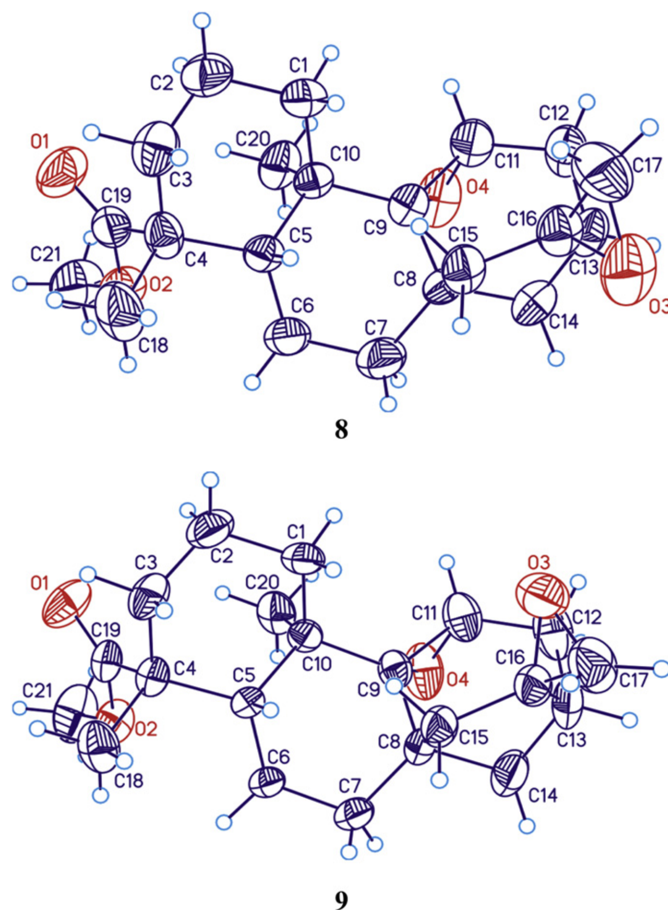


Fig. 1. Molecular plot of the kaurane diepoxides **8** and **9** presenting the relative configuration and the labelling scheme of the non-H atoms (displacement ellipsoids at 50% probability level).

Additionally, allylic oxidation of methyl ester **4** by SeO_2 afforded **22**, while compounds **22**, **23** and **24** were obtained from the photo-oxidation of this ester using Bengal Rose as a photosensitising agent. The configuration of **22**–**24** was deduced by comparison of their NMR data with those of similar *ent*-kaurane derivatives [24,29–33].

2.2. Biological evaluation

2.2.1. Antineoplastic cytotoxicity

Most of the compounds prepared had their antineoplastic cytotoxicity evaluated *in vitro* against a panel of 14 human tumour cell lines representing diverse organs and tissues, including prostate, ovary, breast, melanoma, lung, leukaemia, pancreas, colon and cervical cells. In general, the compound derivatives presented low cytotoxicity, and only those cell lines in which at least one diterpenoid was active are depicted in Table 2 (IGROV, IGROV-ET, K-562, PANC-1, HT-29, LoVo, LoVo-DOX and HELA cell lines). Against the other cell lines tested (DU-145, SK-BR-3, SK-MEL-28, A-549, HT-29-KF and HELA-APL), none of the *ent*-kaurene diterpenoids **1**–**24** demonstrated cytotoxicity at the maximum concentration tested. These cell lines are not shown in Table 2.

It is notable that *ent*-kaurenoic acid (**1**) and most of the evaluated *ent*-kaurane derivatives were inactive against all cell lines assayed displaying GI_{50} values above 8.56 μM. In spite of the low cytotoxicity found in our assays, it is interesting to observe that compound **4** was slightly more cytotoxic against IGROV cells ($GI_{50} = 8.44$ μM) when compared to that of **1** ($GI_{50} > 9.92$ μM) and this effect might be related to the methyl ester function on **4.** A hydroxyl group, either at C-15 or C-17 positions, also seems to increase cytotoxicity against IGROV or IGROV-ET cell lines, but this augmentation is not observed when C-16 or C-11 are hydroxylated or when there is a double bond between C-9/C-11, as can be concluded by comparing the cytotoxicities of **11**, **12**, **22** and **23** with those of **13a/13b**, **14**, **15** and **24**.

As for the cytotoxicities of aldehydes **16** and **17**, the *ent*-16α configuration on **17** appears to favour a slightly higher activity against K-562 ($GI_{50} = 8.18$ μM) and LoVo ($GI_{50} = 8.69$ μM) cells than the *ent*-16β configuration (**16**).

Finally, it is noteworthy to emphasise that the most potent cytotoxicities depicted in Table 2 were displayed by the allylic derivatives **22** and **23**, which were active against IGROV (**22**,

Table 2
Cytotoxicity data (GI_{50}, μM) for some *ent*-kaurane compounds.

Compound	IGROV	IGROV-ET	K-562	PANC-1	HT-29	LoVo	LoVo-DOX	HELA
1	>9.92	>9.92	>9.92	>9.92	>9.92	>9.92	>9.92	>9.92
4	8.44	>9.48	>9.48	>9.48	>9.48	>9.48	>9.48	>9.48
5	>9.48	>9.48	>9.48	>9.48	>9.48	>9.48	>9.48	>9.48
7	>9.02	>9.02	>9.02	>9.02	>9.02	>9.02	>9.02	>9.02
8	>8.66	>8.66	>8.66	>8.66	>8.66	>8.66	>8.66	>8.66
9	>8.66	>8.66	>8.66	>8.66	>8.66	>8.66	>8.66	>8.66
10b	>9.48	>9.48	>9.48	>9.48	>9.48	>9.48	>9.48	>9.48
11	8.34	>9.02	>9.02	>9.02	>9.02	>9.02	>9.02	>9.02
12	>9.02	7.23	>9.02	>9.02	>9.02	>9.02	>9.02	>9.02
13a/13b	>9.02	>9.02	>9.02	>9.02	>9.02	>9.02	>9.02	>9.02
14	>8.56	>8.56	>8.56	>8.56	>8.56	>8.56	>8.56	>8.56
15	>8.56	>8.56	>8.56	>8.56	>8.56	>8.56	>8.56	>8.56
16	>9.02	>9.02	>9.02	>9.02	>9.02	>9.02	>9.02	>9.02
17	>9.02	>9.02	8.18	>9.02	>9.02	8.69	>9.02	>9.02
22	7.52	>9.02	>9.02	>9.02	7.40	8.21	>9.02	7.04
23	8.90	>9.02	>9.02	8.45	>9.02	0.71	4.28	>9.02
24	>8.56	>8.56	>8.56	>8.56	>8.56	>8.56	>8.56	>8.56
DOX HCl[a]	0.11	6.17	0.17	0.24	0.15	0.24	2.21	0.17

[a] Doxorubicin hydrochloride.

GI_{50} = 7.52 μM; **23**, GI_{50} = 8.90 μM), PANC-1 (**23**, GI_{50} = 8.45 μM), HT-29 (**22**, GI_{50} = 7.40 μM), LoVo (**22**, GI_{50} = 8.21 μM; **23**, GI_{50} = 0.71 μM), LoVo-DOX (**23**, GI_{50} = 4.28 μM) and HELA (**22**, GI_{50} = 7.04 μM) cell lines. These results might be interpreted in two ways: the cytotoxicity of such allylic *ent*-kaurane alcohols could be related to alkylation of bionucleophiles, such as DNA, proteins, by derived phosphates/pyrophosphates or by Michael's addition reactions involving donors (protein, DNA, etc.) and α,β-unsaturated carbonyl moieties derived from oxidation of the allylic alcohol of these *ent*-kaurane derivatives in neoplastic cells.

As mentioned before, the GI_{50} values found in our assays indicated that our compounds are non-cytotoxic, however, these values could be in same range of some compounds reported in the literature, such as some naturally occurring *ent*-kaurane diterpenes, including kaurenoic (**1**) and grandiflorenic (**3**) acids, that have been shown to exhibit moderate cytotoxicity against several lines of cultured human neoplastic cells, with a majority of their GI_{50} values in the 10–30 μM range [7,12,34–36]. Furthermore, some *ent*-kaurane derivatives were found to induce apoptosis via the activation of caspases-8 and -9 [37]. Within this context, diterpenes **22** and **23** may represent interesting models for the development of more potent cytotoxic derivatives.

2.2.2. Antimalarial assay

Most of the compounds included in this work were assessed for their *in vitro* antimalarial activity against the chloroquine-resistant W2 clone of *P. falciparum*. These results are shown in Table 3.

According to the criteria recently established [3], the *ent*-kaurane compounds **4**, **5**, **7**, **8**, **9**, **21** and **22** disclosed good antiplasmodial activity against the chloroquine-resistant W2 strain of *P. falciparum*, with IC_{50} values ranging from 5.4 to 10.4 μM, while diterpenes **1** and **3**, as well as derivatives **11** and **13a/13b**, were moderately active, with IC_{50} values from 21.1 to 34.7 μM. Compounds **14**, **15**, **18**, **20**, **23** and **24** presented very low activity, with IC_{50} values above 142.7 μM (Table 3).

Interestingly, methyl kaurenoate (**4**, IC_{50} = 8.2 ± 4.1 μM) and methyl *iso*-kaurenoate (**5**, IC_{50} = 8.8 ± 1.6 μM) displayed a substantially higher activity than kaurenoic (**1**, IC_{50} = 21.1 ± 3.3 μM) and grandiflorenic (**3**, IC_{50} = 23.3 ± 1.7 μM) acids, indicating that esterification of the carboxylic acid group at C-19 improves the antiplasmodial activity of *ent*-kaurane diterpene acids. A similar effect was also observed when trypanocidal activity was evaluated for these diterpenes against *Trypanosoma cruzi*, the causative agent of Chagas' disease (American Trypanosomiasis) [12]. In addition, when the antiplasmodial activities of epoxides **7**, **8** and **9** are compared, one can conclude that an epoxide group at C-16/C17 decreases the activity when it is oriented at the *ent*-β configuration (**7** and **8**, IC_{50} = 9.6 ± 1.8 and 10.4 ± 2.0 μM, respectively). This is opposite to that observed for **9** (IC_{50} = 6.9 ± 2.0 μM), which bears an epoxide group at the *ent*-α configuration. Finally, the perhydropyrimidine derivative **21** was the most potent antiplasmodial compound obtained in this work, and its potent activity suggests that the two secondary amino groups linked to C-17 position may largely contribute to the augmentation of this effect on *ent*-kaurane diterpenes.

Table 3
Inhibitory concentrations (IC_{50}) of some *ent*-kaurane diterpenoids against the *Plasmodium falciparum* chloroquine-resistant clone W2 and their cytotoxicities (CC_{50}) against HepG2 cells.

Compound	W2 (IC_{50}, μM)[a]	HepG2 (CC_{50}, μM)[a]	SI[b]
1	21.1 ± 3.3	192.4 ± 25.5	9.1
3	23.3 ± 1.7	N.D.	–
4	8.2 ± 4.1	32.2 ± 6.6	3.9
5	8.8 ± 1.6	44.9 ± 0.3	5.1
7	9.6 ± 1.8	110.7 ± 0.9	11.5
8	10.4 ± 2.9	1238.5 ± 200.6	119.1
9[c]	6.9 ± 2.0	810.2 ± 105.9	117.4
11	34.7 ± 4.5	N.D.	–
13a/13b	33.1 ± 6.0	N.D.	–
14	>142.7	N.D.	–
15	>142.7	N.D.	–
18	>157.0	N.D.	–
20	>143.5	N.D.	–
21	5.4 ± 1.5	N.D.	–
22	8.4 ± 2.4	N.D.	–
23	>150.4	N.D.	–
24	>142.7	N.D.	–
Chloroquine	0.78 ± 0.03	504.1 ± 30.2	646.3

[a] Values are averages ± standard deviation (n = 3).
[b] Selectivity Index = CC_{50} (HepG2) /IC_{50} (W2).
[c] Caused haemolysis at the concentration of 139 μM; N.D.= not determined.

To investigate the potential of *ent*-kaurane diterpenoids as lead compounds for the development of novel antimalarial drugs, and considering the availability of each diterpenoid, the cytotoxicities of the compounds **1**, **4**, **5**, **7**, **8** and **9** were determined against HepG2 cells, allowing the calculation of their respective selectivity indexes (SI). These results are also presented in Table 3. *ent*-Kaurenoic acid (**1**) displayed a reasonable value of SI (9.1), and the lower SI value observed for its methyl ester **4** (3.9) indicated that methylation of the carboxylic acid group of **1** resulted in a more active derivative with a lower selectivity against the *P. falciparum* W2 clone. On the other hand, the incorporation of epoxide groups along the *ent*-kaurane skeleton substantially increased the selectivity of this class of diterpenes against the *P. falciparum* W2 clone, as can be deduced from the SI values obtained for the monoepoxide **7** (SI = 11.5) and for the diepoxides **8** (SI = 119.1) and **9** (SI = 117.4). None of the *ent*-kaurane diterpenoids **1**–**23** presented an antimalarial activity or a selectivity performance equivalent to chloroquine, but these results are very important for the structure-antimalarial activity relationships studies of *ent*-kaurane diterpenoids and must be considered in the context of the quest for novel derivatives with better and more selective antiplasmodial activities.

As far as the authors are aware, this is the first report on the promising selective antimalarial activity of *ent*-kaurane diterpenoids. The present discovery points to this class of diterpenes as lead compounds for the development of novel antimalarial drugs from some naturally occurring *ent*-kaurenes such as **1** and **3,** which are abundantly found in a few number of plant species, notably in *W. paludosa* D.C. (Asteraceae) and *Xylopia frutescens* (Annonaceae) [9], among others. The *ent*-kaurenes **1** and **3** could then be used as starting material for chemical and/or microbiological semi-synthetic routes aimed at obtaining more potent and selective derivatives.

One concern related to the development of new antimalarials is the development of resistant parasites. Indeed, this has happened with atovaquone that was recently introduced in clinics. The rapid resistance observed for atovaquone may be associated with its action in the parasite electron transport chain [38] that targets the cytochrome bc(1) complex, a key enzyme that catalyses transfer of electrons maintaining the membrane potential of mitochondria. Currently, atovaquone is the only drug in clinical use targeting the *P. falciparum* bc(1) complex [39]. However, when antimalarial drugs do not target enzymes, such as the blood schizonticides chloroquine, mefloquine, halophantrine and artesunate whose site of action is within the digestive vesicle by complexation with hemin and preventing its polymeryzation to the non-toxic pigment hemozoin [40], the arising of resistance seems to happen much less rapidly [41]. We have shown that one of our semi-synthetic kaurane derivatives causes inhibition of the SERCA pump [42]. In this case, a prolonged increase in the levels of cytoplasmic Ca^{2+} is expected what is considered to be an important mode of action for antimalarial drugs, once Ca^{2+} is involved in a wide range

of important process in malarial parasites, including cellular division [43].

Finally, it is important to stress that acute toxicity and *in vivo* efficacy assays need to be undertaken with *ent*-kaurane compounds to confirm them as potential candidates for the development of antimalarial drugs.

3. Conclusion

This paper highlights once again the importance of naturally occurring compounds as synthones in the preparation of semi-synthetic series to be screened for biological activities. A total of eleven novel *ent*-kaurane derivatives were obtained and the complete set of ^{1}H, ^{13}C NMR and crystallographic data for two novel diepoxidised *ent*-kaurane derivatives (**8** and **9**) was established. The allylic alcohols **22** and **23** were shown to be the most potent antineoplastic compounds, while diepoxide **9** and the perhydropyrimidinyl derivative **21** displayed the highest antimalarial activities. Epoxidation along the *ent*-kaurane skeleton led to more selective derivatives against the blood-stage *P. falciparum* chloroquine-resistant W2 clone. These results indicate that *ent*-kaurane derivatives are a promising source of new hits for antimalarial drug research.

4. Experimental section

4.1. General procedures

Melting points were taken with a Microquímica apparatus APF-301 and uncorrected. Optical rotations were measured with a Perkin-Elmer 241 digital polarimeter. IR spectra were obtained on a Shimadzu IR-400 spectrophotometer in diamond film. NMR spectra were recorded either at 200 MHz for ^{1}H and 50 MHz for ^{13}C on a Bruker AC 200 or at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a Bruker Advance DRX 400, in deuterochloroform or deuteromethanol, with added TMS as an internal reference. One-dimensional (1D) ^{1}H and ^{13}C NMR spectra were acquired under standard conditions (5 mm DUAL-^{1}H/^{13}C direct detection). For all two-dimensional NMR experiments, the ^{1}H spectral width was set to 5000 Hz and the ^{13}C spectral width was set to 21.4 for HMQC and to 30 KHz for HMBC, respectively. Chemical shift values are expressed in ppm and coupling constants (J) in Hz. Column chromatography (CC) and flash column chromatography (FCC) were performed with Merck silica gel 60 (0.063–0.200 and 0.040–0.063 mm, respectively). HRMS were run in a VG TS-250 spectrometer working at 70 eV. TLC was carried out on Merck silica gel 60 F254 (0.25 mm thick). The solvents and reagents were purified by standard procedures as necessary.

4.2. Crystallographic analysis

Data collection was performed on a Kappa-CCD-Enraf-Nonius diffractometer using CRYSOM, which was also employed for unit cell determination [44]. X-RAY80 was used for data reduction [45]. The structure was solved by direct methods using the SHELXL97 program [46]. SHELXTL/PC was employed for molecular graphics [47]. The refinement of F^2 against all reflections was conducted until all atomic-parameter shifts were smaller than their standard deviations. Most of the H atoms could be observed in Fourier difference maps; nevertheless, their positions were calculated and refined using a riding model approximation, with distance restraints C—H = 0.96–0.98 Å and with $U_{iso}(H) = 1.5U_{eq}$ (parent atom) for methyl H atoms and $1.2U_{eq}$ (parent atom) for the remaining H atoms. As the data set contained no Friedel pairs, the absolute configuration was assumed from the synthesis. In the final difference Fourier map, there were no peaks greater than 0.34 Å. Crystallographic data (excluding structure factors) for the structures in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC 749702 and 749703. Copies of the data can be obtained, free of charge, upon application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44 (0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk].

4.3. Chemistry

4.3.1. Isolation and methylation of diterpene acids

The mixture of diterpenes **1** + **2** + **3** (1.14 g, proportion 14:8:17, 3.77 mmol of total diterpenes) obtained previously from *W. paludosa* D.C. [11] was methylated by the usual procedure with an ethereal solution of diazomethane (100 mL), giving the respective mixture of methyl esters **4** + **5** + **6** in quantitative yield (1.20 mg, proportion 14:8:17, 3.77 mmol of total esters). Small amounts of **3** (52 mg, 0.17 mmol) and **5** (123 mg, 139 mmol) were previously isolated from these mixtures [11]. Kaurenoic acid (**1**) was also obtained as a pure compound from *W. paludosa* D.C. ethanol extract [8], and its methyl ester derivative (**4**) was quantitatively obtained under diazomethane methylation conditions.

4.3.2. Epoxidation

A portion of the methyl esters mixture (**4** + **5** + **6**, 506 mg; 1.60 mmol total) was treated with MCPBA (55%, 567 mg; 1.83 mmol) in dry CH_2Cl_2 (30 mL), and the system was stirred at room temperature, in the presence of $NaHCO_3$ excess (1 g). After 30 min, the CH_2Cl_2 solution was washed with aq. satd. $Na_2S_2O_3$, water and brine, and dried on Na_2SO_4. The solvent was evaporated and the mixture of products (540 mg) was submitted to Flash Column Chromatography (FCC) over silica gel, eluting with *n*-hexane–EtOAc (95:5), 20 mL per fraction, to yield compounds **7** (frs. 19–24, 217 mg, 78%), **9** (frs. 31–34, 14 mg, 8%) and **8** (frs. 36–39, 31 mg, 17%).

Diterpene **2** (52 mg; 0.17 mmol) was submitted to the same procedure above, yielding a mixture of products (32 mg), which was, in turn, chromatographed (FCC) over silica gel, eluting with *n*-hexane–EtOAc (95:5), 20 mL per fraction, to afford **10a** (fr. 22, 2 mg, <0.01 mmol, 2%) and **10b** (frs. 24–29, 20 mg, 0.06 mmol, 36%) epoxides.

*4.3.2.1. Methyl ent-9β:11,16β-diepoxy-kauran-19-oate (**8**).* Mp 168–170 °C; $\alpha]_D$ −9.5°, $CHCl_3$, *c* 0.9. IR(film) ν_{max}/cm^{-1}: 2934, 2872, 1723, 1458, 1433, 1384, 1226, 1148, 980. ^{1}H NMR and ^{13}C NMR data, Table 1. HRMS (FAB-POSI, M + 1) Calcd. 347.2222; Found 347.2201.

*4.3.2.2. Methyl ent-9β:11,16α-diepoxy-kauran-19-oate (**9**).* Mp 96–99 °C; $\alpha]_D$ −7.2°, $CHCl_3$, *c* 0.5. IR(film) ν_{max}/cm^{-1}: 2928, 2854, 1724, 1458, 1434, 1383, 1227, 1148. ^{1}H NMR and ^{13}C NMR data, Table 1. HRMS (FAB-POSI, M + 1) Calcd. 347.2222; Found 347.2253.

*4.3.2.3. ent-16α-Epoxy-kauran-19-oic acid (**10a**).* Mp 120–123 °C; $[\alpha]_D$ +32.4°, $CHCl_3$, *c* 0.11. IR(film) ν_{max}/cm^{-1}: 3400–3100, 3035, 2928, 2858, 1691, 1651, 1464, 1377, 1264, 1232, 1181, 1162, 977, 966, 796. ^{1}H NMR ($CDCl_3$, 200 MHz) (ppm) δ: 5.36 (br s, 1H, C_{11}-H), 2.86 (d, 1H, C_{17}-Ha, J = 5.3), 2.72 (d, 1H, C_{17}-Hb, J = 5.3), 1.21 (s, 3H, C_4-CH_3), 1.01 (s, 3H, C_{10}-CH_3). ^{13}C NMR ($CDCl_3$, 50 MHz) (ppm) δ: 40.7 (C_1), 20.1 (C_2), 38.2 (C_3), 44.7 (C_4), 46.4 (C_5), 18.3 (C_6), 30.1 (C_7), 41.9 (C_8), 155.7 (C_9), 38.7 (C_{10}), 115.6 (C_{11}), 30.4 (C_{12}), 38.1 (C_{13}), 43.9 (C_{14}), 49.0 (C_{15}), 67.1 (C_{16}), 54.4 (C_{17}), 28.2 (C_{18}), 182.7 (C_{19}), 23.6 (C_{20}). HRMS (FAB-POSI, M + 1) Calcd. 317.2117; Found 317.2149.

*4.3.2.4. ent-16β-Epoxy-kauran-19-oic acid (**10b**).* Mp 158–161 °C; $\alpha]_D$ +6.6°, $CHCl_3$, *c* 0.85. IR(film) ν_{max}/cm^{-1}: 3400–3100, 2946,

1709, 1651, 1460, 1434, 1374, 1254, 1216, 1171, 1151, 976, 815. ^{1}H NMR ($CDCl_3$, 200 MHz) (ppm) δ: 5.24 (br s, 1H, C_{11}-H), 2.85 (d, 1H, C_{17}-Ha, $J = 4.8$), 2.80 (d, 1H, C_{17}-Hb, $J = 4.8$), 1.24 (s, 3H, C_4-CH_3), 1.03 (s, 3H, C_{10}-CH_3). ^{13}C NMR ($CDCl_3$, 50 MHz) (ppm) δ: 40.7 (C_1), 20.1 (C_2), 38.2 (C_3), 44.7 (C_4), 46.7 (C_5), 18.4 (C_6), 30.0 (C_7), 42.9 (C_8), 156.6 (C_9), 38.9 (C_{10}), 114.2 (C_{11}), 32.9 (C_{12}), 40.7 (C_{13}), 43.2 (C_{14}), 50.0 (C_{15}), 69.3 (C_{16}), 50.9 (C_{17}), 28.2 (C_{18}), 183.9 (C_{19}), 23.6 (C_{20}). HRMS (FAB-POSI, M + 1) Calcd. 317.2117; Found 317.2148.

4.3.3. Hydroboration-oxidation

4.3.3.1. Method A. A portion of the mixture of methyl esters (**4** + **5** + **6**, 200 mg; 0.64 mmol) in anhydrous THF (2 mL) was added to a solution of 9-borabicyclo[3.3.1]nonane (9-BBN; 155 mg, 1.27 mmol) in dry THF (2 mL), and the system was stirred at room temperature under inert atmosphere (N_2) for 3 h. After this time, the solution was cooled (0 °C), and EtOH (5 mL), NaOH aq. 20% (5 mL) and H_2O_2 30% v/v (3 mL) were added and then stirred at 50 °C for 1 h before being washed with brine and dried on Na_2SO_4. The solvent was evaporated, and only the mixture of esters (**4** + **5** + **6**) was observed on TLC. No product was observed for this reaction also under ^{1}H NMR analysis.

4.3.3.2. Method B. A portion of the mixture of methyl esters (**4** + **5** + **6**, 504 mg; 1.60 mmol) in anhydrous THF (20 mL) was treated with $NaBH_4$ (610 mg, 16.12 mmol) and $BF_3 \cdot Et_2O$ (2.0 mL, 15.92 mmol). The system was stirred at room temperature under inert atmosphere (Ar) for 1 h. After this time, the solution was cooled (0 °C), and then EtOH (10 mL), NaOH aq. 20% (10 mL) and H_2O_2 30% v/v (5 mL) were added, before the solution was stirred at 50 °C for 1 h and washed with brine and dried on Na_2SO_4. The solvent was evaporated, and the mixture of products (556 mg) was submitted to FCC over silica gel, eluting with *n*-hexane–EtOAc (9:1), 20 mL per fraction, to give alcohols **11** (frs. 2–25, 64 mg, 0.19 mmol, 72%), **13a + 13b** (3:1, frs. 27–29, 26 mg, 0.08 mmol, 15%) and **12** (frs. 32–41, 230 mg, 0.69 mmol, 86%).

4.3.3.3. Method C. A portion of the mixture of methyl esters (**4** + **5** + **6**, 660 mg; 2.09 mmol) in anhydrous THF (25 mL) was treated with $NaBH_4$ (802 mg, 21.20 mmol) and $BF_3 \cdot Et_2O$ (5.0 mL, 39.80 mmol). The system was stirred at room temperature under an inert atmosphere (Ar) for 2 h. After this time, the solution was cooled (0 °C), and then EtOH (10 mL), NaOH aq. 20% (10 mL) and H_2O_2 30% v/v (7 mL) were added, before being stirred at 50 °C for 2 h and washed with brine and dried on Na_2SO_4. The solvent was evaporated, and the mixture of products (894 mg) was submitted to FCC over silica gel, eluting with *n*-hexane–EtOAc (9:1), 20 mL per fraction, to yield alcohols **11** (frs. 19–21, 81 mg, 0.24 mmol, 77%), **12** (frs. 25–31, 278 mg, 0.83 mmol, 80%), **14** (frs. 42–44, 25 mg, 0.07 mmol, 10%) and **15** (frs. 45–46, 87 mg, 0.25 mmol, 36%).

*4.3.3.4. Methyl ent-15β-hydroxy-16α-kauran-19-oate (**11**).* Mp 139–141 °C; $[\alpha]_D$ −62.6°, $CHCl_3$, *c* 1.00. IR(film) ν_{max}/cm^{-1}: 3421, 2923, 2851, 1726, 1464, 1375, 1232, 1216, 1193, 1158, 1097, 1078, 1056, 1032, 997, 807, 737. ^{1}H NMR ($CDCl_3$, 200 MHz) (ppm) δ: 2.17 (d, 1H, C_{13}-H, $J = 14.2$), 3.23 (d, 1H, C_{15}-H, $J = 4.4$), 1.78 (m, 1H, C_{16}-H), 1.12 (d, 3H, C_{16}-CH_3, $J = 7.3$), 1.18 (s, 3H, C_4-CH_3), 0.84 (s, 3H, C_{10}-CH_3), 3.65 (s, 3H, C_{19}-OCH_3). ^{13}C NMR ($CDCl_3$, 50 MHz) (ppm) δ: 40.7 (C_1), 19.1 (C_2), 38.1 (C_3), 43.8 (C_4), 57.1 (C_5), 21.4 (C_6), 38.0 (C_7), 47.6 (C_8), 54.5 (C_9), 39.6 (C_{10}), 18.5 (C_{11}), 24.9 (C_{12}), 38.2 (C_{13}), 35.8 (C_{14}), 88.3 (C_{15}), 47.2 (C_{16}), 13.6 (C_{17}), 28.7 (C_{18}), 178.1 (C_{19}), 15.5 (C_{20}), 51.2 (C_{21}). HRMS (FAB-POSI, M + 1) Calcd. 335.2586; Found 335.2555.

*4.3.3.5. Methyl ent-17-hydroxy-16α-kaur-9(11)-en-19-oate/ent-17-hydroxy-16β-kaur-9(11)-en-19-oate (**13a/13b**, 3:1).* Amorphous solid; $[\alpha]_D$ +41.7°, $CHCl_3$, *c* 0.77. IR(film) ν_{max}/cm^{-1}: 3364, 3060, 2928, 2867, 1723, 1651, 1463, 1221, 1147, 980. ^{1}H NMR ($CDCl_3$, 200 MHz) (ppm) δ: 5.22/5.20 (br s, 1H, C_{11}-H), 2.35 (m, 1H, C_{16}-H), 3.58 (d, 2H, C_{17}-Ha, $J = 7.9$), 3.45 (m, 2H, C_{17}-Hb), 1.15 (s, 3H, C_4-CH_3), 0.90/0.89 (s, 3H, C_{10}-CH_3), 3.63 (s, 3H, C_{19}-OCH_3). ^{13}C NMR ($CDCl_3$, 50 MHz) (ppm) δ: 41.2 (C_1), 20.2 (C_2), 38.3 (C_3), 45.0 (C_4), 46.6 (C_5), 18.5 (C_6), 30.0/29.7 (C_7), 44.8 (C_8), 158.1/156.4 (C_9), 38.6 (C_{10}), 114.9/114.7 (C_{11}), 30.0/37.2 (C_{12}), 35.0/35.9 (C_{13}), 44.8/42.6 (C_{14}), 45.6/47.0 (C_{15}), 45.1/48.1 (C_{16}), 65.8/67.5 (C_{17}), 28.0 (C_{18}), 178.0 (C_{19}), 23.3 (C_{20}), 51.2 (C_{21}). HRMS (FAB-POSI, M + 1) Calcd. 333.2430; Found 333.2443.

*4.3.3.6. Methyl ent-11β,17-dihydroxy-9-epi-16α-kauran-19-oate (**14**).* Mp 159–162 °C; $[\alpha]_D$ −63.5°, $CHCl_3$, *c* 0.95. IR(film) ν_{max}/cm^{-1}: 3363, 2986, 2932, 2856, 1722, 1464, 1450, 1384, 1371, 1334, 1233, 1154, 1096, 1049, 1032, 1021, 998. ^{1}H NMR ($CDCl_3$, 200 MHz) (ppm) δ: 4.00 (m, 1H, C_{11}-H), 2.38 (m, 1H, C_{16}-H), 3.67 (dd, 2H, C_{17}-2H, $J = 4.0$, 8.3), 1.17 (s, 3H, C_4-CH_3), 0.90 (s, 3H, C_{10}-CH_3), 3.62 (s, 3H, C_{19}-OCH_3). ^{13}C NMR ($CDCl_3$, 50 MHz) (ppm) δ: 38.8 (C_1), 19.4 (C_2), 38.2 (C_3), 44.1 (C_4), 51.3 (C_5), 20.2 (C_6), 37.9 (C_7), 42.5 (C_8), 59.4 (C_9), 37.3 (C_{10}), 68.8 (C_{11}), 36.1 (C_{12}), 35.7 (C_{13}), 37.9 (C_{14}), 40.2 (C_{15}), 46.4 (C_{16}), 63.6 (C_{17}), 28.9 (C_{18}), 178.4 (C_{19}), 22.7 (C_{20}), 51.2 (C_{21}). HRMS (FAB-POSI, M + 1) Calcd. 351.2535; Found 351.2491.

*4.3.3.7. Methyl ent-11α,17-dihydroxy-16α-kauran-19-oate (**15**).* Mp 88–91 °C; [ ]$_D$ −51.3°, $CHCl_3$, *c* 0.90. IR(film) ν_{max}/cm^{-1}: 3363, 2925, 2872, 2854, 1724, 1465, 1446, 1376, 1325, 1235, 1191, 1156, 1095, 1030, 997, 975, 733. ^{1}H NMR ($CDCl_3$, 200 MHz) (ppm) δ: 3.86 (m, 3H, C_{11}-H, C_{17}-2H), 2.15 (m, 1H, C_{16}-H), 1.17 (s, 3H, C_4-CH_3), 0.70 (s, 3H, C_{10}-CH_3), 3.63 (s, 3H, C_{19}-OCH_3). ^{13}C NMR ($CDCl_3$, 50 MHz) (ppm) δ: 40.2 (C_1), 19.0 (C_2), 37.9 (C_3), 43.8 (C_4), 56.7 (C_5), 21.8 (C_6), 39.9 (C_7), 42.8 (C_8), 65.4 (C_9), 38.3 (C_{10}), 66.8 (C_{11}), 35.4 (C_{12}), 37.7 (C_{13}), 39.9 (C_{14}), 42.1 (C_{15}), 42.7 (C_{16}), 63.0 (C_{17}), 28.7 (C_{18}), 177.9 (C_{19}), 14.8 (C_{20}), 51.1 (C_{21}). HRMS (FAB-POSI, M + 1) Calcd. 351.2535; Found 351.2533.

*4.3.4. Synthesis of oxidised compounds from either epoxide **7** or alcohol **12***

The synthesis of aldehydes **16** and **17** (1:1) was previously performed starting from epoxide **7**, as well as the syntheses of compounds **17**, **18**, **19** and **20** from alcohol **12** [20].

*4.3.5. Synthesis of the perhydropyrimidinyl derivative **21***

A solution of aldehyde **17** (50 mg, 0.15 mmol) in anhydrous CH_2Cl_2 (3 mL) was added (dropwise) to 1,3-propanediamine (375 μL, 4.5 mmol) containing $MgSO_4$ (50 mg, 0.42 mmol), and the system was stirred at room temperature under an inert argon atmosphere for 6 h. Next, the solution was filtered and concentrated exhaustively under reduced pressure to give **21** (45 mg, 0.12 mmol) at 77% yield.

*4.3.5.1. Methyl ent-16α-(hexahydropyrimidin-2-yl)-17-nor-kauran-19-oate (**21**).* Amorphous solid; $[\alpha]_D$ −58.9°, $CHCl_3$, *c* 2.30. IR(film) ν_{max}/cm^{-1}: 3315, 2933, 2852, 1724, 1461, 1449, 1384, 1347, 1324, 1234, 1192, 1150, 1024, 980, 774, 732. ^{1}H NMR ($CDCl_3$, 200 MHz) (ppm) δ: 3.22 (br s, 1H, C_{16}-H), 3.46 (d, 1H, C_{17}-H, $J = 9.6$), 1.24 (s, 3H, C_4-CH_3), 0.95 (s, 3H, C_{10}-CH_3), 3.63 (s, 3H, C_{19}-OCH_3), 2.80 (m, 4H, $NC_{1'}$-2H, $NC_{3'}$-2H), 3.14 (m, 2H, –NH–). ^{13}C NMR ($CDCl_3$, 50 MHz) (ppm) δ: 40.7 (C_1), 19.1 (C_2), 38.0 (C_3), 43.8 (C_4), 56.9 (C_5), 22.1 (C_6), 42.0 (C_7), 44.1 (C_8), 56.5 (C_9), 39.4 (C_{10}), 19.6 (C_{11}), 26.2 (C_{12}), 36.7 (C_{13}), 40.3 (C_{14}), 43.6 (C_{15}), 47.0 (C_{16}), 73.2 (C_{17}), 28.6 (C_{18}), 178.1 (C_{19}), 15.4 (C_{20}), 51.1 (C_{21}), 46.0 ($C_{1'}$), 27.8 ($C_{2'}$), 45.9 ($C_{3'}$). HRMS (FAB-POSI, M + 1) Calcd. 389.3168; Found 389.3193.

*4.3.6. Synthesis of the alcohols **22–24** from the ester **4***

4.3.6.1. Method D. The methyl ester **4** (101 mg, 0.32 mmol) in dry CH_2Cl_2 (5 mL) was treated with SeO_2 (30 mg, 0.27 mmol) and *t*-BuOOH in CH_2Cl_2 (5 mL, 7.94 mmol). After stirring for 45 min at room temperature under an inert argon atmosphere, the solution was concentrated, and the yellowish mixture of products was submitted to FCC over silica gel, eluting with *n*-hexane–EtOAc (95:5), 10 mL per fraction, to yield alcohol **22** (frs. 10–11, 74 mg, 0.22 mmol, 70%).

4.3.6.2. Method E. The methyl ester **4** (120 mg, 0.38 mmol) in *i*-PrOH (50 mL) was treated with Bengal Rose (20 mg), and the mixture was stirred for 16 days at room temperature and exposed to sunlight. The solvent was removed under vacuum. The residue was dissolved in MeOH (25 mL), and the solution was stirred for 1 h at room temperature after being treated with $NaBH_4$ (294 mg, 3.42 mmol). The solution was then concentrated, and the mixture of products was dissolved in Et_2O, washed with brine and dried on Na_2SO_4. The organic solution was concentrated under reduced pressure, and the mixture of products (108 mg) was submitted to FCC over silica gel, eluting with *n*-hexane–EtOAc (85:15), 20 mL per fraction, to afford alcohols **22** (frs. 26–28, 10 mg, 0.03 mmol, 10%) and **23** (frs. 30–34, 38 mg, 0.11 mmol, 36%). Final fractions 44–46, eluted with EtOAc, yielded alcohol **24** (12 mg, 0.03 mmol, 11%).

*4.3.6.3. Methyl ent-15β-hydroxy-kaur-16-en-19-oate (**22**).* Mp 62–65 °C; $[\alpha]_D$ −95.2°, $CHCl_3$, *c* 0.50. IR(film) ν_{max}/cm^{-1}: 3419, 2925, 2851, 1726, 1652, 1463, 1376, 1233, 1192, 1154, 1093, 1070, 1054, 1019, 1001, 897, 772. ^1H NMR ($CDCl_3$, 200 MHz) (ppm) δ: 2.74 (br s, 1H, C_{13}-H), 3.80 (s, 1H, C_{15}-H), 5.21 (s, 1H, C_{17}-Ha), 5.08 (s, 1H, C_{17}-Hb), 1.19 (s, 3H, C_4-CH_3), 0.84 (s, 3H, C_{10}-CH_3), 3.65 (s, 3H, C_{19}-OCH_3). ^{13}C NMR ($CDCl_3$, 50 MHz) (ppm) δ: 40.7 (C_1), 19.1 (C_2), 38.0 (C_3), 43.8 (C_4), 57.0 (C_5), 21.0 (C_6), 36.2 (C_7), 47.7 (C_8), 53.3 (C_9), 39.6 (C_{10}), 18.3 (C_{11}), 32.6 (C_{12}), 42.3 (C_{13}), 35.2 (C_{14}), 82.7 (C_{15}), 160.3 (C_{16}), 108.3 (C_{17}), 28.7 (C_{18}), 178.1 (C_{19}), 15.6 (C_{20}), 51.1 (C_{21}). HRMS (FAB-POSI, M + 1) Calcd. 333.2430; Found 333.2472.

*4.3.6.4. Methyl ent-17-hydroxy-kaur-15-en-19-oate (**23**).* Mp 80–82 °C; $[\alpha]_D$ −51.1°, $CHCl_3$, *c* 1.35. IR(film) ν_{max}/cm^{-1}: 3436, 2930, 2848, 1727, 1674, 1469, 1446, 1377, 1367, 1326, 1233, 1192, 1158, 1131, 1090, 1024, 952. ^1H NMR ($CDCl_3$, 200 MHz) (ppm) δ: 2.54 (br s, 1H, C_{13}-H), 5.36 (s, 1H, C_{15}-H), 4.19 (s, 2H, C_{17}-2H), 1.17 (s, 3H, C_4-CH_3), 0.85 (s, 3H, C_{10}-CH_3), 3.64 (s, 3H, C_{19}-OCH_3). ^{13}C NMR ($CDCl_3$, 50 MHz) (ppm) δ: 40.7 (C_1), 19.1 (C_2), 38.0 (C_3), 43.8 (C_4), 56.7 (C_5), 20.8 (C_6), 39.2 (C_7), 48.8 (C_8), 47.5 (C_9), 39.6 (C_{10}), 18.8 (C_{11}), 25.4 (C_{12}), 41.0 (C_{13}), 43.8 (C_{14}), 135.4 (C_{15}), 146.1 (C_{16}), 61.2 (C_{17}), 28.7 (C_{18}), 178.0 (C_{19}), 15.2 (C_{20}), 51.1 (C_{21}). HRMS (FAB-POSI, M + 1) Calcd. 333.2430; Found 333.2397.

*4.3.6.5. Methyl ent-16α,17-dihydroxy-kauran-19-oate (**24**).* Mp 65–67 °C; $\alpha]_D$ −45.4°, $CHCl_3$, *c* 0.90. IR(film) ν_{max}/cm^{-1}: 3363, 2925, 2872, 2854, 1724, 1465, 1446, 1376, 1325, 1235, 1191, 1156, 1095, 1030, 997, 975, 733. ^1H NMR and ^{13}C NMR data were very similar to those described in the literature [28]. HRMS (FAB-POSI, M + 1) Calcd. 351.2535; Found 351.2533.

4.4. Biological evaluation

4.4.1. Antineoplastic cytotoxicity assay

The cell lines assayed were DU-145, prostate carcinoma; IGROV and IGROV-ET, ovarian; SK-BR-3, breast adenocarcinoma; SK-MEL-28, malignant melanoma; A-549, lung carcinoma NSCL; K-562, chronic myelogenous leukaemia; PANC-1, pancreatic epithelioid carcinoma; HT-29, HT-29-KF and LoVo colon adenocarcinoma, and LoVo-Dox, colon adenocarcinoma resistant to doxorubicin; and HELA and HELA-APL, cervix epithelioid carcinoma. A conventional colorimetric assay [48] was set up to estimate GI_{50} values, the drug concentration that causes 50% cell growth inhibition after 72 h of continuous exposure to the test molecules.

Cells were seeded into 16-mm wells (multidishes, NUNC 42001) at concentrations of 2×10^4 cells/well, in 1-mL aliquots of MEM10FCS medium containing the compound to be evaluated at the concentrations tested. In each case, a set of control wells was incubated in the absence of sample and counted daily to ensure the exponential growth of cells. After 3 days at 37 °C, under a 10% CO_2, 98% humidity atmosphere, the cells were stained with crystal violet, observed through an inverted microscope and the degree of inhibition was determined by comparison with the controls. All calculations represent the average of duplicated wells. For Jurkat-77 cell-line assays, the cells were seeded into 25-mm wells (multidishes, NUNC 150318) at concentrations of 13×10^4 cells/mL, in RPMI 1604-SBF10% medium containing the compound to be evaluated at the concentrations tested. In each case, a set of control wells was incubated in the absence of sample and counted daily to ensure the exponential growth of cells. After 4 days at 37 °C, under a 5% CO_2, 90% humidity atmosphere, the cells were stained with trypan blue, observed through an inverted microscope and the degree of inhibition was determined by comparison with the controls. All calculations represent the average of triplicate wells.

4.4.2. Antimalarial assay

Infected red blood cells were maintained in continuous culture with human erythrocytes (blood group: 0 negative) in RPMI medium supplemented with 10% human plasma (complete medium), as previously described [49]. The synchronisation of the parasites was achieved by sorbitol treatment [50], and parasitemia was determined microscopically with Giemsa-stained smears.

The antimalarial effects of *ent*-kaurane compounds and controls were measured with the [^3H]-hypoxanthine incorporation assay performed as described previously [51] with minor modifications [52]. Briefly, ring-stage parasites in sorbitol-synchronised blood cultures were added to 96-well culture plates at 1% parasitemia and 1% haematocrit and then incubated with the test drugs alone. The compounds were diluted in complete medium without hypoxanthine, from 10 mg/mL stock solutions in DMSO at a final concentration of 0.002% (vol/vol) and stored at −20 °C. After a 24-h incubation period, 25 μL of medium containing [^3H]-hypoxanthine (0.5 μCi/well) was added per well, followed by another 18-h incubation at 37 °C. The plates were frozen (−70 °C for 24 h) and thawed, and the cells were harvested (Tomtec 96-Harvester) on glass fibre filters (Wallac, Turku, Finland) and then placed into sample bags (Wallac) and immersed in scintillation fluid (Optiphase super mix; Wallac). Radioactive emission was counted with a 1450 Microbeta reader (Wallac). The inhibition of parasite growth was evaluated from the levels of [^3H]-hypoxanthine incorporation; i.e., the IC_{50} values were evaluated by comparing the incorporation in drug-free control cultures and estimated by linear interpolation [51] using curve-fitting software (Microcal Origin software 5.0). All experiments were performed three times, and each sample was tested in triplicate.

4.4.3. Cytotoxicity assay against HepG2 cells

Hep G2A16 hepatoma cells were stored at 37 °C, 5% CO_2 in 75-cm^3 sterile culture flasks (Corning®) in RPMI1640 culture medium (Sigma®) supplemented with 5% FBS, gentamicin (40 mg/mL), with medium changes twice a week. The assays were performed as described previously [53] with slight modifications. Briefly, the cells were maintained with weekly passages and grown to confluence (ATCC). The monolayers were trypsinised (0.05% trypsin/0.5 mM EDTA), washed, counted, diluted in complete

medium, distributed in 96-well microliter plates (4×10^4 cells/well), then incubated for another 18 h at 37 °C. The test compounds and control drugs (DMSO, chloroquine diphosphate), assayed in triplicate, were diluted to a final concentration of 0.01% DMSO in culture medium to yield four drug concentrations obtained in serial dilutions starting at 1000 μg/mL. After a 24-h incubation at 37 °C, the supernatant was removed and 18 μL of MTT solution (6 mg/mL in PBS) was added to each well, followed by 1 h, 30 min of incubation at 37 °C. Then, DMSO (180 μL) was added to each well and the plate was shaken for 15 min. The culture plates were read in a spectrophotometer with a 570-nm filter, and the cytotoxic concentrations were determined based on a dose–response curve.

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