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Synthesis and induction of apoptosis signaling pathway of *ent*-kaurane derivatives

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ABSTRACT

Thirty one *ent*-kaurane derivatives were prepared from kaurenoic acid (**1**), grandiflorenic acid (**16**), 15α-acetoxy-kaurenoic acid (**26**) and 16α-hydroxy-kaurenoic acid (**31**). They were tested for their ability to inhibit cell viability in the mouse leukemic macrophagic RAW 264.7 cell line. The most effective compounds were **12**, **20**, **21**, and **23**. These were selected for further evaluation in other human cancer cell lines such as Hela, HepG2, and HT-29. Similar effects were obtained although RAW 264.7 cells were more sensitive. In addition, these compounds were significantly less cytotoxic in non-transformed cells. The apoptotic potential of the most active compounds was investigated and they were able to induce apoptosis with compound **12** being the best inducer. The caspase-3, -8 and -9 activities were measured. The results obtained showed that compounds **12**, **21**, and **23** induce apoptosis via the activation of caspase-8, whereas compound **20** induces apoptosis via caspase-9. Immunoblot analysis of the expression of p53, Bax, Bcl-2, Bcl-xl, and IAPs in RAW 264.7 cells was also carried out. When cells were exposed to 5 μM of the different compounds, expression levels of p53 and Bax increased whereas levels of antiapoptotic proteins such as Bc1-2, Bc1-x1, and IAPs decreased. In conclusion, kaurane derivatives (**12**, **20**, **21**, and **23**) induce apoptosis via both the mitochondrial and membrane death receptor pathways, involving the Bcl-2 family proteins. Taken together these results provide a role of kaurane derivatives as apoptotic inducers in tumor cells.

1. Introduction

Apoptosis is an evolutionarily conserved cell death program executed by cysteine proteases (caspases) that is essential for normal tissue homeostasis and development of organisms.[1] Two main activation pathways have been identified, termed the extrinsic and intrinsic pathways.[2] The extrinsic pathway is initiated at the plasma membrane by activation of cell surface death receptors such as Fas/CD95, TNF-R1, and TRAIL. Ligand-activated death receptors recruit the adaptor molecule Fas-associated death domain protein (FADD), which in turn recruits and activates an initiator enzyme, usually caspase-8, in the death-inducing signaling complex (DISC).[3] Active caspase-8 activates downstream apoptotic effectors such as caspases 3, 6, and 7.[4] The intrinsic pathway is mediated by mitochondria, and involves the release of proapoptotic factors such as cytochrome *c* from the intramembrane space to the cytosol. Once released, cytochrome *c* and dATP bind to apoptotic proteinase-activating factor-1 (Apaf-1), and this complex, together with adenine nucleotides, promotes the autoactivation of procaspase-9 to caspase 9. This then activates the effector caspases 2, 3, 6, 8, and 10.[5] Release of apoptosis-related proteins from mitochondria is controlled by proapoptotic members of the Bcl-2 family such as Bax and Bid. Caspase-8 can activate Bid by cleavage to generate truncated Bid, providing a link between the extrinsic and intrinsic pathways.[6]

One of the most important advances in cancer research is the recognition that apoptosis is an important phenomenon in cancer chemotherapy. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. This ideal situation is achievable by selectively inducing apoptosis in cancer cells. Thus, targeting critical apoptosis regulators is a promising strategy in cancer therapy.[7]

Natural products have been the single most productive source of leads for the development of drugs. Approximately, 65% of the anticancer drugs commercially available in Europe and the US

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are of natural origin.[8] Natural products have properties distinguishing them from other medicinal chemistry compounds.[9] A large component of biologically relevant chemical space is occupied by natural products which can be regarded as prevalidated by nature.[10] They have a unique and vast chemical diversity and have been optimized for optimal interactions with biological macromolecules through evolutionary selection. Virtually, all of the biosynthesized compounds have a biological activity with (from an evolutionary perspective) beneficial purpose for the organism that produces it, thus fulfilling the requirement for biological relevance. Taken together, these facts make them exceptional as design resources in drug discovery and interest in natural products remains considerable.[11]

The secondary metabolites of diverse life forms have potent biological activities and have provided lead compounds in drug discovery. Terpenoids represent a chemical defense against environmental stress and provided a repair mechanism for wound and injuries. Plant terpenoids have demonstrated excellent anticancer properties by suppression of tumor proliferation and induction of apoptosis.[12] Diterpenes with different skeletons have been described in the literature as inducers of apoptosis. Four xenicane-type diterpenoids isolated from the choral *Xenia elongata* induced apoptosis in precancerous mammalian epithelial cells.[13] Macrocyclic lathyrane-type diterpenes from *Euphorbia lagascae* showed high apoptosis-inducing activity.[14] Andrographolide, a labdane diterpenoid isolated from *Andrographis paniculata* also triggers apoptosis and suppresses cancer cell growth.[15] It has also been reported the apoptosis-inducing properties in a human leukemia cell line (HL-60) of *ent*-kaurane-type diterpenoids from the liverwort *Jungermannia truncata*.[16] The proapoptotic effect of *ent*-15-oxo-kaur-16-en-19-oic acid on the human prostate carcinoma epithelial cell line PC-3 has been also described.[17]

We have recently published that two kaurane diterpenes, foliol and linearol, interact with the mitochondrial pathway protecting macrophages from NO-dependent apoptosis. In addition, these diterpenes can reduce phagocytosis in cultured peritoneal macrophages and in macrophage cell lines.[18]

Continuing with our interest in the evaluation of the biological potential of natural diterpenes,[19] in the present study we report the preparation of 31 kaurane derivatives from the natural diterpenes kaurenoic acid (**1**), grandiflorenic acid (**16**), 15α-acetoxy-kaurenoic acid (**26**), and 16α-hydroxy-kaurenoic acid (**31**), their induction of apoptosis signaling pathway in mouse and human cancer cells and some conclusions about structure–activity relationships. Our results clearly demonstrate that four of these compounds (**12**, **20**, **21**, and **23**) induced apoptosis in a dose-dependent manner through a caspase-dependent mechanism, which may contribute to the chemopreventive functions.

2. Results and discussion

2.1. Chemistry

Most of the derivatives were obtained from the natural compounds kaurenoic acid (**1**), grandiflorenic acid (**16**), 15α-acetoxy-kaurenoic acid (**26**), and 16α-hydroxy-kaurenoic acid (**31**). These diterpenes are the main secondary metabolites of the *Espeletia* species, resinous plants, popularly known as frailejón, that are characteristic of the high Andean plateaus of Venezuela, Colombia, and Ecuador.[20]

Their structures are constituted by a perhydrophenantrene unit (A–C rings) fused with a cyclopentane unit (D ring) formed by a bridge of two carbons between C-8 and C-13, this skeleton confers lipophilic properties which is essential to cross membranes and occupy hydrophobic pockets in the target.

2.1.1. Synthesis of kaurenoic acid derivatives (2–15)

From kaurenoic acid (**1**) or kaurenoic acid methyl ester (**2**), we have carried out several transformations on the carboxylic acid function and also on the exocyclic double bond (Scheme 1).

Compound **2** was obtained from **1** after treatment with CH_2N_2/Et_2O. Compound **2** under hydrogenation with Pd/C 10% in THF yielded **3**. Epoxidation of the double bond in **2** using MCPBA produced the epoxide isomers (**4**) and (**5**). The main epoxide derivative (**5**) was converted into the aldehyde **6** when treated with BF_3Et_2O in THF. The reaction of compound **6** with $NH_2OH{\cdot}HCl$ provided compound **7**. To evaluate the influence in the activity of a voluminous group in the ring A, we obtained the benzyl ester **8**. Compound **1** was reduced to the corresponding alcohol **9** using $LiAlH_4$. The allylic oxidation of **2** with SeO_2 afforded the allylic alcohol **10** and the α,β-unsaturated aldehyde **11**. The posterior oxidation of the derivative **10** produced the α,β-unsaturated ketone **12** which was converted into compound **13** under treatment with ethyl vinyl ether and toluene in a sealed tube at 100 °C. The dihydroxylated ester **14** was obtained from **2** under treatment with OsO_4 while derivative **15** was synthesized using $SOCl_2$/MeOH.

2.1.2. Synthesis of grandiflorenic acid derivatives (17–25)

Grandiflorenic acid (**16**) was subjected to similar transformations that those in kaurenoic acid (**1**) (Scheme 2). Thus, **16** was reduced with $LiAlH_4$ to form the alcohol **25**. Compound **16** was esterified with BnBr yielding derivative **24** and the corresponding methyl ester **17** was obtained under treatment with CH_2N_2/Et_2O. The reaction of **17** with OsO_4 afforded **18** in a regioselective form. Compounds **19**–**21** were obtained when **17** was treated with SeO_2. The unexpected compound **21** was formed probably via a [2,3] sigmatropic rearrangement. The structure of **21** was unequivocally established on the basis of the correlations observed in the HMBC spectrum and the α-orientation of the hydroxyl group was determined by the NOE effects detected between the hydrogen H-11 and the methyl group Me-20 and between H-15 and H-5. The derivative **22** was obtained from **19** using usual acetylation conditions and compound **23** was also obtained from **19** after oxidation with PDC.

2.1.3. Synthesis of kaurenoic acid derivatives (27–30)

Derivatives (**27–30**) were obtained from natural 15α-acetoxy-kaurenoic acid (**26**) following the reactions shown in Scheme 3. Basic hydrolysis of **26** yielded the hydroxyl derivative **27**, which was subsequently oxidized with Jones reagent to afford the corresponding carbonyl compound **28**. The treatment of **28** with CH_2N_2 yielded the pirazoline (**29**), which was formed through a 1,3-dipolar cycloaddition reaction between the activated double bond and the diazo compound.

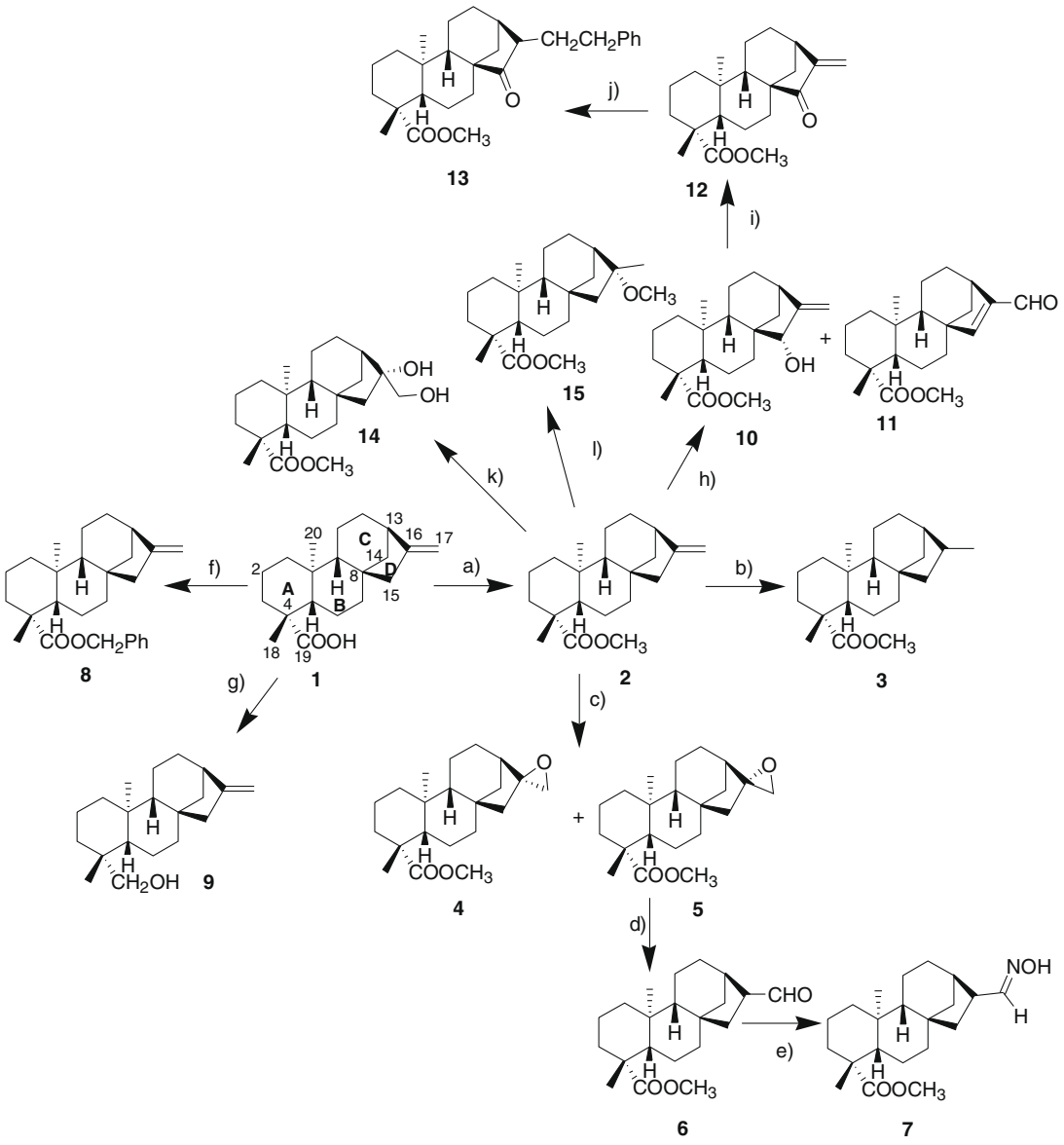
2.1.4. Synthesis of kaurenoic acid derivatives (32–34)

Compounds **32–34** were obtained from natural 16α-hydroxy-kaurenoic acid (**31**) (Scheme 4). Derivative **32** was formed by methylation of **31** which under treatment with thionyl chloride yielded compounds **2**, **33**, and **34**.

2.2. Biological assays and SAR

2.2.1. *ent*-Kaurane derivatives decrease cell viability in tumor cells

To establish the potential use of these compounds, the obtained derivatives were tested for their ability to inhibit cell viability in the mouse leukemic macrophagic RAW 264.7 cell line. The IC_{50} values are shown in Table 1. Twelve compounds were able to inhibit cell viability in the range of 1–25 μM. The most effective compounds were compound **12** (IC_{50} = 1 μM) and compounds **20**, **21**, and **23** with an IC_{50} value of 5 μM.

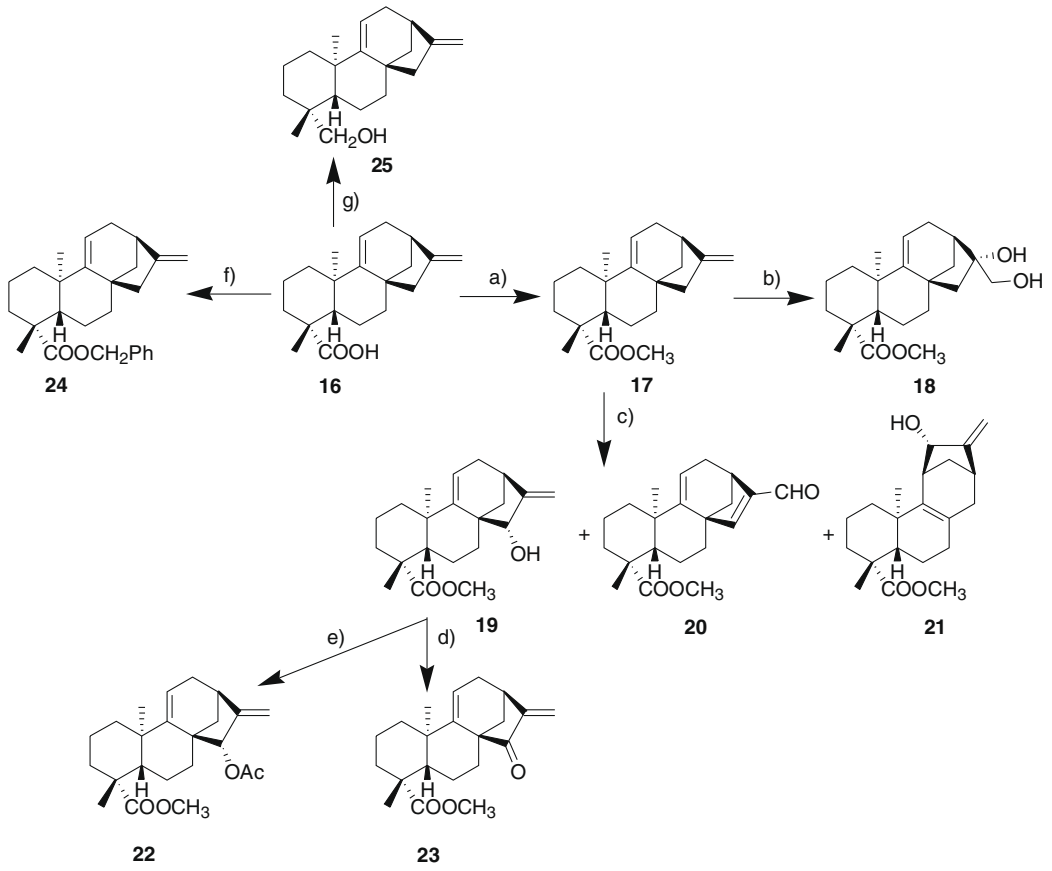


Scheme 1. Reagents and conditions: (a) CH_2N_2, Et_2O, 24 h; (b) H_2, Pd/C 10%, THF, 24 h; (c) MCPBA 70%, DCM, $NaHCO_3$, 25 min; (d) BF_3Et_2O, THF; (e) $H_2NOH?HCl$, AcONa, H_2O, EtOH, 16 h; (f) BnBr, acetone, 6 h; (g) $LiAlH_4$, Et_2O, 3 h; (h) SeO_2, DCM, 24 h; (i) PDC, DCM, 24 h; (j) toluene, sealed tube 100 °C, 70 h;(k) OsO_4, NMO, $^tBuOH/THF/H_2O$ 7:2:1, 23 h; (l) $SOCl_2$/MeOH, reflux, 15 h.

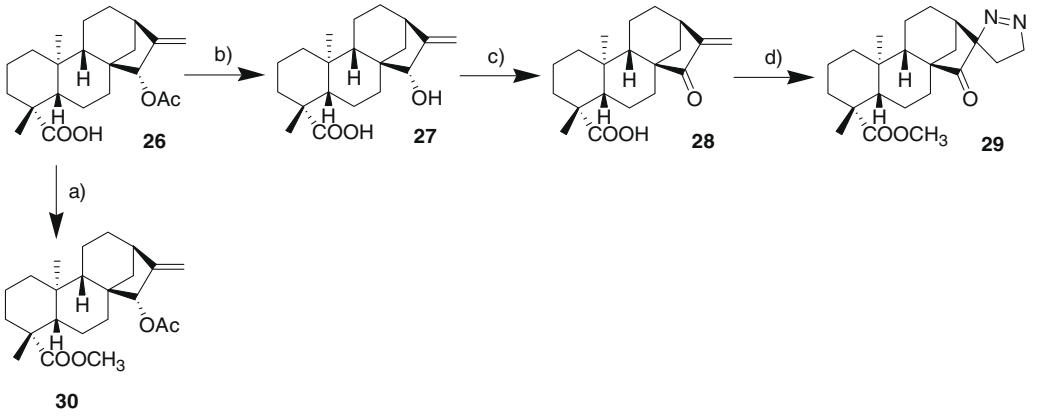
In addition, compounds **12, 20, 21**, and **23** were also tested in several human and mouse tumor cell lines. As we can observe in Figure 1 and Table 1, similar effects were observed in all cancer cell lines, although RAW 264.7 cells were more sensitive.

In order to verify the selectivity of these compounds on cancer cell lines, we examine their effects on non-tumor cells. Primary cultures of peritoneal macrophages obtained from thioglycolate-injected mice were stimulated for 24 h with a range of concentrations of compounds**12, 20, 21**, and **23** and apoptosis and cell viability were determined (Fig. 2). Only compound **20** affected cell viability and induced apoptosis in peritoneal macrophages, whereas compounds **12, 21**, and **23** were not cytotoxic for peritoneal macrophages. These data thus suggest that e*nt*-kaurane derivatives induce apoptosis in various cancer cell lines but are significantly less cytotoxic in non-transformed cells, providing evidence for the potential use of these compounds as antitumor agents.

From the obtained results some structure–activity relationships can be established. Concerning the functionalization at C-4 of the ring A, the best results were achieved with compounds having an ester group. The hydroxyl derivatives **9** and **25** obtained from the reduction of the corresponding carboxylic function were inactive. The esterification of the parent carboxylic acids led to an increase in the activity (i.e., **12** vs **28**, **1** vs **8**, **1** vs **2**). These results indicated that a hydrogen-bond-donor (HBD) group at ring A was not an important requirement for the inhibitory activity which is favoured by the increase of lipophilicity. The role of the double bond at C-9–C-11 of the ring C is not too clear. Depending on the type of substituents at the ring D, this double bond increases or decreases the activity. When the exocyclic double bond C-16–C-17 is only present in the ring D, the activity is increased with the additional double bond C-9–C-11 (e.g., compare the activity of **1** vs **16**, **2** vs **17**, and **9** vs **25**). If an oxygenated function is present at C-15 or at C-16 the more active compounds are those that do not have the double bond at ring C (e.g., compare the activity of **14** vs **18**, **12** vs **23**, and **8** vs **24**) while compound **20** having an aldehyde group at C-17 and the C-9–C-11 double bond was more

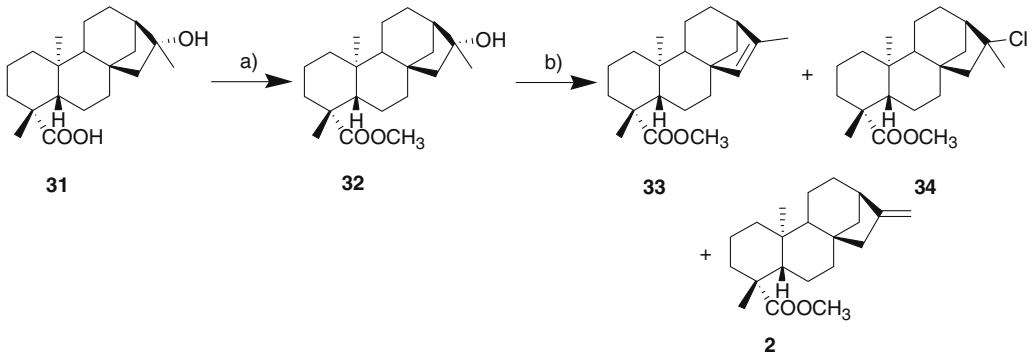


Scheme 2. Reagents and conditions: (a) CH_2N_2, Et_2O, 24 h; (b) OsO_4, NMO, $^tBuOH/THF/H_2O$ 7:2:1, 23 h; (c) SeO_2, DCM, 17 h; (d) PDC, DCM, 24 h; (e) Ac_2O, py, DMAP, 24 h; (f) BnBr, acetone, 6 h; (g) $LiAlH_4$, Et_2O, 3 h.



Scheme 3. Reagents and conditions: (a) Na_2CO_3 1 M, MeOH, 24 h; (b) Jones reagent, acetone, 0 °C, 6 h; (c) CH_2N_2, Et_2O, 5 h; (d) CH_2N_2, Et_2O, 17 h.

active than compound **11**, without the mentioned double bond. In order to evaluate the influence on the activity of substituents of different nature in the cyclopentane ring D, we carried out several modifications. The best activities were obtained with those compounds having a α,β-unsaturated carbonyl group (compounds **12**, **20**, and **23**). The replacement of the carbonyl group of compounds **12**, **20**, and **23** by hydroxy or acetoxy groups (compounds **19**, **22**, **26**, **27**, and **30**) led to a loss of activity. When the double bond of the α,β-unsaturated carbonyl moiety was modified, the proliferative activity decreased (e.g., compare the activity of **12** vs **29**, **12** vs **13**, and **11** vs **6**). These results highlighted the importance of this moiety, probably acting as a Michael acceptor with nucleophilic residues present in the target. This conclusion is in agreement with the results found for other kaurenoic derivatives from Asian species belonging to the *Isodon* genus (Labiatae) and containing an α,β-unsaturated ketone moiety at the D ring. These compounds are poly-hydroxylated and most of them have been found to be cytotoxic against several cancer cell lines.[21] The active compound **21** presents a different skeleton, because of it was formed through an oxidative rearrangement from **17**. The cyclopentane ring in compound **21** is formed by a bridge between C-11 and C-13 instead of between C-8 and C-13. We have not found similar structures described previously in the chemical literature.[22]



Scheme 4. Reagents and conditions: (a) Me_3SiCHN_2, MeOH, Et_2O, 15 h; (b) $SOCl_2$, DCM, reflux, 3 h.

Table 1
IC_{50} values[a] (μM) of compounds **1–34** in cell viability

Compound	RAW 264.7	Hela	HepG2	HT-29
1	>100	—	—	—
2	75 ± 2.3	—	—	—
3	75 ± 7.6	—	—	—
4	>100	—	—	—
5	>100	—	—	—
6	>100	—	—	—
7	35 ± 5.2*	—	—	—
8	25 ± 2.1*	—	—	—
9	>100	—	—	—
10	>100	—	—	—
11	25 ± 4.3*	—	—	—
12	1 ± 0.2***	50 ± 6.3*	75 ± 8.3*	30 ± 2.7*
13	>100	—	—	—
14	10 ± 0.5**	—	—	—
15	>100	—	—	—
16	15 ± 1.3**	—	—	—
17	35 ± 4.3*	—	—	—
18	25 ± 3.7*	—	—	—
19	>100	—	—	—
20	5 ± 0.4**	10 ± 1.4*	25 ± 3.3*	40 ± 4.5*
21	5 ± 0.3**	>100	>100	>100
22	35 ± 3.6*	—	—	—
23	5 ± 0.7**	25 ± 1.2*	>100	25 ± 2.7*
24	65 ± 2.0*	—	—	—
25	35 ± 1.5*	—	—	—
26	25 ± 0.3*	—	—	—
27	>100	—	—	—
28	35 ± 1.9*	—	—	—
29	100	—	—	—
30	25 ± 4.3*	—	—	—
31	Not tested	Not tested	Not tested	Not tested
32	25 ± 0.9*	—	—	—
33	100	—	—	—
34	>100	—	—	—

[a] IC_{50} values refer to the concentration needed to inhibit 50% of cell viability in the presence of the compounds. RAW 264.7 cells were treated with different concentrations of derivatives (1–100 μM) for 24 h and Hela, HepG2, and HT-29 cells were treated with different concentrations of derivatives **12**, **20**, **21**, and **23** (1–100 μM) for 24 h. The effects on cell viability of the compounds are reported as IC_{50} values (μM) determined using the MTT assay. *P <0.05, **P < 0.01, and ***P >0.001 with respect to the non-treated cells.

2.2.2. Induction of apoptosis in RAW 264.7 cells

Many anticancer drugs induce apoptosis. To investigate the apoptotic potential of these compounds, the mouse leukemic macrophagic RAW 264.7 cell line was treated for 24 h with compounds over a concentration range from 1 to 25 μM (Fig. 3A) and apoptotic cells detected by flow cytometry. All compounds were able to induce apoptosis; although the most effective compound was compound **12** which induced apoptosis in 50% of cells at 1 μM. Apoptotic cells undergo characteristic morphological changes that depend on a cascade of proteolytic enzymes called caspases. Caspase 8 is the main executer of the extrinsic pathway initiated at the plasma membrane by activation of cell surface death receptors, whereas caspase 9 is activated in the intrinsic pathway mediated by mitochondria. In addition, both caspases activate downstream apoptotic effectors such as caspase 3. To analyze caspase activation, we used the fluorogenic peptide substrates, Ac-DEVD-AMC, Ac-IETD-AFC, and Ac-LEHD-AFC specific for caspase-3, -8, and -9, respectively. Caspase activities were measured following treatment of cells with 5 μM of compound for 24 h (Fig. 3B). Treatment with compounds **12**, **21**, and **23** increased the enzymatic activities of caspases 8, 9, and 3, whereas compound **20** only activated caspases 9 and 3. Both caspase activity and apoptosis were efficiently inhibited after treatment with the general caspase inhibitor z-VAD (Fig. 3B and C). These data demonstrate that compounds **12**, **21**, and **23** induce apoptosis via the activation of caspase 8, which can also activate the intrinsic pathway, whereas in compound **20**-dependent apoptosis, mitochondria seems to play a key role.

An analysis of the structures of compounds **12**, **20**, **21**, and **23** indicates that compounds that activate caspase 9 present an accessible exocyclic double bond which probably efficiently binds some residues present in caspase-9. Compound **20** does not have the mentioned exocyclic double bond, it presents an aldehyde group conjugated with a trisubstituted endocyclic double bond which may interact better with other residues present in caspase 9.

2.2.3. Gene expression during apoptosis

Several gene products are known to be important in controlling the apoptotic process. To determine whether our compounds have an effect on the expression levels of these gene products, cells were incubated with 5 μM of the kaurane derivatives (**12**, **20**, **21**, and **23**) and cellular lysates were analyzed by Western blotting. These Western blots were probed with antibodies against p53, Bcl-2, Bax, Bcl-xl, XIAP, IAP-1, and IAP-2 proteins (Fig. 4). When cells were exposed to 5 μM of the different compounds, expression levels of p53 and Bax increased whereas levels of antiapoptotic proteins such as Bcl-2, Bcl-xl, and IAPs decreased. These results are very interesting due to the fact that an important area of future research is the identification of the genes that are involved in the apoptotic program of cell death. For example, the ratio between Bcl-2 and Bax determines cell survival or death. In our study, we found that the expression of the Bcl-2 and Bcl-xl decreased, whereas the amount of Bax protein increased under the same conditions.

3. Conclusion

Apoptosis has been recognized as a tightly controlled mechanism involving death factors and death receptors in the control of cell proliferation. Induction of apoptosis in cancer cells or malig-

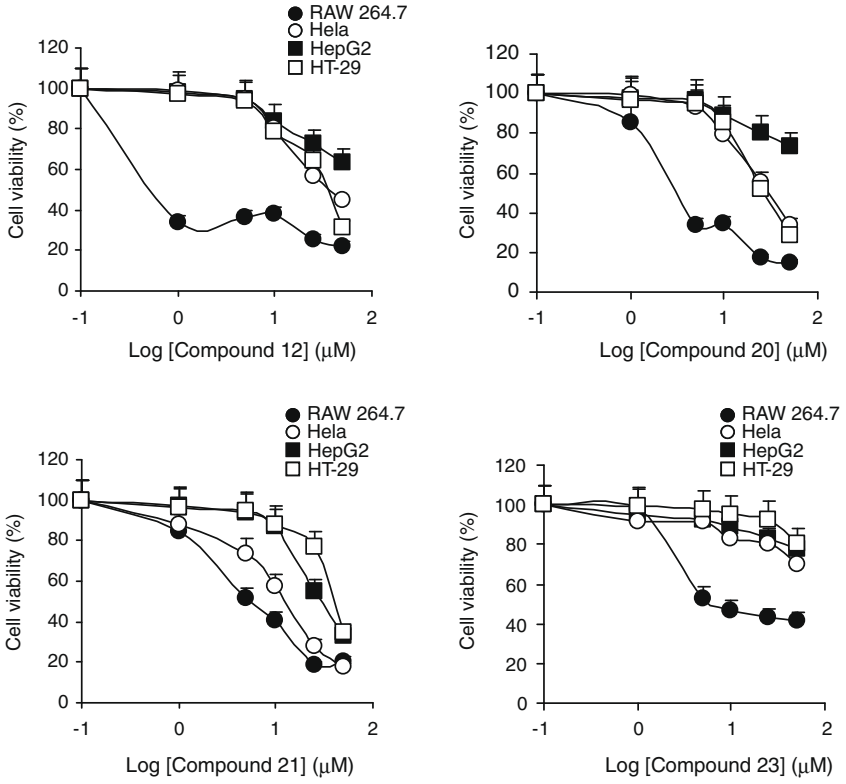


Figure 1. Cell viability on tumor cells. RAW 264.7, Hela, HepG2, and HT-29 cells were treated with different concentrations (1–50 μM) of compounds for 24 h. Cell viability was determined by MTT assay, and results are reported as mean of cell viability (%) of at least three replicate wells ± SD. Results are normalized to cell viability of non-treated cells at 24 h.

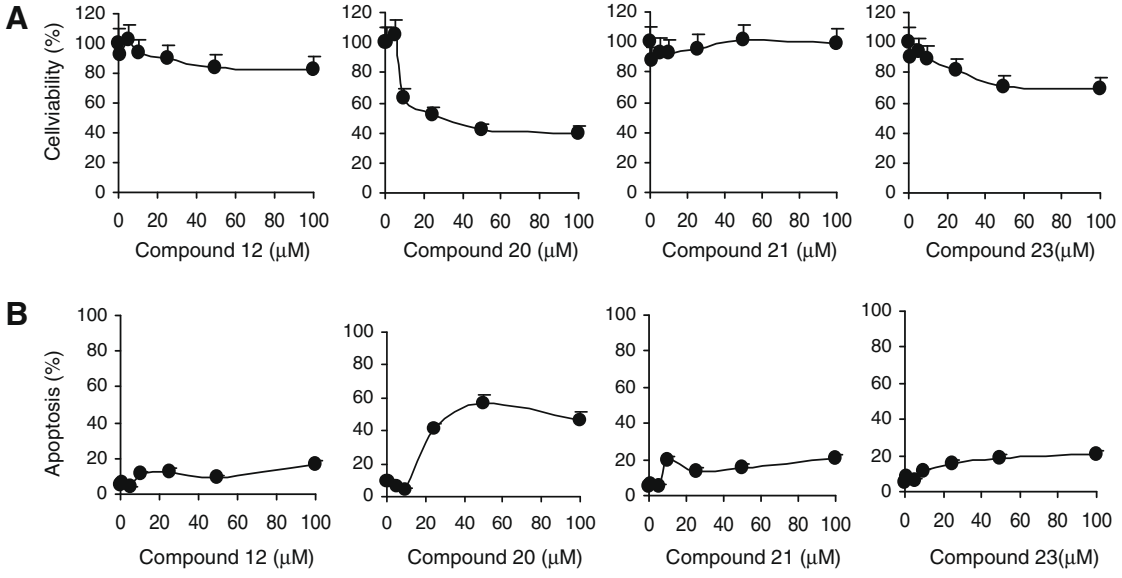


Figure 2. Effects of *ent*-kauranes on non-tumor cells. (A) Peritoneal macrophages (PM) isolated from thioglycolate-injected mice were treated with the indicated concentrations of compounds for 24 h. Cell viability was determined by MTT assay. (B) PM were treated with the indicated concentrations of compounds for 24 h. The percentage of apoptotic cells was determined by flow cytometry after staining with PI. Data are the means ± SD of three independent experiments carried out in duplicate.

nant tissues is recognized as an efficient strategy for cancer chemotherapy. Natural products have been the source of many medically beneficial drugs, and their importance in the prevention and treatment of cancer is becoming increasingly apparent. In this work, a set of *ent*-kaurane derivatives were synthesized with the aim of identifying the structural requirements to achieve good activity and to establish structure–activity relationships. The biological activities of the obtained kaurane derivatives were focused on apoptotic activity and revealed that compounds **12**, **20**, **21**, and **23** act as apoptosis inducers. These compounds exhibited growth inhibition and cytotoxicity in different cancer cells. Of particular interest in this regard is our finding that these compounds have a stronger proapoptotic action on tumor cells whereas no cytotoxic effects were observed for compounds **12**, **21**, and **23** in peritoneal

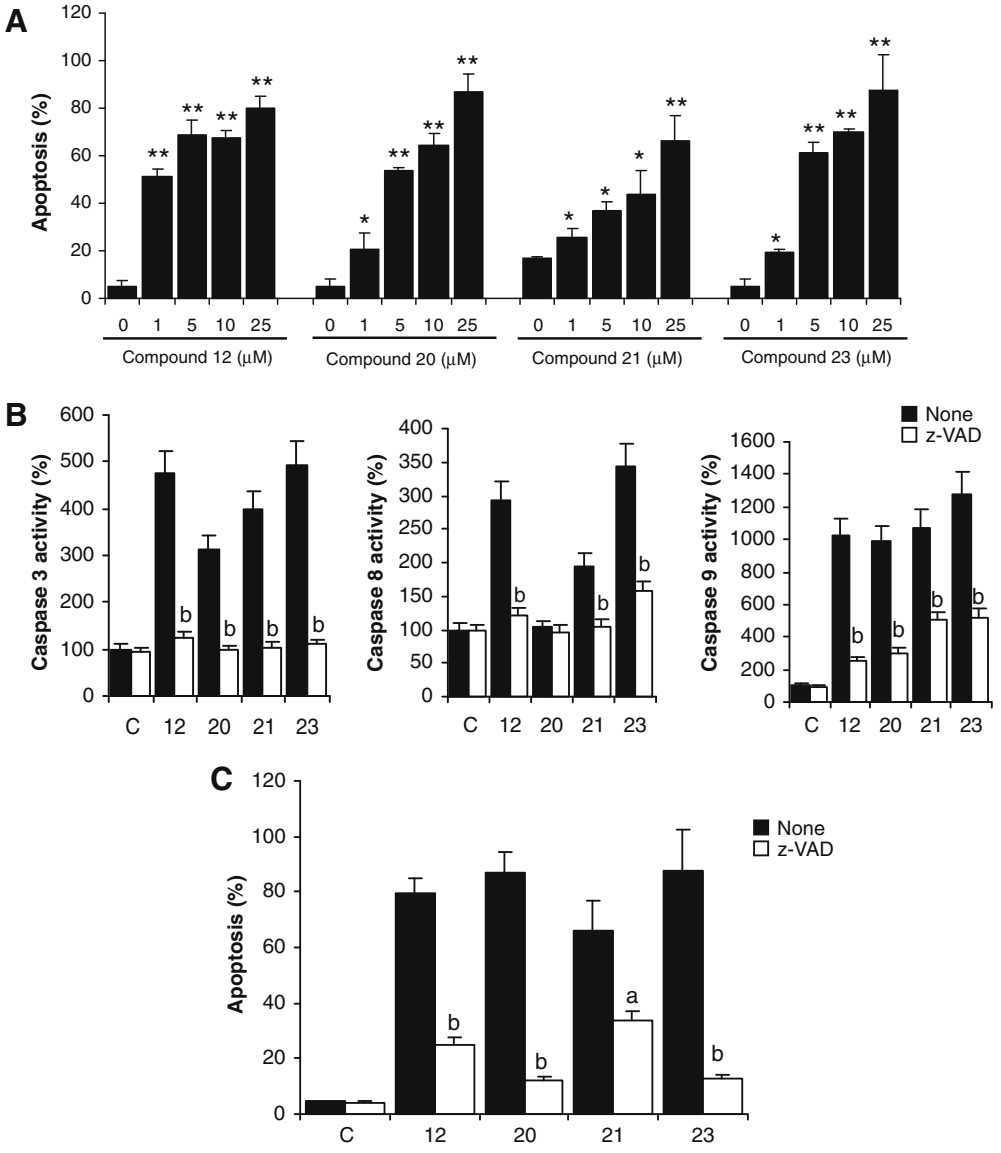


Figure 3. Induction of apoptosis and caspase activation. (A) Cells were treated with different concentrations of compounds (1–25 μM) for 24 h. The percentage of apoptotic cells was determined by flow cytometry after staining with propidium iodide (PI). (B) Caspase activities were fluorimetrically determined in cell extracts after treatment of RAW cells with compounds (5 μM) in the presence or absence of z-VAD (40 μM). C. Cells were treated with compounds (5 μM) in the presence or absence of z-VAD (40 μM) for 24 h. The percentage of apoptotic cells was determined by flow cytometry after staining with propidium iodide (PI). Data are the means ± SD of three independent experiments carried out in triplicate. $^{*}P$ <0.05, $^{**}P$ < 0.01 with respect to the control condition and *a* <0.05 and *b* <0.01 with respect to the cells incubated in absence of z-VAD.

macrophages. This selective apoptosis of transformed cells requires further study, but might indicate a greater resistance of normal cells to apoptosis than tumor cells, and suggests a promising future for these compounds as therapeutic agents.

ent-Kaurane derivatives-induced apoptosis was characterized by the activation of caspase-3, -8, and -9. Exposure of cells to *ent*-kaurane derivatives resulted in up-regulation of Bcl-2-associated protein (Bax) and down-regulation of the expression of the survival protein Bcl-2 and Bcl-xl.

The presence of a α,β-unsaturated carbonyl moiety seems to play an important role in the activity since it is present in three of the four apoptosis inducers, and replacement of the carbonyl group of compounds **12**, **20**, and **23** by a different group (hydroxy or acetoxy) resulted in the loss of activity. This moiety could act as Michael acceptor with nucleophilic residues, especially cysteine sulfhydryl groups.[23]

In short, we believe that the findings presented here demonstrate that *ent*-kaurane derivatives are potent inducers of apoptosis in tumor cells and will provide a basis for constructing new chemotherapeutic agents for the treatment or clinical management of some types of human cancers. Thus, further investigation of *ent*-kaurane derivatives will contribute to additional understanding of their in vitro activity toward malignant cells and their potential toxicity toward normal tissues.

4. Experimental

4.1. Chemistry

4.1.1. General

All solvents and reagents were purified by standard techniques reported in Perrin, D. D.; Amarego, W. L. F. *Purification of Laboratory Chemicals, 3rd ed.;* Pergamon Press: Oxford, 1988 or used as supplied from commercial sources as appropriate. Reactions were monitored by TLC (on silica gel POLYGRAM SIL G/UV_{254} foils). Purification by column flash-chromatography used Merck Kiesel 60-H

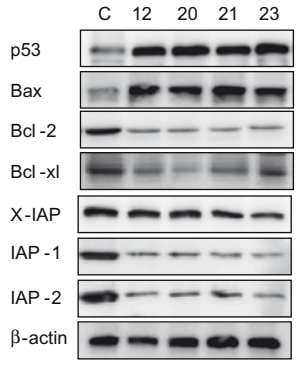


Figure 4. Immunoblot analysis of the expression of p53, Bax, Bcl-2, Bcl-xl, and IAPs in RAW 264.7 cells. RAW 264.7 cells were incubated with compounds (5 μM) and the expression of the apoptosis-related proteins p53, Bax, Bcl-2, Bcl-xl, X-IAP, IAP-1, and IAP-2 was determined at the indicated times by Western blot in cytosolic extracts. Protein loading was normalized to the expression of β-actin. A representative experiment is shown of three performed.

(0.063–0.2 mm) as adsorbent and different mixtures of hexanes–ethyl acetate as eluent. Pre-coated TLC plates SIL G-100 UV_{254} (Machery-Nagel) were used for preparative-TLC purification. ^1H NMR spectra were recorded in $CDCl_3$ or C_6D_6 at 300 or 400 MHz, using a Bruker AMX300 or Bruker AMX400 instruments. For ^1H spectra, chemical shifts are given in parts per million (ppm) and are referenced to the residual solvent peak. The following abbreviations are used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Proton assignments and stereochemistry are supported by ^1H–1H COSY and ROESY where necessary. Data are reported in the following manner: chemical shift (multiplicity, coupling constant if appropriate, integration). Coupling constants (J) are given in hertz (Hz) to the nearest 0.5 Hz. ^{13}C NMR spectra were recorded at 75 and 100 MHz using a Bruker AMX300 or Bruker AMX400 instruments. Carbon spectra assignments are supported by DEPT-135 spectra, ^{13}C–1H (HMQC) and ^{13}C–1H (HMBC) correlations were necessary. Chemical shifts are quoted in ppm and are referenced to the appropriate residual solvent peak. MS and HRMS were recorded on a VG Micromass ZAB-2F. IR spectra were taken on a Bruker IFS28/55 spectrophotometer. Kaurenoic acid (**1**), grandiflorenic acid (**16**), 15α-acetoxy-kaurenoic acid (**26**), and 16α-hydroxy-kaurenoic acid (**31**) were used as starting material to synthesize the diterpenes. These diterpenes were isolated from *Espeletia chardonii* using the habitual previously described methodology.[20]

4.1.2. Kaurenoic acid methyl ester (2)

292.8 mg (0.970 mmol) of kaurenoic acid dissolved in 10 mL of ethyl ether were treated with 1 mL of solution of CH_2N_2 in Et_2O. The reaction mixture was left at room temperature until the disappearance of the starting material. After elimination of solvent a white residue was obtained (305.0 mg, 100%), which showed identical spectroscopic data to those of kaurenoic acid methyl ester (**2**).[24]

4.1.3. Dihydro kaurenoic acid methyl ester (3)

23.5 mg (0.074 mmol) of compound **2** dissolved in 4 mL of dry THF were hydrogenated in the presence of catalytic amount of Pd/C 10%. The reaction mixture was stirred for 24 h until disappearance of the starting material. After elimination of solvent the resulting residue was purified by preparative-TLC using Hex/AcOEt (3:2) to yield 8.7 mg of compound **3** (37%) as white amorphous solid. Compound **3** showed identical spectroscopic data to those of dihydro kaurenoic acid methyl ester.[25]

4.1.4. Preparation of epoxy derivatives (4) and (5)

To 346.8 mg (1.097 mmol) of compound **2** dissolved in DCM (12 mL) were added 324.5 mg of 70% AMCPB (1.2 equiv) and 405 mg of $NaHCO_3$ (4.4 equiv). The reaction mixture was left to room temperature for 25 min, then the organic phase was separated and treated with a saturated solution of sodium thiosulfate. The organic phase was separated again and dried over anhydrous magnesium sulfate, the mixture was filtered, the solvent was removed under reduced pressure and the crude was purified by chromatotron using Hex/AcOEt 5% to yield 41.4 mg of **4** (11%) and 240.7 mg of **5** (66%) as yellow oils. Compounds **4** and **5** showed spectroscopic data identical to those reported.[26]

4.1.5. Preparation of the aldehyde (6)

75.4 mg (0.227 mmol) of epoxy derivative **5** dissolved in 5 mL of dry THF under nitrogen atmosphere were treated with 0.27 mL of BF_3Et_2O (9.4 equiv). The reaction was followed by TLC and when the starting material disappeared the solvent was removed, the residue was redissolved in ethyl acetate and treated with saturated solution of NaCl. The organic phase was dried over anhydrous $MgSO_4$, filtered and concentrated to afford 75.4 mg of compound **6** as pale yellow oil (100%). This compound resulted be highly unstable. ^1H NMR (300 MHz, $CDCl_3$) δ: 9.64 (1H, d, J = 1.89 Hz, H-17), 3.63 (3H, s, H-21), 2.59 (1H, t, J = 7.5 Hz, H-16), 2.53 (1H, s, H-13), 2.16 (1H, d, J = 13 Hz, H-15a), 1.16 (3H, s, H-18), 0.80 (3H, s, H-20), 2.05–0.85 (19H, m). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 203.8 (d, C-17), 177.9 (s, C-19), 56.7 (d, C-5), 54.9 (d, C-9), 53.4 (d, C-16), 51.0 (q, C-21), 44.9 (s, C-8), 43.5 (s, C-4), 40.8 (t, C-7), 40.5 (t, C-1), 39.9 (t, C-15), 39.2 (s, C-10), 37.8 (t, C-3), 37.5 (t, C-14), 37.5 (d, C-13), 30.7 (t, C-12), 28.5 (q, C-18), 22.2 (t, C-6), 18.9 (t, C-2), 18.5 (t, C-11), 15.1 (q, C-20).

4.1.6. Preparation of the oxime derivative (7)

To a solution of hydroxylamine hydrochloride (7.2 mg, 3 equiv) and sodium acetate (5.7 mg, 1.7 equiv) in 0.4 mL of H_2O, 11.5 mg of aldehyde **6** in 3 mL of EtOH was added. The reaction mixture was stirred under reflux for 16 h. Then the EtOH was removed, and the residue was extracted with DCM (3 × 10 mL). The organic phases were collected and dried over anhydrous $MgSO_4$, then were filtered and concentrated. The residue was purified by preparative-TLC using *n*-Hex/AcOEt (4:1) and 4.4 mg of **7** (37%) were obtained as a yellow oil in a *E*:*Z* ratio of 1.5:1. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.37 (1H, d, J = 7.1 Hz, H-17*E*), 6.67 (1H, d, J = 6.9 Hz, H-17*Z*), 3.64 (3H, s, H-21), 1.16 (3H, s, H-18), 0.80 (3H, s, H-20), 3.18–0.85 (23H, m). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 177.9 (s, C-19), 156.7 (d, C-17), 56.7 (d, C-5), 54.9 (d, C-9), 50.9 (q, C-21), 45.2 (d, C-16), 45.1 (s, C-8), 43.5 (s, C-4), 40.6 (t, C-7), 40.5 (t, C-1), 40.5 (t, C-15), 39.1 (s, C-10), 37.8 (t, C-3), 37.4 (d, C-13), 30.6 (t, C-14), 29.5 (t, C-12), 28.5 (q, C-18), 22.2 (t, C-6), 18.9 (t, C-2), 18.4 (t, C-11), 15.1 (q, C-20). EIMS *m/z* (%): 347 ([M]$^+$, 23), 330 (44), 288 (72), 276 (48), 270 (100), 149 (28), 131 (21), 123 (45), 121 (37), 119 (25), 109 (53), 107 (39), 101 (21), 97 (20), 95 (34), 93 (30), 91 (32), 83 (31), 81 (44), 79 (32), 72 (34), 71 (30), 69 (68). HREIMS: 347.2451 (calcd for $C_{21}H_{33}NO_3$ [M]$^+$ 347.2460). IR ν_{max} 3276, 2928, 2853, 1726, 1459, 1233, 1150, 771 cm^{-1}. UV($CHCl_3$) λ_{max} (log ε): 202.2 (3.42) nm. $[\alpha]_D^{20}$ −83.41 (c 0.4, $CHCl_3$).

4.1.7. Kaurenoic acid benzyl ester (8)

100 mg (0.331 mmol) of kaurenoic acid (**1**) and 82 μL of benzyl bromide (1 equiv) were added to a solution of 74.7 mg of KOH (4 equiv) in 5 mL of dry acetone (5 mL). The reaction mixture was stirred at room temperature for 6 h. Then, 40 mL of H_2O were poured and the mixture was extracted with AcOEt. The combined organic extracts were washed with brine and dried over $MgSO_4$. After removal of solvent the residue was purified by flash chromatography using *n*-Hex/AcOEt 5% to afford 54.3 mg of compound **8**

(54%) as an amorphous white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.35 (5H, m, H-23, 24, 25, 26, 27), 5.14 (1H, d, J = 12.5 Hz, H-21a), 5.03 (1H, d, J = 12.5 Hz, H-21b), 4.79 (1H, s, H-17a), 4.73 (1H, s, H-17b), 2.62 (1H, s, H-13), 1.20 (3H, s, H-18), 0.79 (3H, s, H-20), 2.23–0.83 (20H, m). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 177.1 (s, C-19), 155.6 (s, C-16), 136.0 (s, C-22), 128.8 (d, C-25), 128.6 (d, C-24), 128.2 (s, C-26), 128.0 (s, C-23), 127.8 (d, C-27), 102.8 (t, C-17), 65.7 (t, C-21), 57.0 (d, C-5), 54.8 (d, C-9), 48.7 (t, C-15), 44.0 (s, C-8), 43.7 (s, C-4), 43.6 (d, C-13), 41.1 (t, C-7), 40.5 (t, C-1), 39.3 (t, C-14), 39.3 (s, C-10), 37.9 (t, C-3), 32.9 (t, C-12), 28.7 (q, C-18), 21.7 (t, C-6), 19.0 (t, C-2), 18.2 (t, C-11), 15.4 (q, C-20). EIMS m/z (%): 392 ([M]$^+$, 41), 301 (27), 255 (11), 147 (9), 133 (9), 123 (10), 105 (12), 93 (12), 91 (100), 81 (16), 79 (11), 55 (11). HREIMS: 392.2706 (calcd for $C_{27}H_{36}O_2$ [M]$^+$ 392.2715). IR ν_{max} 3064, 2930, 2853, 1723, 1652, 1540, 1458, 1228, 1144, 873, 735, 671 cm^{-1}. UV(EtOH) λ_{max} (log ε): 203.8 (3.71) nm. $[\alpha]_D^{20}$ −83.42 (c 0.8, $CHCl_3$).

4.1.8. Preparation of compound 9

To a solution of 100 mg of **1** (0.331 mmol) in 25 mL of Et_2O were slowly added 30 mg of $LiAlH_4$ (2.4 equiv). The reaction mixture was left under stirring at room temperature for 3 h, then it was treated with a saturated solution of ammonium sulfate. The mixture was filtered through Celite, the aqueous phase was several times extracted with AcOEt. The combined organic extracts were dried over $MgSO_4$ and after removal of solvent 27 mg (28%) of **9** were obtained. Compound **9** showed identical spectroscopic data to those reported.[27]

4.1.9. Preparation of compounds 10 and 11

To 46.8 mg of methyl ester kaurenoic acid (**2**) in 2.6 mL of dry DCM under N_2 atmosphere were added 20.6 mg of SeO_2 (1.2 equiv). The reaction mixture was stirred at room temperature for 24 h until disappearance of the starting material. After removal of solvent the residue was redissolved in AcOEt, treated with brine and the aqueous phase extracted several times with AcOEt. The combined organic extracts were dried over $MgSO_4$, filtered and concentrated. The residue was purified by preparative-TLC using n-Hex/AcOEt (4:1) to obtain 18.5 mg of compound **10** (38%) and 5.6 mg of compound **11** (11%) as amorphous yellow solids. Compound **10**: ^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.20 (1H, s, H-17a), 5.07 (1H, s, H-17b), 3.80 (1H, s, H-15), 3.64 (3H, s, H-21), 2.74 (1H, s, H-13), 1.18 (3H, s, H-18), 0.83 (3H, s, H-20), 1.94–0.76 (19H, m).^{13}C NMR (75 MHz, $CDCl_3$) δ: 177.9 (s, C-19), 160.1 (s, C-16), 108.1 (t, C-17), 82.4 (d, C-15), 56.7 (d, C-5), 53.1 (d, C-9), 50.9 (q, C-21), 47.5 (s, C-8), 43.6 (s, C-4), 42.0 (d, C-13), 40.5 (t, C-1), 39.3 (s, C-10), 37.8 (t, C-3), 35.9 (t, C-7), 35.0 (t, C-14), 32.3 (t, C-12), 28.5 (q, C-18), 20.8 (t, C-6), 18.9 (t, C-2), 18.0 (t, C-11), 15.4 (q, C-20). EIMS m/z (%): 332 ([M]$^+$, 55), 317 (31), 314 (49), 299 (51), 274 (70), 273 (47), 272 (38), 257 (58), 255 (100), 249 (32), 239 (51), 199 (22), 189 (37), 173 (29), 164 (28), 159 (30), 150 (32), 147 (36), 145 (29), 137 (26), 135 (40), 133 (38), 123 (70), 121 (90), 119 (30), 117 (29), 109 (85), 107 (76), 105 (58). HREIMS: 332.2349 (calcd for $C_{21}H_{32}O_3$ [M]$^+$ 332.2351). IR ν_{max} 3501, 2933, 2855, 1725, 1463, 1234, 1192, 1154, 1000, 755 cm^{-1}. UV(EtOH) λ_{max} (log ε): 202.0 (3.43) nm. $[\alpha]_D^{20}$ −92.00 (c 0.5, $CHCl_3$). Compound **11**: ^{1}H NMR (300 MHz, $CDCl_3$) δ: 9.71 (1H, s, H-17), 6.57 (1H, s, H-15), 3.63 (3H, s, H-21), 3.01 (1H, m, H-13), 1.16 (3H, s, H-18), 0.86 (3H, s, H-20), 2.18–0.74 (18H, m). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 189.2 (d, C-17), 177.5 (s, C-19), 161.4 (d, C-15), 148.3 (s, C-16), 56.3 (d, C-5), 51.0 (q, C-21), 50.7 (s, C-8), 45.6 (d, C-9), 43.5 (s, C-4), 42.7 (t, C-7), 40.4 (t, C-1), 39.6 (s, C-10), 37.9 (t, C-14), 37.7 (t, C-3), 37.5 (d, C-13), 28.5 (q, C-18), 24.8 (t, C-12), 20.1 (t, C-6), 18.8 (t, C-2), 18.4 (t, C-11), 15.1 (q, C-20). EIMS m/z (%): 330 ([M]$^+$, 100), 315 (32), 271 (77), 270 (62), 255 (31), 253 (21), 237 (20), 175 (25), 163 (57), 162 (28), 149 (26), 147 (24), 135 (22), 133 (30), 123 (78), 121 (56), 109 (69), 107 (57), 101 (27), 95 (36), 93 (37), 91 (73), 81 (54), 79 (54), 77 (32), 67 (40), 55 (55). HREIMS: 330.2185 (calcd for $C_{21}H_{30}O_3$ [M]$^+$ 330.2195). IR ν_{max} 2937, 2852, 2703, 1724, 1676, 1603, 1447, 1232, 1158, 987, 709 cm^{-1}. UV(EtOH) λ_{max} (log ε): 200.6 (3.30) and 251.8 (3.53) nm. $[\alpha]_D^{20}$ −97.09 (c 0.8, $CHCl_3$).

4.1.10. Preparation of the α,β-unsaturated ketone 12 from compound 10

To 74.4 mg (0.224 mmol) of compound **12** in 2.5 mL of dry DCM were added 150.1 mg of PDC (1.9 equiv) dissolved in 0.55 mL of DCM. The reaction mixture was stirred for 24 h, then it was filtered through florisil and the solvent removed. The residue was purified by flash chromatography using n-Hex/AcOEt 10% to yield 47.7 mg of **12** (65%) as amorphous white solid. Compound **12** showed identical spectroscopic data to those reported.[28]

4.1.11. Preparation of derivative 13

22.8 mg of α,β-unsaturated-ketone **12** in 20 mL of toluene were treated with 40 μL of ethyl vinyl ether (6 equiv) in a sealed tube at 100 °C for 70 h. Then the solvent was removed and the crude was purified by preparative-TLC using n-Hex/AcOEt (12%) to yield 6.7 mg of compound **13** (23%) as yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.21 (5H, m, H-3′, H-4′, H-5′, H-6′, H-7′), 3.64 (3H, s, H-21), 2.74 (2H, m, H-16, H-1′a), 2.49 (1H, s, H-13), 2.41 (1H, d, J = 12 Hz, H-1′b), 1.17 (3H, s, H-18), 0.87 (3H, s, H-20) 2.39–0.78 (18H, m).^{13}C NMR (75 MHz, $CDCl_3$) δ: 223.8 (s, C-15), 177.6 (s, C-19), 141.6 (s, C-2′), 130.1 (d, C-4′, C-6′), 128.1 (d, C-3′, C-7′), 125.6 (d, C-5′), 55.8 (d, C-16), 53.3 (s, C-8), 52.7 (d, C-5), 51.2 (d, C-9), 51.0 (q, C-21), 43.5 (s, C-4), 39.5 (s, C-10), 39.4 (t, C-1), 37.7 (t, C-3), 36.9 (t, C-1′), 34.1 (t, C-7), 33.9 (t, C-14), 32.8 (d, C-13), 28.5 (q, C-18), 27.2 (t, C-17), 24.5 (t, C-12), 20.1 (t, C-6), 18.6 (t, C-2), 18.2 (t, C-11), 15.0 (q, C-20). EIMS m/z (%): 422 ([M]$^+$, 43), 409 (30), 408 (100), 319 (21), 318 (55), 275 (32), 274 (95), 215 (21), 215 (20), 131 (20), 121 (50), 107 (37), 105 (25), 93 (25), 91 (97). HREIMS: 422.2801 (calcd for $C_{28}H_{38}O_3$ [M]$^+$ 422.2821). IR ν_{max} 2928, 2859, 1726, 1454, 1231, 1156, 699 cm^{-1}. UV(EtOH) λ_{max} (log ε): 204.2 (3.62). $[\alpha]_D^{20}$ −87.46 (c 0.7, $CHCl_3$).

4.1.12. Preparation of compound 14

To 40.8 mg of compound **2** (0.129 mmol) dissolved in a mixture of ^{t}BuOH/ THF/H_2O (7:2:1) was added 2 mg of OsO_4 (6 mol %) and 49.6 mg of NMO (3.3 equiv). The reaction mixture was stirred at room temperature until disappearance of the starting material, then it was treated with a saturated solution of $NaHSO_3$ and extracted several times with AcOEt. The combined organic layers were successively washed with saturated solution of $Na_2S_2O_3$ and brine. Then the organic extracts were dried over $MgSO_4$, filtered and concentrated to yield 44.2 mg (98%) of **14** as an amorphous white solid. Compound **14** showed spectroscopic data identical to those reported.[29]

4.1.13. Grandiflorenic acid methyl ester (17)

Following the same experimental procedure for compound **2**, the methyl ester **17** was obtained quantitatively from **16**, and it showed spectroscopical data identical to those reported.[30]

4.1.14. Preparation of compound 18

Following the same experimental procedure for compound **14**, 247.6 mg of compound **17** (0.789 mmol) dissolved in 5 mL of ^{t}BuOH: THF: H_2O (7:2:1) were treated with 5 mg de OsO_4 and 304.45 mg of NMO (3.3 equiv). The reaction mixture was stirred for 24 h, and it was quenched with 8 mL of saturated solution of $NaHSO_3$. After the same treatment describe for **14**, 243.7 mg of **18** were obtained (89%). **18** showed identical spectroscopic data to those published in reference 31.[31]

4.1.15. Preparation of compounds 19–21

To 277.2 mg of compound **17** (0.883 mmol) dissolved in 12 mL of dry DCM under N_2 atmosphere were added 250.8 mg of SeO_2 (2.6 equiv) and the mixture was stirred for 17 h at room temperature. Then the mixture was treated following the same procedure for compounds **10** and **11**. The crude was purified by chromatotron using 2 mm plate and *n*-Hex/AcOEt 10% as solvent to yield 152.2 mg of **19** (53%), 23.2 mg of **20** (8%), and 16.0 mg of **21** (6%) as amorphous solids. Compound **19** showed identical spectroscopic data to those reported.[32]

Compound **20** ^{1}H NMR (300 MHz, $CDCl_3$) δ: 9.58 (1H, s, H-17), 7.06 (1H, s, H-15), 5.13 (1H, s, H-11), 3.65 (3H, s, H-21), 3.14 (1H, s, H-13), 1.21 (3H, s, H-18), 0.95 (3H, s, H-20), 2.50–0.77 (15H, m).^{13}C NMR (75 MHz, $CDCl_3$) δ: 189.4 (d, C-17), 177.3 (s, C-19), 160.4 (d, C-15), 153.9 (s, C-16), 145.4 (s, C-9), 116.4 (d, C-11), 51.2 (q, C-21), 47.6 (d, C-5), 47.5 (t, C-14), 47.3 (s, C-4), 44.8 (s, C-8), 39.8 (t, C-1), 38.0 (t, C-3), 37.9 (s, C-10), 34.9 (d, C-13), 28.0 (q, C-18), 27.8 (t, C-12), 25.9 (t, C-7), 22.8 (q, C-20), 19.8 (t, C-2), 18.1 (t, C-6). EIMS *m/z* (%): 328 ([M]$^+$, 45), 269 (29), 253 (81), 235 (25), 234 (100), 202 (19), 197 (23), 187 (21), 175 (37), 174 (69), 173 (21), 159 (80), 147 (39), 145 (22), 129 (24), 121 (27), 117 (26), 107 (28), 105 (40), 93 (20), 91 (50). HREIMS: 328.2039 (calcd for $C_{21}H_{28}O_3$ [M]$^+$ 328.2038). IR ν_{max} 2942, 2871, 1723, 1672, 1459, 1218, 1145, 981, 706 cm^{-1}: UV(EtOH) λ_{max} (log ε): 202.2 (3.55) and 251.0 (3.47) nm. $[\alpha]_D^{20}$ +61.26 (*c* 0.9, $CHCl_3$). Compound **21**.^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.19 (1H, s, H-17a), 5.17 (1H, s, H-17b), 4.22 (1H, s, H-15), 3.62 (3H, s, H-21), 2.80 (1H, s, H-13), 2.42 (1H, d, *J* = 4.1 Hz, H-11), 2.26 (1H, d, *J* = 4.6 Hz, H-14a), 2.22 (1H, m, H-3a), 2.07 (1H, m, H-12a), 1.95 (1H, m, H-1a), 1.91 (1H, m, H-6a), 1.87 (1H, d, *J* = 3.8 Hz, H-7a), 1.82 (1H, *J* = 3.8 Hz, H-7b), 1.79 (1H, m, H-2a), 1.76 (1H, m, H-6b), 1.69 (1H, m, H-14b), 1.54 (1H, m, H-2b), 1.41 (1H, d, *J* = 10.8 Hz, H-12b), 1.32 (1H, d, *J* = 12.1 Hz, H-5), 1.21 (1H, m, H-1b), 1.17 (3H, s, H-18), 1.03 (1H, m, H-3b), 0.78 (3H, s, H-20).^{13}C NMR (75 MHz, $CDCl_3$) δ: 177.8 (s, C-19), 161.6 (s, C-16), 140.3 (s, C-9), 125.7 (s, C-8), 109.4 (t, C-17), 78.9 (d, C-15), 53.0 (d, C-5), 50.9 (q, C-21), 43.6 (s, C-4), 42.2 (d, C-11), 41.2 (t, C-14), 40.4 (d, C-13), 38.6 (s, C-10), 37.6 (t, C-3), 35.5 (t, C-1), 32.9 (t, C-12), 31.6 (t, C-7), 28.1 (q, C-18), 20.3 (t, C-6), 19.0 (t, C-2), 16.9 (q, C-20). EIMS *m/z* (%): 330 ([M]$^+$, 2); 315 (3), 235 (19), 189 (32), 175 (34), 173 (55), 159 (29), 145 (25), 135 (24), 129 (29), 117 (54), 105 (43), 95 (36). HREIMS 330.2184 (calcd for $C_{21}H_{30}O_3$ [M]$^+$ 330.2195). IR ν_{max} 3386, 2929, 1726, 1653, 1231, 1159, 1015, 897 cm^{-1}. UV($CHCl_3$) λ_{max} (log ε): 203.0 (3.62). $[\alpha]_D^{20}$ −35.85 (*c* 0.5, $CHCl_3$).

4.1.16. 15α-Acetoxy-grandiflorenic acid methyl ester (22)

17.7 mg (0.054 mmol) of compound **19** dissolved in the minimum amount of pyridine (105 μL) was treated with an excess of Ac_2O (1 mL, 197 equiv) in the presence of catalytic amount of DMAP. The reaction mixture was stirred for 24 h, after elimination of solvent, compound **22** (19.9 mg, 100%) was obtained as an amorphous solid.^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.45 (1H, s, H-15), 5.37 (1H, s, H-11), 5.20 (1H, s, H-17a), 5.16 (1H, s, H-17b), 3.63 (3H, s, H-21), 2.77 (1H, s, H-13), 2.07 (3H, s, $OCOCH_3$), 1.17 (3H, s, H-18), 0.94 (3H, s, H-20), 2.42–0.99 (15H, m). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 177.8 (s, C-19), 170.3 (s, $OCOCH_3$), 158.1 (s, C-9), 153.4 (s, C-16), 118.1 (d, C-11), 112.0 (t, C-17), 79.1 (d, C-15), 51.0 (q, C-21), 46.3 (d, C-5), 45.1 (s, C-4), 44.5 (s, C-8), 41.0 (t, C-14), 40.9 (t, C-1), 39.1 (d, C-13), 38.3 (s, C-10), 37.8 (t, C-3), 37.4 (t, C-12), 27.8 (q, C-18), 23.7 (q, C-20), 23.2 (t, C-7), 21.1 (q, $OCOCH_3$), 20.0 (t, C-2), 17.7 (t, C-6).EIMS *m/z* (%): 372 ([M]$^+$, 18), 357 (32), 313 (28), 312 (100), 297 (85), 253 (54), 237 (61), 197 (23), 183 (21), 173 (36), 172 (40), 157 (20), 145 (26), 143 (20), 131 (21), 129 (25), 105 (28), 93 (15). HREIMS: 372.2311 (calcd for $C_{23}H_{32}O_4$ [M]$^+$ 372.2301). IR ν_{max} 2926, 2873, 1731, 1457, 1370, 1235, 1146, 1016, 979, 903 cm^{-1}. UV(EtOH) λ_{max} (log ε): 201.8 (3.71). $[\alpha]_D^{20}$ +49.24 (c 0.8, $CHCl_3$).

4.1.17. 15-Oxo-grandiflorenic acid (23)

Compound **19** was oxidized following the same procedure described for **12**. Thus 42.3 mg of **19** (0.128 mmol) dissolved in DCM were treated with 84.7 mg of PDC (1.9 equiv) under N_2 atmosphere. The reaction mixture was stirred at room temperature for 24 h. After filtration through florisil and evaporation of solvent the residue was purified by preparative-TLC using *n*-Hex/AcOEt 15% to render 13.7 mg of compound **23** (33%) as amorphous pale yellow solid. **23** showed. identical spectroscopic data to those published.[32]

4.1.18. Grandiflorenic acid benzyl ester (24)

To a solution of 75 mg of KOH (4 equiv) in dry acetone (5 mL) were added 100 mg (0.333 mmol) of grandiflorenic acid (**16**), and 82 μL of benzyl bromide (1 equiv). The reaction mixture was stirred at room temperature until disappearance of the starting acid (6 h). Then 40 mL of H_2O were added and the aqueous phase was extracted several times with AcOEt. The organic phases were combined, dried over $MgSO_4$ and concentrated under reduced pressure. The crude was purified by column chromatography using *n*-Hex/AcOEt 5% to yield 119.5 mg of **24** (88%) as a pale yellow solid.^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.34 (5H, m, H-23–H-27), 5.22 (1H, s, H-11), 5.10 (2H, s, H-21), 4.91 (1H, s, H-17a), 4.79 (1H, s, H-17b), 2.76 (1H, s, H-13), 2.60 (1H, d, *J* = 15.6 Hz, H-15), 1.21 (3H, s, H-18), 0.89 (3H, s, H-20), 2.50–0.96 (16H, m).^{13}C NMR (75 MHz, $CDCl_3$) δ: 176.9 (s, C-19), 158.2 (s, C-9), 155.7 (s, C-16), 136.0 (s, C-22), 128.8 (d, C-25), 128.6 (d, C-24), 128.3 (d, C-26), 127.9 (d, C-23), 127.8 (d, C-27), 114.6 (d, C-11), 105.3 (t, C-17), 65.9 (t, C-21), 50.1 (t, C-15), 46.5 (d, C-5), 44.7 (t, C-14), 44.7 (s, C-4), 42.0 (s, C-8), 41.1 (d, C-13), 40.6 (t, C-1), 38.5 (s, C-10), 38.3 (t, C-3), 37.7 (t, C-12), 29.5 (t, C-7), 28.0 (q, C-18), 23.5 (q, C-20), 20.0 (t, C-2), 18.4 (t, C-6). EIMS *m/z* (%): 390 ([M]$^+$, 27), 375 (43), 299 (32), 239 (31), 211 (8), 173 (8), 155 (7), 145 (7), 131 (11), 117 (6), 105 (11), 91 (100). HREIMS: 390.2534 (calcd for $C_{27}H_{34}O_2$ [M]$^+$ 390.2559). IR ν_{max} 2930, 2868, 1721, 1457, 1214, 1138, 874, 696 cm^{-1}; UV(EtOH) λ_{max} (log ε): 202.8 (3.67). $[\alpha]_D^{20}$ +27.47 (*c* 0.9, $CHCl_3$).

4.1.19. Preparation of compound 25

The reduction of grandiflorenic acid was achieved following the same experimental procedure described for the obtention of derivative **9**. 61 mg (0.20 mmol) of grandiflorenic acid (**16**) were treated with a large excess of $LiAlH_4$ (250 mg, 32.4 equiv) to obtain 47 mg (81%) of the hydroxylated derivative **25** which showed identical spectroscopic data to those published.[30]

4.1.20. 15α-Hydroxy-kaurenoic acid (27)

To a solution of 172.4 mg of **26** (0.461 mmol) in 3 mL of MeOH was added 1.28 mL of 0.5 M Na_2CO_3 solution (1.5 equiv). The reaction mixture was refluxed for 24 h. Then the MeOH was removed, brine was added to the aqueous solution and it was extracted with DCM (3x 10 mL). The organic phases were collected and dried over anhydrous $MgSO_4$, then were filtered and concentrated. The resulting product (**27**) was obtained as an amorphous white solid (132.0 mg, 90%) and it showed spectroscopic data identical to grandifloric acid.[33]

4.1.21. 15-Oxo-kaurenoic acid (28)

To 149.0 mg of **27** (0.469 mmol) in 10 mL of acetone at 0° C were added 180 μL of Jones reagent. The reaction was followed by TLC and it was quenched after 6 h with 2 mL of isopropanol. The reaction mixture was filtered through florisil and washed several times with AcOEt. Then the solvent was removed and the residue was purified by preparative-TLC using *n*-Hex/AcOEt (30%) to yield 42.3 mg of **28** (29%) as an amorphous white solid. **28** showed identical spectroscopic data to those reported.[17]

4.1.22. Preparation of derivative 29

To a solution of **28** (22.2 mg, 0.070 mmol) in Et_2O was added an excess of ethereal solution of CH_2N_2 (1 mL). The reaction mixture was stirred at room temperature for 5 h. After elimination of solvent 26 mg of **29** were obtained (100%) as amorphous white solid. ^1H NMR (300 MHz, $CDCl_3$) δ: 4.62 (2H, m, H-17a, H-22a), 3.65 (3H, s, H-21), 2.60 (2H, s, H-14), 2.38 (1H, s, H-13), 1.18 (3H, s, H-18), 0.93 (3H, s, H-20), 2.19–0.76 (16H, m).^{13}C NMR (75 MHz, $CDCl_3$) δ: 216.7 (s, C-15), 177.6 (s, C-19), 105.2 (s, C-16), 77.7 (t, C-22), 55.8 (d, C-5), 52.6 (s, C-8), 51.5 (d, C-9), 51.1 (q, C-21), 43.5 (s, C-4), 39.7 (s, C-10), 39.6 (t, C-1), 39.1 (d, C-13), 37.6 (t, C-3), 35.6 (t, C-7), 33.6 (t, C-14), 28.4 (q, C-18), 26.9 (t, C-12), 21.8 (t, C-6), 20.0 (t, C-17), 18.9 (t, C-2), 18.6 (t, C-11), 15.1 (q, C-20). EIMS *m/z* (%): 344 ([M]$^+$-28, 100), 284 (43), 269 (28), 251 (17), 163 (43), 123 (50), 121 (56), 93 (33),. HREIMS: 344.2353 (calcd for $C_{22}H_{32}O_3$ [M]$^+$ −28(N_2) 344.2451). IR ν_{max} 2928, 2870, 1724, 1447, 1232, 1158, 885, 462. UV(EtOH) λ_{max} (log ε): 211.0 (3.33). $[\alpha]_D^{20}$ −436.2 (*c* 0.5 $CHCl_3$,) cm^{-1}.

4.1.23. 15-Acetoxy-kaurenoic acid methyl ester (30)

A solution of 300 mg of compound **30** (0.833 mmol) in 10 mL of ethyl ether was treated with 1 mL of solution of CH_2N_2 in Et_2O. The reaction mixture was left at room temperature until disappearance of the starting material. After elimination of solvent a pale yellow oil was obtained (311.5 mg, 100%). ^1H NMR (300 MHz, $CDCl_3$) δ: 5.23 (1H, s, H-17a), 5.07 (2H, s, H-17b, H-15), 3.61 (3H, s, H-21), 2.76 (1H, s, H-13), 2.04 (3H, s, $OCOCH_3$), 1.13 (3H, s, H-18), 0.80 (3H, s, H-20), 2.16–0.77 (18H, m).^{13}C NMR (75 MHz, $CDCl_3$) δ: 177.6 (s, C-19), 170.7 (s, $OCOCH_3$), 155.1 (s, C-16), 109.7 (t, C-17), 82.6 (d, C-15), 56.5 (d, C-5), 52.5 (d, C-9), 50.9 (q, C-21), 47.1 (s, C-8), 43.4 (s, C-4), 42.2 (d, C-13), 40.3 (t, C-1), 39.3 (s, C-10), 37.6 (t, C-3), 36.9 (t, C-7), 34.4 (t, C-14), 32.3 (t, C-12), 28.4 (q, C-18), 21.0 (q, $OCOCH_3$), 20.6 (t, C-6), 18.8 (t, C-2), 18.1 (t, C-11), 15.3 (q, C-20). EIMS *m/z* (%): 374 ([M]$^+$, 18), 359 (25), 332 (37), 315 (45), 314 (100), 299 (74), 273 (43), 257 (28), 255 (85), 254 (34), 239 (65), 173 (26), 159 (30), 147 (45), 145 (37), 135 (36), 133 (40), 131 (37), 123 (51), 121 (86), 119 (37), 117 (37), 109 (72), 107 (71), 105 (68), 95 (50), 93 (63). HREIMS: 374.2474 (calcd for $C_{23}H_{34}O_4$ [M]$^+$ 374.2457). IR ν_{max} 2935, 2868, 1729, 1462, 1370, 1238, 1192, 1151, 1021, 993, 903 cm^{-1}. UV(EtOH) λ_{max} (log ε): 202.0 (3.48) nm. $[\alpha]_D^{20}$ −53.96 (*c* 1.0, $CHCl_3$).

4.1.24. 16α-Hydroxy-kaurenoic acid methyl ester (32)

Following the same procedure for compound **30**, 145 mg of **31** in Et_2O were treated with an excess of CH_2N_2/Et_2O. The reaction mixture was stirred at room temperature for 15 h. Then the solvent was removed and 151.3 mg (100%) of compound **32** were obtained. **32** showed identical spectroscopic data to those reported.[34]

4.1.25. Preparation of compounds 33 and 34

To a solution of 178.5 mg of **32** (0.534 mmol) in dry DCM were added 46.7 μL of freshly distilled $SOCl_2$ (1.2 equiv) and 52 μL of pyridine (1.2 equiv) under N_2. The reaction mixture was stirred under reflux for 3 h, until disappearance of the starting ester. Then H_2O it was added and the mixture was extracted three times with DCM. The organic phases were combined, dried over $MgSO_4$ and concentrated under reduced pressure. The crude was purified by chromatotron using *n*-hex/AcOEt 2%. 3.6 mg of **34** (2%) was isolated as amorphous white solid and a mixture of compounds **2** and **33**. This mixture was resolved using a silica gel column impregnated with $AgNO_3$, and hexanes/toluene 60% as solvent. Thus, 16.9 mg of **2** (10%) and 42.6 mg of **34** (25%) were isolated as amorphous white solids.

Compound **33**: ^1H NMR (300 MHz, $CDCl_3$) δ: 3.64 (3H, s, H-21), 2.31 (1H, br s, H-13), 1.74 (3H, s, H-17), 1.16 (3H, s, H-18), 0.83 (3H, s, H-20), 2.19–0.87 (20H, m). EIMS *m/z* (%): 352 ([M]$^+$, 1), 316 (48), 301 (19), 273 (26), 257 (50), 241 (29), 187 (29), 149 (51), 133 (18), 123 (37), 121 (37), 109 (36), 106 (53), 105 (53), 97 (31), 94 (100), 91 (48). HREIMS: 352.2177 (calcd for $C_{21}H_{33}O_2{}^{35}Cl$ [M]$^+$ 352.2169).

Compound **34**: ^1H NMR (300 MHz, $CDCl_3$) δ: 5.06 (1H, s, H-15), 3.63 (3H, s, H-21), 2.30 (1H, s, H-13), 1.69 (3H, s, H-17), 1.16 (3H, s, H-18), 0.84 (3H, s, H-20), 2.17–0.73 (18H, m). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 177.9 (s, C-19), 142.3 (s, C-16), 134.9 (d, C-15), 56.6 (d, C-5), 50.8 (q, C-21), 49.0 (s, C-8), 47.8 (d, C-9), 44.5 (d, C-13), 43.6 (s, C-4), 43.6 (t, C-7), 40.6 (t, C-1), 39.4 (s, C-10), 39.3 (t, C-14), 37.9 (t, C-3), 28.5 (q, C-18), 24.7 (t, C-12), 20.7 (t, C-6), 18.9 (t, C-2), 18.7 (t, C-11), 15.2 (q, C-20), 15.0 (q, C-17). EIMS *m/z* (%): 316 ([M]$^+$, 58), 301 (12), 273 (14), 257 (28), 207 (22), 187 (24), 119 (19), 106 (40), 105 (33), 94 (100); 91 (25), 81 (15). HREIMS: 316.2411 (calcd for $C_{21}H_{32}O_2$ [M]$^+$ 316.2402). IR ν_{max} 2928, 2850, 1727, 1443, 1231, 1191, 1156, 814, 671 cm^{-1}. UV(EtOH) λ_{max} (log ε): 205.0 (3.36) nm. $[\alpha]_D^{20}$ −46.92 (*c* 0.9, $CHCl_3$).

4.2. Cell culture conditions

RAW 264.7, mouse leukemic macrophagic cells were maintained in RPMI 1640 medium supplemented with 10% FBS (fetal bovine serum), L-glutamine, and antibiotics. HT-29, human colorectal adenocarcinoma; HepG2, human hepatocellular carcinoma, and HeLa, human cervical adenocarcinoma cells were maintained in DMEM supplemented with 10% FBS, L-glutamine, and antibiotics.

4.3. Assay of cell viability

Cells were incubated in the presence of different concentrations of derivatives for 24 h, before they were then reacted with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) at 37 °C for 4 h. The reaction product, formazan, was extracted with dimethyl sulphoxide (DMSO) and the absorbance was read at 540 nm. Assays were performed in triplicate, and results are expressed as the percent reduction in cell viability compared to untreated control cultures for at least three independent experiments. IC_{50} values refer to the concentration needed to inhibit 50% of cell viability in the presence of the compounds.

4.4. Flow cytometry analysis of apoptosis and necrosis

After treatment with the appropriate stimuli, cells were stained with 0.005% (w/v) propidium iodide (PI) and immediately analyzed in a FACSCanto II flow cytometer (Becton Dickinson) according to a previously described protocol.[35] The percentages of apoptotic and necrotic cells were determined from a dot plot of forward scatter against PI fluorescence.[36,37]

4.5. Cytosolic extracts and Western blot

Cytosolic extracts were prepared as previously described.[38] Protein content was estimated by the Bio-Rad protein assay. Protein extracts were subjected to SDS–PAGE (10–15% gels) and blotted onto polyvinylidene difluoride membranes, which were incubated with the following antibodies: anti-β-actin, anti Bcl-xl, anti-Bcl-2, anti-p53, (all from Santa Cruz Biotechnology), anti-XIAP, anti-IAP-1, and anti-IAP-2 (all from BD Biosciences). After incubation with HRP-conjugated secondary antibody, protein bands were revealed with an enhanced chemiluminescence kit (GE Healthcare). β-Actin was used as a loading control.[38]

4.6. Caspase assays

The activities of caspase-3, -8, and -9 were determined fluorimetrically in cytosolic protein extracts, using the substrates

Ac-DEVD-AMC, Ac-IETD-AFC, and Ac-LEHD-AFC, respectively, according to the suppliers' instructions (BD Biosciences).

4.7. Statistical analysis

The data presented are shown as means ± standard deviations (SD) of three independent experiments. Statistical significance was estimated by Student's *t* test for unpaired observations, with $P < 0.05$ considered significant. For Western blots, a linear correlation was observed between increasing amounts of input protein and signal intensity.

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Supplementary data

Supplementary data (elemental analyses for kaurane derivatives) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.11.064.

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