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Short communication

Novel oleanolic vinyl boronates: Synthesis and antitumor activity

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ABSTRACT

A series of novel oleanane-type pentacyclic triterpenoids bearing a boronate ester moiety at C3 have been synthesized by palladium-catalyzed cross-coupling of bis(pinacolato)diboron with vinyl triflates, in the presence of base, and these compounds were fully characterized by 1D and 2D NMR techniques. Evaluation of their antiproliferative effects on a panel of hematological-based and solid tumor cell lines identified three active oleanolic vinyl boronates that inhibited the growth of leukemia (Jurkat, K562), Burkitt's lymphoma (Jijoye), cervix (Hela), colon (SW480), and ovary (SKOV-3) cancer cells without concomitant inhibition of non-tumoral human fibroblasts. Their mechanisms of action were investigated on the leukemia Jurkat cell line. The results show that the incorporation of boron in the oleanolic acid core combined with the presence of amide bonds afforded compounds with desirable biological effects such as apoptosis induction and inhibition of proteasomal activity on tumor cells, which makes them potential templates for further development in the anticancer drug setting.

1. Introduction

Pentacyclic triterpenoids are secondary plant metabolites found in fruit peel, leaves and stem bark [1–3]. Their potential interest in cancer has now been well demonstrated by the successful clinical utilities of oleanolic acid (OA), glycyrrhetic acid, asiaticoside, and carbenoxolone as market drugs [4]. OA bears an impressive myriad of reported biological activities [5–7] including antibacterial [8], antiparasitic [9], antiosteoporotic [10], antifertility [11], anti-hypertensive [12], antihyperlipidemic [13], antidiabetic [14], immunomodulatory [15], anti-inflammatory [16], antinociceptive [17], gastro and hepatoprotective [18], and antiviral [19]. Chemical modifications of OA to produce derivatives have been successful in improving not only its activity but also its pharmacokinetic properties. Although quite many OA derivatives have now been reported in the literature, none have been synthesized bearing boron. Boron chemistry remained relatively unexploited in the medicinal chemistry arena until the FDA approved the boronic acid proteasome inhibitor bortezomib (Velcade®) for the treatment of multiple myeloma and non-Hodgkin lymphoma, in 2003 [20]. Since then, some promising compounds have been developed and tested among which is PT-100 (Val-boroPro, Talabostat) that targets dipeptidyl peptidases, such as fibroblast activation protein. In 2004, a phase II clinical trial of PT-100 was launched for the treatment of advanced non-small cell lung cancer [21]. However, it has been placed on hold in 2007 due to efficacy issues. The compound has also been recently studied on Phase I and II clinical trials in other cancers including melanoma and brain tumors.

The physical, chemical and biological properties of boron offer medicinal chemists an excellent opportunity to explore and pioneer new areas of drug discovery [22]. Boron is non-toxic, has Lewis acid behavior due to its empty p-orbital, and bears electrophilic character. Its empty p-orbital can be occupied by a lone pair of electrons, allowing it to form dative bonds with biological nucleophiles of enzyme residues (such as serine) and hydroxyl groups

Abbreviations: AA, amino acid; B_2pin_2, bis(pinacolato)diboron; ChT-L, chymotryptic-like; $PdCl_2(PPh_3)_2$, dichlorobis(triphenylphosphine)palladium(II); DMEM, Dulbecco's modified Eagle medium; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DTBMP, di-*t*-butyl-methylpyridine; FCC, flash column chromatography; HMBC, heteronuclear multiple-bond correlation; HMQC, heteronuclear multiple quantum coherence; IR, infra-red; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; PBS, phosphate saline buffer; PI, propidium iodide; Tf_2O, triflic anhydride; THF, tetrahydrofuran; T-L, tryptic-like; SAR, structure–activity relationship.

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from carbohydrates and nucleic acids. Thus, several boron-containing compounds, including analogs of natural products such as β-lactamic antibiotics and curcumin, have been reported to be inhibitors of serine and aspartic proteases, metalloproteases, γ-glutamyl transpeptidase, arginase, surfactin synthetase C-terminal thioesterase, cysteine proteases, tyrosine kinases, as well as to be threonine-based inhibitors [22]. In continuation of our efforts to design new triterpenoids as anticancer agents [2,23–29], we have successfully prepared a series of novel oleanane-type pentacyclic triterpenoids bearing a boronate ester moiety. We have screened the compounds for cytotoxic activity against a panel of hematological-based and solid tumor cell lines and against non-tumoral human fibroblasts. We have also studied their effects on cell cycle, ability to induce apoptosis and to inhibit proteasomal activity in leukemia Jurkat cells.

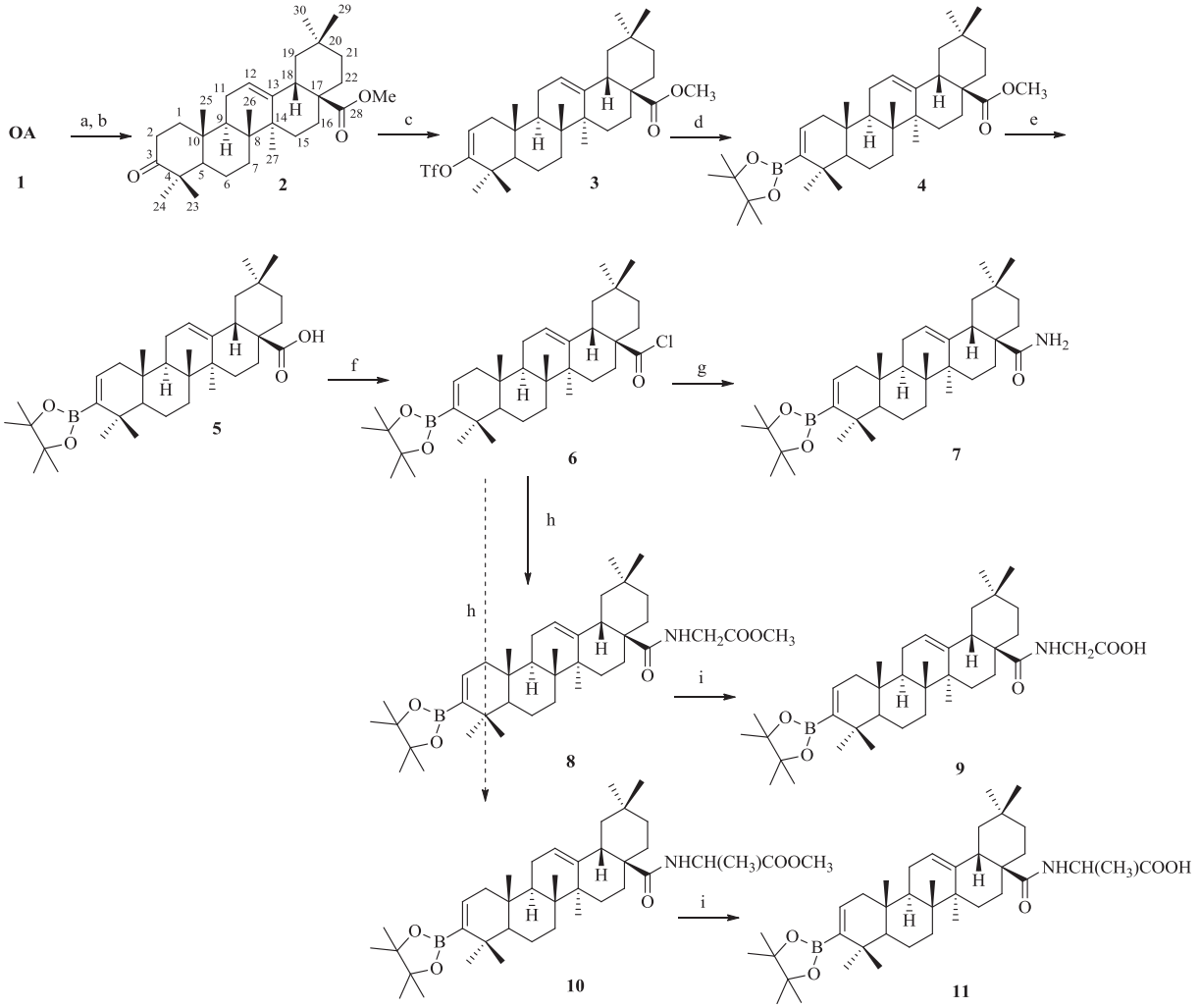
2. Results and discussion

2.1. Chemistry

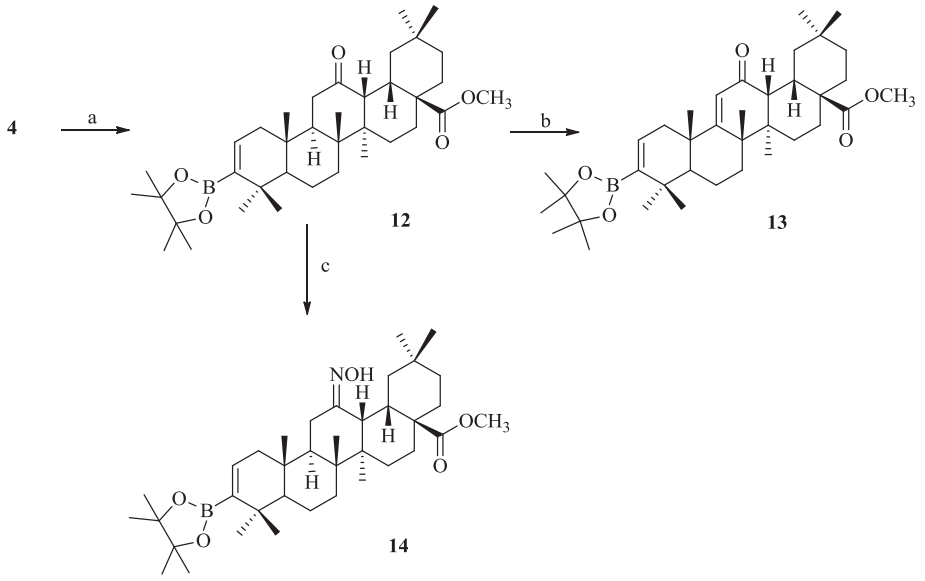
The synthesis of the oleanolic vinyl boronates **4**–**11** started from oleanolic acid **1** (Scheme 1) and exploited the palladium-catalyzed cross-coupling reaction of bis(pinacolato)diboron (B_2pin_2) with 1-alkenyl (vinyl) triflates in the presence of di-*t*-butyl-methylpyridine (DTBMP) [30]. The 28-carboxyl group was first protected as the methyl ester [31] to afford compound **2** which was further reacted with triflic anhydride (Tf_2O) in the presence of base to give the vinyl triflate intermediate **3**, in 83% yield after purification by flash column chromatography (FCC) [32,33]. This compound was coupled with bis(pinacolato)diboron using bis(triphenylphosphine)palladium(II) chloride ($PdCl_2(PPh_3)_2$) as catalyst to afford the oleanolic vinyl boronate **4**, in 89% yield.

Deprotection of the carboxylate ester was then performed using lithium iodide in refluxing DMF to give compound **5** [34]. Reaction of compound **5** with oxalyl chloride gave the acyl chloride intermediate **6** [35] which was reacted either with ammonia or with an amino acid methyl ester hydrochloride to afford compounds **7**, and **8** and **10**, respectively [36]. Deprotection of the carboxyl group of the amino acid side chain was finally performed by alkaline hydrolysis and compounds **9** and **11** were obtained in 98% yield. The synthesis of the oleanolic vinyl boronates **12**–**14** started from compound **4** (Scheme 2).

Thus, oxidation of compound **4** with the $KMnO_4/FeSO_4 \cdot nH_2O$ system [37] gave the 12-oxo derivative **12**, in 92% yield, which by reaction with bromine and hydrobromic acid in refluxing acetonitrile afforded the enone **13** [38]. The oxime **14** was prepared by reaction of compound **4** with hydroxylamine hydrochloride in pyridine, at reflux [39]. Reaction of oleanonic acid **15** with oxalyl chloride afforded the acyl chloride derivative intermediate **16** [35] which was reacted with ammonia to give the amide **17** (Scheme 3).



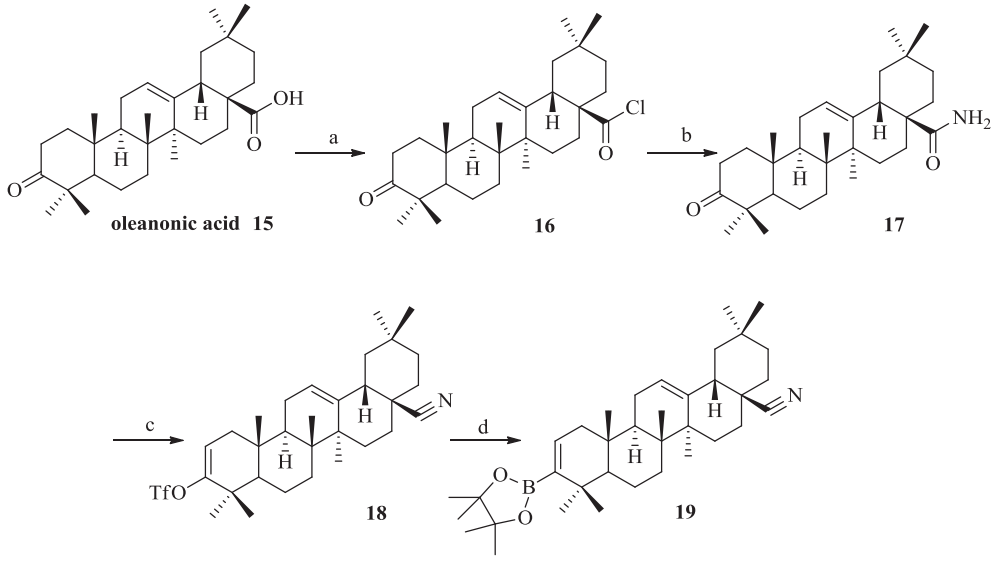
Scheme 1. Synthesis of the oleanolic boronates **4**–**11**. Reagents and conditions: a. Jones reagent; b. CH_3I, K_2CO_3, DMF, r.t.; c. Tf_2O, DTBMP, CH_2Cl_2, reflux; d. B_2pin_2, $PdCl_2(PPh_3)_2$, Na_2CO_3, THF, reflux; e. LiI, DMF, reflux; f. $(COCl)_2$, $CHCl_3$, r.t.; g. NH_3 (aq), THF, 0 °C; h. AA methyl ester hydrochloride, NEt_3, CH_2Cl_2, r.t.; i. NaOH (4 N), THF:MeOH, r.t.



Scheme 2. Synthesis of the oleanolic boronates **12**–**14**. Reagents and conditions: a. $KMnO_4/FeSO_4{\cdot}nH_2O$, *t*-BuOH, H_2O, CH_2Cl_2, r.t.; b. HBr/Br_2, CH_3CN, reflux; c. $NH_2OH.HCl$, pyr, reflux.

Reaction of **17** with triflic anhydride in the presence of base afforded the oleanolic vinyl triflate **18** bearing a nitrile group at C-28 [40]. Reaction of compound **18** with bis(pinacolato)diboron in the presence of bis(triphenylphosphine)palladium(II) chloride afforded compound **19**, in 91% yield.

Elucidation of the final structures of the synthesized compounds was performed by infra-red (IR), 1D and 2D nuclear magnetic resonance (NMR) spectroscopy. The specific IR absorptions for the asymmetric and symmetric SO_2-vibrations of the oleanolic vinyl triflates **3** and **18** were observed around 1400–1430 and 1200–1250 cm^{-1} as well as the respective C–F absorptions at 1190–1130 cm^{-1}. The 2-H signal was seen as a double doublet at 5.56 and 5.57 ppm, on the ^1H NMR spectra of compounds **3** and **18**, respectively. In addition, the ^{13}C NMR signal for the CF_3 carbon was observed as a pair of peaks at 116.8 and 120 ppm with the coupling constant of 319 Hz [41,42]. For the oleanolic vinyl boronates **4**–**14** and **19**, the 2-H multiplet was consistently found at 6.35–6.43 ppm on the ^1H NMR spectra. The pinacolborane ring methyl group protons were observed as a singlet at 1.24–1.25 ppm on the ^1H NMR spectra of the compounds [30]. The corresponding methyl carbon signals were found at 24.6 and 24.8 ppm whereas the signals for the two quaternary carbons of the pinacolborane ring were found at 82.6–82.8 ppm. No signal for the quaternary carbon attached to the boron atom was ever observed on the ^{13}C NMR spectra of the oleanolic vinyl boronates **4**–**14** and **19**. COSY analysis of compound **10** showed the expected correlations between the proton signals of the amino acid side chain. Thus, correlations were found for the amide proton signal at 6.60 ppm and the signal at 4.45 ppm belonging to the single proton of the adjacent carbon. This signal at 4.45 ppm also correlated with the signal at 1.37 ppm belonging to the methyl protons of the same carbon. Analysis of the heteronuclear multiple quantum coherence (HMQC) spectroscopy



Scheme 3. Synthesis of the oleanolic boronate **19**. Reagents and conditions: a. $(COCl)_2$, $CHCl_3$, r.t.; b. NH_3 (aq), THF, 0 °C; c. Tf_2O, DTBMP, CH_2Cl_2, reflux; d. B_2pin_2, $PdCl_2(PPh_3)_2$, Na_2CO_3, THF, reflux.

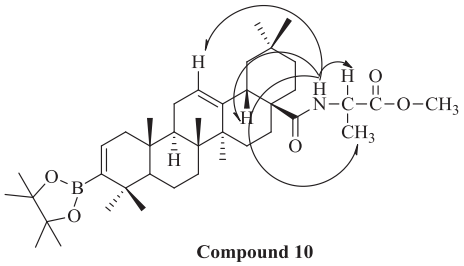


Fig. 1. NOESY correlations of the amide proton of the side chain of compound **10**.

spectra allowed the assignment of C12 at 123.5 ppm and C2 at 138.5 ppm as well as confirmation of the methyl carbon signals of the pinacolborane ring which were found at 24.6 and 24.7 ppm. The correlation observed on the heteronuclear multiple-bond correlation (HMBC) spectroscopy spectrum of compound **10** between the 18β-H signal at 2.63 ppm and the ^{13}C signal at 177.5 ppm allowed the assignment of this signal to C28. The signal of the 18β-H also displayed the expected correlations with the signals of C12 and C13 (143.6 ppm). The other quaternary ^{13}C signal at 173.6 ppm was assigned to the carboxylic group carbon of the methyl ester side chain. Consistently, this signal displayed correlations with all the proton signals of the amino acid side chain. The nuclear Overhauser effect spectroscopy (NOESY) spectrum of compound **10** displayed the selected correlations shown in Fig. 1 for the amide proton of the amino acid side chain. Similar correlations were seen after 2D NMR analysis of compound **11**.

2.2. Biology

The ability of the novel synthesized oleanolic vinyl boronates **4**–**14** and **19** and of oleanolic acid **1** to inhibit the proliferation of a panel of tumor cell lines comprising both hematological-based (Jurkat, K562, and Jijoye) and solid (Hela, SKOV-3, and SW480) tumor cells was evaluated by MTT assay (Table 1). Compounds **7**, **9**, and **11** were active. Thus, compounds **9** and **11** inhibited the proliferation of the tumor cell lines tested with IC_{50} values ranging from 13.4 to 28 μM and 19.4 to 31.2 μM, respectively. In addition, this inhibition of proliferation was selective for malignant cells seeing that the compounds were inactive ($IC_{50} > 50$ μM) on non-tumoral human fibroblasts. A very interesting cytotoxicity profile was observed for compound **7**. The compound preferentially inhibited the proliferation of hematological-based cell lines with IC_{50} values of 21.6, 8.3, and 8.4 μM for Jurkat, K562, and Jijoye cells, respectively, when compared to solid tumor cell lines. Thus, an IC_{50} value of 34.7 μM could be determined for Hela cells alone with compound **7** (Table 1). Moreover, its effects on cell proliferation were also selective as the compound was inactive against the human fibroblasts.

This cytotoxicity screening allowed us to not only identify the active vs. inactive compounds but also provided important structure–activity relationships (SAR) information. Only the oleanolic vinyl boronates bearing an amide bond at the C28 position **7**, **9**, and **11** were active. All other modifications at C28 such as the methyl ester, the nitrile or even the free carboxyl group, or at ring C such as the 12-oxo group, the enone and the oxime moieties, combined with the vinylic boronate structure of ring A failed to provide compounds with cytotoxic activity. In addition, for compounds **9** and **11**, bearing an amino acid residue at C28, activity was only observed when the terminal carboxyl group was unprotected. Thus, the corresponding methyl ester amino acid derivatives **8** and **10** were inactive against the cell lines tested. We next examined the effects of the active compounds on apoptosis. We found that treatment of Jurkat cells with 20 μM of compounds **7**, **9** and **11** resulted in activation of the executioner caspases 3 and 7 (Fig. 2A).

Accordingly, expansion of a sub-G_0 fraction on the cell cycle profile after propidium iodide (PI) staining which was consistent with the increase in the percentage of cells in late apoptosis/necrosis observed after double staining occurred (Fig. 3A and B, respectively). Treatment of Jurkat cells with 20 μM of compound **7** overnight did not, however, produce significant changes in the cell cycle (data not shown).

To try to further shed some light into the possible mechanisms of action of compounds **7**, **9** and **11**, we decided to investigate their effects on proteasomal activity [43]. Although the vast majority of proteasome inhibitors are small peptides (either boron-containing or not) [44–47], a number of natural products including tyropeptin A, epoxomicin, lactacystin, salinosporamide A, belactosins, argyrin A, and withaferin A, chalcones [48] as well as some pentacyclic triterpenoids [49–56] have also been reported to inhibit the proteasome. A first screening using proteasomes semi-purified from Jurkat cells pellets revealed that compound **9** inhibited the chymotrypsin-like (ChT-L) proteasomal activity at both 50 and 100 μM, in 36 and 41%, respectively (Table 2). The trypsin-like (T-L) activity was only inhibited at 100 μM by this compound, in about 50%. Compound **11** was an effective inhibitor of both proteasomal activities only at 100 μM, whereas compound **7** was not active at the concentrations tested. Thus, although an amide bond at C28 common to all the compounds was important for the cytotoxic activity of the oleanolic vinyl boronates, in order to have proteasome inhibition the presence of the amino acid moiety seemed mandatory.

The effects on compound **9** and **11** on the proteasomes of intact Jurkat cells were also examined (Fig. 4). The natural product epoxomicin, a potent inhibitor of the ChT-L activity of the proteasome [57] was used as a positive control. After pre-incubation of intact Jurkat cells with compounds **9** and **11** at 50 μM, inhibition of the ChT-L and T-L activities of the proteasome occurred (Fig. 4A and B). Impairment of the ChT-L activity was more pronounced after treatment with compound **9** than with compound **11** (25 and 37% of control, respectively), and the effect of the compounds on the T-L activity was similar. Additional confirmation of proteasomal inhibition was observed by accumulation of ubiquitinated proteins after incubation of Jurkat cells with compounds **9** and **11** (Fig. 2B).

Table 1
Cell viability and proliferation assay for the oleanolic boronates **7**, **9**, and **11**.

Compound	Cell line (IC_{50}, μM)[a,b]						
	Jurkat	K562	Jijoye	Hela	SKOV-3	SW480	Human fibroblasts
7	21.6 ± 2.6	8.3 ± 1.8	8.4 ± 2.9	34.7 ± 2.1	> 50	> 50	>50
9	15.5 ± 2.4	18.2 ± 0.4	13.4 ± 2.6	28.0 ± 0.9	15.4 ± 2.1	17.9 ± 1.8	>50
11	21.4 ± 2.9	20.1 ± 0.2	19.4 ± 0.4	30.5 ± 1.1	31.2 ± 2.4	28.7 ± 2.1	>50

[a] IC_{50} is the concentration that inhibits 50% of cellular growth. IC_{50} values were determined by MTT assay, after 48 h of treatment. The reported values are expressed as a mean ± SD of three independent experiments.
[b] IC_{50} values for compounds **4**, **5**, **8**, **10**, **12**–**14**, and **19** (>50 μM).

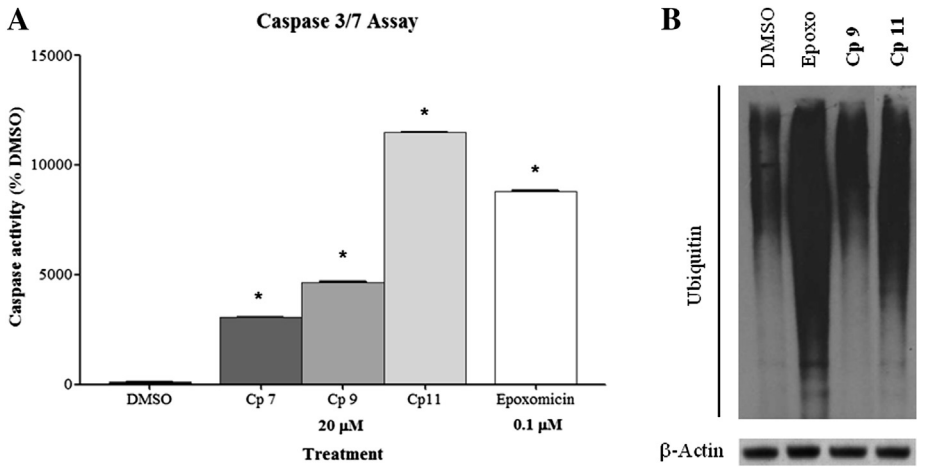


Fig. 2. A. Caspase 3/7 activation after overnight treatment of Jurkat cells with compounds **7**, **9**, **11** (20 μM), and epoxomicin (0.1 μM). A representative example of three independent experiments is shown. One-way ANOVA with a Dunnett post-test was used to analyze the data: $^{*}p < 0.01$ vs. control. B. Effect of compounds **9** and **11** on the levels of poly-ubiquitinated proteins in Jurkat cells. Jurkat cells were exposed to the compounds (50 μM). Lysates were prepared and assayed for ubiquitinated protein levels by western blot. Epoxomicin (0.1 μM) was used as positive control. A representative example of three independent experiments is shown.

These results show that compounds **9** and **11** are apoptosis inducers and inhibit the proteasomal activity of Jurkat cells pellet and are in addition cell-permeable bearing a similar effect on the proteasomes of intact Jurkat cells. Nonetheless, the fact that this proteasomal inhibition is somewhat weaker than that of cell viability and proliferation suggests that additional targets may be important for the activity of these compounds.

3. Conclusions

We herein report the synthesis and structural elucidation of a series of novel oleanane-type pentacyclic triterpenoids bearing a boronate ester moiety, and the study of their antitumoral effects. We have found that the oleanolic vinyl boronates bearing an amide bond at C28 **7**, **9**, and **11** could inhibit the proliferation of the solid- and hematological-based tumor cell lines tested and that this inhibition was selective toward tumor cell lines. In addition, the compounds induced caspase-dependent apoptosis of leukemia Jurkat cells. Further insights on their possible mechanisms of action have been provided. Compounds **9** and **11**, bearing an amino acid moiety at C28, were inhibitors of the proteasomal activity of both Jurkat cells pellets and intact Jurkat cells. To the best of our knowledge, our results show for the first time the potential of boron-containing oleanane-type derivatives as templates that can be further modified in the pursuit of anticancer agents. Efforts are currently underway to optimize these compounds and further precise their mechanisms of action.

4. Experimental

4.1. Chemistry

All reagents were obtained from Sigma–Aldrich Co. For thin layer chromatography (TLC) analysis, Kieselgel 60HF254/Kieselgel 60G was used. IR spectra were obtained using a JASCO FT/IR-420 spectrophotometer (FTIR-ATR). NMR spectra were obtained using a Brucker Digital NMR—Avance 400 apparatus spectrometer, in $CDCl_3$ with Me_4Si as the internal standard. NMR data was obtained at the Nuclear Magnetic Resonance Laboratory of the Coimbra Chemistry Centre (www.nmrccc.uc.pt), Universidade de Coimbra, supported in part by grant REEQ/481/QUI/2006 from FCT, POCI-2010 and FEDER, Portugal. Mass spectra were recorded on a Finnigan PolarisQGC/MS Benchtop Ion Trap mass spectrometer. Melting points were recorded on a BUCHI Melting point B-540 apparatus and are uncorrected. The purity (>95%) of the tested compounds was determined by elemental analysis performed at the Centro de Apoio Científico e Tecnolóxico á Investigación (C.A.C.T.I.), Universidade de Vigo, Campos Lagoas – Marcosende, 15, 36310 Vigo.

4.2. Methyl 3-oxoolean-12-en-28-oate (*2*)

Compound **2** was prepared according to the literature staring from OA (**1**) [31].

4.3. Methyl oleana-2,12-dien-28-oate 3-trifluoromethanesulfonate (*3*)

To a solution of compound **2** (1.0 g, 2.14 mmol) in dichloromethane (20 mL) stirred at room temperature under N_2 atmosphere Tf_2O (438 μL, 2.6 mmol) was added followed by DTBMP (616 mg, 3 mmol). The solution darkened and temperature was then raised to reflux. After 4 h 30 the reaction was complete and water (75 mL) and ethyl acetate (150 mL) were added with stirring. The aqueous phase was extracted twice with ethyl acetate (2 × 100 mL) and washed with 10% aqueous HCl (2 × 50 mL), 10% aqueous $NaHCO_3$ (2 × 50 mL), 10% aqueous Na_2SO_3 (2 × 50 mL), water (50 mL) and brine (50 mL), dried over anhydrous Na_2SO_4, filtered, and the solvent was removed under reduced pressure to afford a yellow oil which turned into a yellow solid with drying (1.20 g). The solid was purified by FCC using ethyl acetate:petroleum ether 1:10 to afford compound **3** (1.06 g; 83%). Mp (ethyl acetate:petroleum ether 40–60): 98–100 °C. IR 1727, 1412, 1244, 1209, 1142 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.77 (s, 3H), 0.90 (s, 3H), 0.93 (s, 3H), 0.99 (s, 3H), 1.03 (s, 3H), 1.13 (s, 6H), 2.87 (m, 1H, 18β-H), 3.62 (s, 3H, 28-OCH_3), 5.31 (m, 1H, 12-H), 5.56 (dd, 1H, 2-H, $J_1 = 1.77$ Hz and $J_2 = 6.60$ Hz); ^{13}C NMR δ 113.7 (C2), 116.8 and 120 ($J = 319$ Hz, CF_3), 122 (C12), 143.8 (C13), 155.4 (C3), 178.2 (C28). EI-MS m/z (%) 600 (3) M^+, 262 (23), 248 (20), 203 (100), 189 (66), 133 (43), 119 (23), 105 (17). Anal. Calcd. for $C_{32}H_{47}F_3O_5S$: C, 63.97; H; 7.89. Found: C, 63.57; H, 7.84.

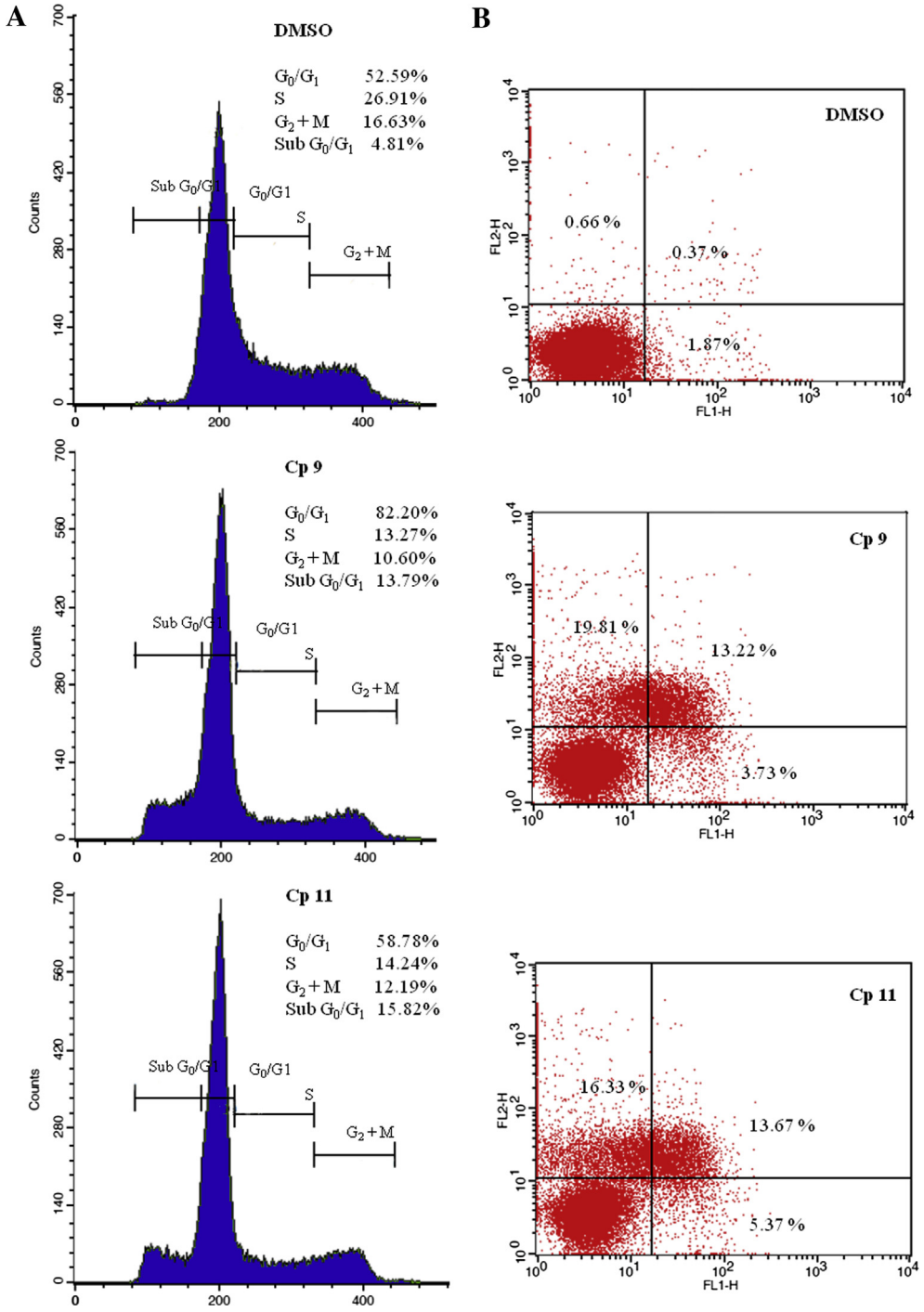


Fig. 3. Effect of compounds **9** and **11** on the cell cycle. FACS analysis by single (PI alone, *A*) staining and double (PI and annexin V, B) staining of Jurkat cells after overnight treatment with DMSO or compounds **9** and **11** at 20 μM. A representative example of three independent experiments is shown.

*4.4. Methyl 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-oleana-2,12-dien-28-oate (**4**)*

To a mixture of compound **3** (600 mg, 1 mmol) and $PdCl_2(PPh_3)_2$ (50 mg, 0.07 mmol), THF (12 mL) was added at room temperature followed by B_2pin_2 (380 mg, 1.5 mmol). A 0.8 M solution of Na_2CO_3 (4.8 mL; 3.84 mmol) was then charged dropwise into the reaction flask. The solution darkened and temperature was then raised to reflux. After 1 h the reaction was complete. The reaction mixture was concentrated under reduced pressure and ethyl acetate (200 mL) and water (50 mL) were added. The aqueous phase was further extracted with ethyl acetate (2 × 100 mL). The organic phase was then filtered through a Celite pad, washed with 10% aqueous HCl (50 mL), 10% aqueous $NaHCO_3$ (50 mL), 10% aqueous Na_2SO_3 (50 mL), water (50 mL) and brine (50 mL), dried over anhydrous Na_2SO_4, filtered, and the solvent was removed under reduced pressure to afford compound **4** (516 mg, 89%) as a yellow solid. Mp (THF:methanol): 208–210 °C. IR 1726, 1622 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.76 (s, 3H), 0.89 (s, 6H), 0.92 (s, 3H), 1.03 (s, 3H), 1.08 (s, 3H), 1.13 (s, 3H), 1.24 (s, 12H, pinacol-[$C(CH_3)_2]_2$), 2.87 (m, 1H, 18β-

Table 2
Proteasomal activity inhibition screening with the oleanolic boronates **7**, **9**, and **11**.

Compound	Proteasomal activity (%)[a,b]					
	ChT-L activity			T-L activity		
	25 μM	50 μM	100 μM	25 μM	50 μM	100 μM
7	95.8 ± 3.8	92.4 ± 3.4	85.6 ± 2.7	81.6 ± 1.1	77.9 ± 1.9	72.5 ± 2.9
9	83.6 ± 2.7	64 ± 2.1	59.3 ± 2.1	85.3 ± 1.2	84.1 ± 2.8	51.4 ± 2.1
11	91.7 ± 2.8	84.3 ± 2.2	47.2 ± 2.9	85.1 ± 1.8	70.7 ± 2.1	50.8 ± 2.3

[a] Semi-purified proteasomes from Jurkat cells pellets. Values are expressed in % of control (DMSO) and represent residual proteasome activity after treatment with the compounds. The reported values are expressed as a mean ± SD of three independent experiments.
[b] After treatment with MG132 (10 μM), used as a positive control, ≤20% of both ChT-L and T-L activities remained.

H), 3.62 (s, 3H, 28-OCH_3), 5.31 (m, 1H, 12-H), 6.37 (m, 1H, 2-H); ^{13}C NMR δ 82.6 (pinacol-$[C(CH_3)_2]_2$), 122.6 (C12), 138.7 (C2), 143.6 (C13), 178.3 (C28); EI-MS *m/z* (%) 578 (3) M^+, 315 (23), 262 (44), 203 (100), 189 (19), 173 (16), 133 (24), 119 (13). Anal. Calcd. for $C_{37}H_{59}BO_4$: C, 76.80; H, 10.28. Found: C, 77.07; H, 10.68.

*4.5. 3-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)-oleana-2,12-dien-28-oic acid (**5**)*

To a mixture of compound **4** (200 mg, 0.35 mmol) and lithium iodide (816 mg, 6.1 mmol), dry DMF (4 mL) was added under N_2 atmosphere and the mixture was heated to reflux. After 52 h the reaction was complete and water (25 mL) and ethyl acetate (75 mL) were added with stirring. The aqueous phase was extracted twice with ethyl acetate (2 × 50 mL) and washed with 10% aqueous HCl (25 mL), water (25 mL) and brine (25 mL), dried over anhydrous Na_2SO_4, filtered, and the solvent was removed under reduced pressure to afford a yellow solid (215 mg). The compound was purified by FCC using ethyl acetate:petroleum ether 40–60 (1: 10) to afford compound **5** (120 mg; 62%). Mp (acetone:*n*-hexane): 319–321 °C. IR 3377, 1697, 1622 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.78 (s, 3H), 0.89 (s, 6H), 0.92 (s, 3H), 1.00 (s, 3H), 1.07 (s, 3H), 1.13 (s, 3H), 1.25 (s, 12H, pinacol-$[C(CH_3)_2]_2$), 2.81 (m, 1H, 18β-H), 5.30 (m, 1H, 12-H), 6.38 (m, 1H, 2-H); ^{13}C NMR δ 82.6 (pinacol-$[C(CH_3)_2]_2$), 122.8 (C12), 138.8 (C2), 143.4 (C13), 184.2 (C28). EI-MS *m/z* (%) 565 (2) M^+, 316 (18), 248 (64), 203 (100), 187 (21), 133 (50), 119 (30), 91 (19). Anal. Calcd. for $C_{36}H_{57}BO_4$: C, 76.58; H, 10.17. Found: C, 76.51; H, 10.57.

*4.6. 3-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)-oleana-2,12-dien-28-oyl chloride (**6**)*

Oxalyl chloride (0.36 mL; 4.16 mmol) was added dropwise to a solution of compound **5** (90 mg; 0.16 mmol) in chloroform (2 mL), at room temperature, and the resulting mixture was stirred for 30 min. The solvent was evaporated to dryness and the resulting solid was washed with *n*-hexane (3 × 1 mL). The product was used without further purification for the following steps. Compound **6**: 90 mg; 97%. Mp 287–289 °C. IR 1785, 1367 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.81 (s, 3H), 0.90 (s, 3H), 0.91 (s, 3H), 0.92 (s, 3H), 1.03 (s, 3H), 1.08 (s, 3H), 1.15 (s, 3H), 1.25 (s, 12H, pinacol-$[C(CH_3)_2]_2$), 2.83 (m, 1H, 18β-H), 5.36 (m, 1H, 12-H), 6.38 (m, 1H, 2-H); ^{13}C NMR δ 82.7 (pinacol-$[C(CH_3)_2]_2$), 124 (C12), 138.6 (C2), 141.8 (C13), 179.8 (C28). EI-MS *m/z* (%) 582 (2) M^+, 518 (20), 214 (15), 201 (95), 189 (100), 144 (63), 118 (69), 90 (55). Anal. Calcd. for $C_{36}H_{56}BClO_3$: C, 74.15; H, 9.68. Found: C, 74.36; H, 9.96.

*4.7. 3-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)-oleana-2,12-dien-28-amide (**7**)*

A solution of compound **6** (400 mg, 0.68 mmol) in THF (20.8 mL) was added dropwise to ammonia (0.4 mL) cooled in an ice bath, under magnetic stirring. After 3 h the reaction was complete. The reaction mixture was evaporated to dryness and ethyl acetate (125 mL) and water (25 mL) were added. The aqueous phase was further extracted with ethyl acetate (2 × 50 mL) and the resulting organic phase was washed with 10% aqueous HCl (25 mL), 10% aqueous $NaHCO_3$ (25 mL), water (25 mL) and brine (25 mL), dried over anhydrous Na_2SO_4, filtered, and the solvent was removed under reduced pressure to afford a brown solid (355 mg; 92%) which was purified by FCC using ethyl acetate:petroleum ether 40–60 4:1 to 2:1 as eluent, to afford compound **7** (220 mg; 62%). Mp (ethyl acetate:petroleum ether 40–60): 294–296 °C. IR 3160, 1670, 1362 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.86 (s, 3H), 0.90 (s, 3H), 0.90 (s, 3H), 0.91 (s, 3H), 1.03 (s, 3H), 1.08 (s, 3H), 1.18 (s, 3H), 1.25 (s, 12H, pinacol-$[C(CH_3)_2]_2$), 2.52 (m, 1H, 18β-H), 5.40 (m, 1H, 12-H), 5.70 (br s, 1H), 5.97 (br s, 1H), 6.37 (m, 1H, 2-H); ^{13}C NMR δ 82.7 (pinacol-$[C(CH_3)_2]_2$), 123.2 (C12), 138.4 (C2), 144.6 (C13), 181.8 (C28). EI-MS *m/z* (%) 563 (4) M^+, 259 (18), 246 (20), 202 (100), 188 (27), 132 (48), 118 (26), 104 (20). Anal. Calcd. for $C_{36}H_{58}BNO_3$: C, 76.71; H, 10.37; N, 2.48. Found: C, 77.01; H, 10.83; N, 2.59.

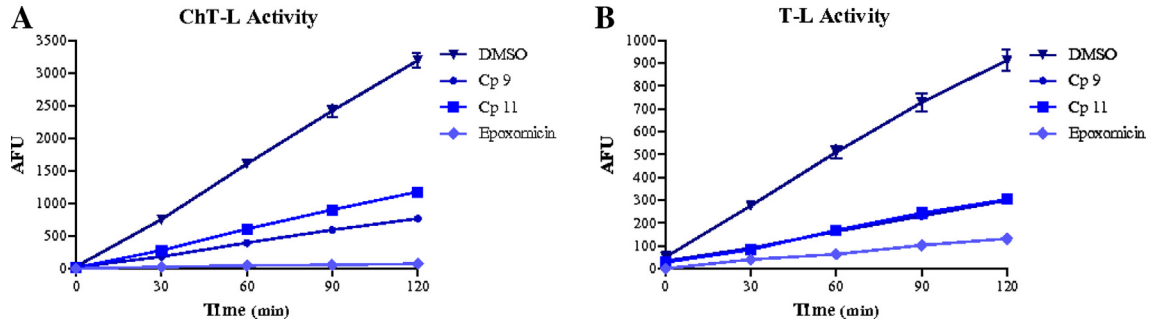


Fig. 4. Inhibition of the ChT-L (A) and T-L (B) proteasomal activities after pre-treatment of Jurkat cells with compounds **9** and **11** (50 μM), or epoxomicin (0.1 μM), for 30 min, followed by collection of the pellet and semi-purification of the proteasome. A representative example of three independent experiments is shown (AFU = arbitrary fluorescence units).

4.8. Methyl N-[3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-oleana-2,12-dien-28-oyl]glycinate (**8**)

To a solution of compound **6** (600 mg; 1.02 mmol) and glycine methyl ester hydrochloride (174 mg; 1.38 mmol) in dichloromethane (6.5 mL), triethylamine (0.3 mL; 2.22 mmol) was added, and the mixture was stirred at room temperature, for 6 h. Additional glycine methyl ester hydrochloride (58 mg; 0.46 mmol), triethylamine (0.1 mL; 0.74 mmol), and dichloromethane (0.5 mL) were then added and after 2 h the reaction was complete. The reaction mixture was evaporated to dryness and ethyl acetate (150 mL) and water (30 mL) were added. The aqueous phase was further extracted with ethyl acetate (2 × 75 mL) and the resulting organic phase was washed with 10% aqueous HCl (30 mL), 10% aqueous $NaHCO_3$ (30 mL), water (30 mL) and brine (30 mL), dried over anhydrous Na_2SO_4, filtered, and the solvent was removed under reduced pressure to afford a brown solid (595 mg; 91%) which was purified by FCC using ethyl acetate:petroleum ether 40–60 from 6:1 to 3:1 to afford compound **8** (385 mg; 65%). Mp (ethyl acetate:petroleum ether 40–60): 149–151 °C. IR 1752, 1646, 1362 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.73 (s, 3H), 0.88 (s, 3H), 0.90 (s, 3H), 0.91 (s, 3H), 1.01 (s, 3H), 1.07 (s, 3H), 1.16 (s, 3H), 1.24 (s, 12H, pinacol-$[C(CH_3)_2]_2$), 2.61 (m, 1H, 18β-H), 3.78 (s, 3H, OCH_3), 3.82 and 4.13 (m, 1H and m, 1H, $-NCH_2-$), 5.48 (m, 1H, 12-H), 6.37 (m, 1H, 2-H), 6.55 (m, 1H, NH); ^{13}C NMR δ 82.7 (pinacol-$[C(CH_3)_2]_2$), 123.6 (C12), 138.5 (C2), 144.1 (C13), 170.5 ($COOCH_3$), 178.4 (C28). EI-MS *m/z* (%) 635 (30) M^+, 162 (26), 158 (35), 144 (47), 132 (100), 118 (93), 104 (67), 100 (53). Anal. Calcd. for $C_{39}H_{62}BNO_5$: C, 73.68; H, 9.83; N, 2.20. Found: C, 73.70; H, 10.23; N, 2.30.

4.9. N-[3-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)-oleana-2,12-dien-28-oyl] glycine (**9**)

To a solution of compound **8** (150 mg; 0.24 mmol) in methanol (1.1 mL) and THF (1.5 mL), NaOH 4 N (0.6 mL; 2.4 mmol) was added and the reaction mixture was stirred, at room temperature, for 1 h. The reaction mixture was evaporated to dryness and ethyl acetate (100 mL) and water (25 mL) were added. The aqueous phase was further extracted with ethyl acetate (2 × 50 mL) and the resulting organic phase was washed with 10% aqueous HCl (25 mL), 10% aqueous $NaHCO_3$ (25 mL), water (25 mL) and brine (25 mL), dried over anhydrous Na_2SO_4, filtered, and the solvent was removed under reduced pressure to afford compound **9** (144 mg; 98%). Mp (ethyl acetate:petroleum ether 40–60): 268–270 °C. IR 1733, 1651, 1366 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.74 (s, 3H), 0.88 (s, 3H), 0.90 (s, 6H), 1.02 (s, 3H), 1.08 (s, 3H), 1.17 (s, 3H), 1.25 (s, 12H, pinacol-$[C(CH_3)_2]_2$), 2.57 (m, 1H, 18β-H), 3.89 and 4.09 (m, 1H and m, 1H, $-NCH_2-$), 5.49 (m, 1H, 12-H), 6.37 (m, 1H, 2-H), 6.77 (m, 1H, NH); ^{13}C NMR δ 82.7 (pinacol-$[C(CH_3)_2]_2$), 124 (C12), 138.5 (C2), 143.8 (C13), 172.2 (COOH), 179.8 (C28). EI-MS *m/z* (%) 621 (6) M^+, 162 (20), 158 (24), 144 (30), 132 (100), 118 (70), 100 (50), 94 (35). Anal. Calcd. for $C_{38}H_{60}BNO_5$: C, 73.41; H, 9.73; N, 2.25. Found: C, 73.19; H, 10.07; N, 2.29.

4.10. Methyl N-[3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-oleana-2,12-dien-28-oyl]alaninate (**10**)

The method followed that of compound **8** using compound **6** (600 mg; 1.02 mmol), L-alanine methyl ester hydrochloride (192 mg; 1.38 mmol), dichloromethane (6 mL) and triethylamine (0.3 mL; 2.22 mmol), at room temperature, for 6 h, after which additional L-alanine methyl ester hydrochloride (64 mg; 0.46 mmol), dichloromethane (0.5 mL) and triethylamine (0.1 mL; 0.74 mmol) were added, and after 2 h the reaction was complete and afforded a brown solid (600 mg; 90%) which was purified by FCC using ethyl acetate:petroleum ether 40–60 from 6:1 to 3:1 to afford compound **10** (400 mg; 67%). Mp (ethyl acetate:petroleum ether 40–60): 209–211 °C. IR 1741, 1663, 1366 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.72 (s, 3H), 0.87 (s, 3H), 0.90 (s, 3H), 0.91 (s, 3H), 1.01 (s, 3H), 1.07 (s, 3H), 1.16 (s, 3H), 1.24 (s, 12H, pinacol-$[C(CH_3)_2]_2$), 1.37 (d, 3H, $-NCH(CH_3)-$), 2.63 (m, 1H, 18β-H), 3.73 (s, 3H, OCH_3), 4.45 (m, 1H, $-NCH(CH_3)-$), 5.46 (m, 1H, 12-H), 6.37 (m, 1H, 2-H), 6.60 (m, 1H, NH); ^{13}C NMR δ 82.7 (pinacol-$[C(CH_3)_2]_2$), 123.5 (C12), 138.5 (C2), 143.6 (C13), 173.6 ($COOCH_3$), 177.5 (C28). EI-MS *m/z* (%) 649 (20) M^+, 189 (27), 158 (33), 144 (45), 132 (100), 118 (95), 104 (80), 90 (67). Anal. Calcd. for $C_{40}H_{64}BNO_5$: C, 73.94; H, 9.93; N, 2.16. Found: C, 74.19; H, 10.25; N, 2.22.

4.11. N-[3-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)-oleana-2,12-dien-28-oyl]alanine (**11**)

The method followed that of compound **9** using compound **10** (150 mg; 0.23 mmol), methanol (1.1 mL), THF (1.5 mL), and NaOH 4 N (0.6 mL; 2.4 mmol), at room temperature, for 1 h, to afford compound **11** (144 mg; 98%). Mp (ethyl acetate:petroleum ether 40–60): 244–246 °C. IR 1733, 1622, 1367 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.73 (s, 3H), 0.87 (s, 3H), 0.91 (s, 6H), 1.01 (s, 3H), 1.07 (s, 3H), 1.16 (s, 3H), 1.25 (s, 12H, pinacol-$[C(CH_3)_2]_2$), 1.43 (d, 3H, $-NCH(CH_3)-$), 2.56 (m, 1H, 18β-H), 4.43 (m, 1H, $-NCH(CH_3)-$), 5.48 (m, 1H, 12-H), 6.37 (m, 1H, 2-H), 6.71 (m, 1H, NH); ^{13}C NMR δ 82.7 (pinacol-$[C(CH_3)_2]_2$), 124 (C12), 138.5 (C2), 143.7 (C13), 175.5 (COOH), 179.7 (C28). EI-MS *m/z* (%) 635(6) M^+, 214(20), 202 (65), 189 (100), 172 (60), 118 (60), 90 (60), 82 (35). Anal. Calcd. for $C_{39}H_{62}BNO_5$: C, 73.68; H, 9.83; N, 2.20. Found: C, 73.96; H, 10.23; N, 2.11.

4.12. Methyl 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-12-oxoolean-2-en-28-oate (**12**)

To a solution of compound **4** (100 mg; 0.17 mmol) in dichloromethane (2 mL) a mixture of $KMnO_4$ (361 mg; 2.28 mmol) and $FeSO_4 \cdot nH_2O$ (180 mg) previously grounded in a mortar was added, at room temperature, under strong magnetic stirring, followed by *t*-butanol (0.09 mL) and water (18 μL). After 6 h the reaction was complete. The reaction mixture was diluted with ethyl ether (100 mL), filtered through a Celite pad with further washing with ethyl ether (2 × 75 mL). The resulting organic phase was washed with 10% aqueous $NaHCO_3$ (30 mL), water (30 mL), and brine (30 mL), dried over anhydrous Na_2SO_4, filtered, and the solvent was removed under reduced pressure to afford compound **12** (95 mg, 92%). Mp (acetonitrile): 179–181 °C. IR 1727, 1622, 1367 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.83 (s, 3H), 0.90 (s, 3H), 0.94 (s, 3H), 0.98 (s, 3H), 0.98 (s, 3H), 1.03 (s, 3H), 1.08 (s, 3H), 1.25 (s, 12H, pinacol-$[C(CH_3)_2]_2$), 2.63 (m, 13β-H), 2.79 (m, 1H, 18β-H), 3.68 (s, 3H, 28-OCH_3), 6.35 (m, 1H, 2-H); ^{13}C NMR δ 82.7 (pinacol-$[C(CH_3)_2]_2$), 138 (C2), 178.4 (C28), 212 (C12). EI-MS *m/z* (%) 594 (5) M^+, 277 (70), 217 (100), 202 (64), 174 (54), 132 (38), 118 (44), 104 (37). Anal. Calcd. for $C_{37}H_{59}BO_5$: C, 74.73; H, 10.00. Found: C, 74.61; H, 10.40.

4.13. Methyl 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-12-oxooleana-2,9(11)-dien-28-oate (**13**)

To a solution of compound **12** (150 mg; 0.25 mmol) in acetonitrile (7.3 mL), 48% aqueous HBr (12.2 μL) was added followed by bromine (1 M solution in acetonitrile, 0.35 mL; 0.35 mmol) and the mixture was heated to reflux. After 1 h, additional bromine (0.1 mL; 0.1 mmol) was added and after 1.5 h the reaction was complete. The reaction mixture was evaporated to dryness and ethyl acetate (75 mL) and water (20 mL) were added. The aqueous phase was further extracted with ethyl acetate (2 × 50 mL) and the resulting organic phase was washed with 10% aqueous $NaHCO_3$ (25 mL), 10%

aqueous Na_2SO_3 (25 mL), and water (25 mL), dried over anhydrous Na_2SO_4, filtered, and the solvent was removed under reduced pressure to afford compound **13** as a yellowish solid (140 mg; 94%). Mp (acetonitrile): 224–226 °C. IR 1719, 1657, 1628, 1371 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.89 (s, 3H), 0.99 (s, 6H), 1.08 (s, 3H), 1.12 (s, 3H), 1.16 (s, 3H), 1.24 (s, 3H), 1.25 (s, 12H, pinacol-[$C(CH_3)_2]_2$), 2.87 (m, 1H, 13β-H), 3.02 (m, 1H, 18β-H), 3.69 (s, 3H, 28-OCH_3), 5.78 (m, 1H, 11-H), 6.43 (m, 1H, 2-H), ^{13}C NMR δ 82.8 (pinacol-[$C(CH_3)_2]_2$), 123.9 (C11), 137.8 (C2), 178.3 and 178.3 (C10 and C28), 200.4 (C12). EI-MS *m/z* (%) 592 (15) M^+, 278 (11), 200 (10), 186 (20), 174 (100), 132 (67), 104 (93), 90 (65). Anal. Calcd. for $C_{37}H_{57}BO_5$: C, 74.98; H, 9.69. Found: C, 74.85; H, 10.09.

4.14. Methyl 12-hydroxyimino-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-olean-2-en-28-oate (**14**)

To a solution of compound **12** (80 mg; 0.13 mmol) in dry pyridine (1.3 mL), hydroxylamine hydrochloride (60 mg; 0.86 mmol) was added. The mixture was warmed to reflux under N_2 atmosphere and after 5 h the reaction was complete. Ethyl acetate (50 mL) and 5% aqueous HCl (20 mL) were added and the aqueous phase was further extracted with ethyl acetate (2 × 50 mL). The resulting organic phase was washed with 5% aqueous HCl (25 mL), 10% aqueous $NaHCO_3$ (25 mL), water (25 mL), and brine (25 mL), dried over anhydrous Na_2SO_4, filtered, and the solvent was removed under reduced pressure to afford compound **14** (74 mg, 90%). Mp (acetonitrile): 177–179 °C. IR 1717, 1628, 1362 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.84 (s, 3H), 0.85 (s, 3H), 0.88 (s, 3H), 0.93 (s, 3H), 0.93 (s, 3H), 1.02 (s, 3H), 1.07 (s, 3H), 1.25 (s, 12H, pinacol-[$C(CH_3)_2]_2$), 2.57 (m, 1H, 13β-H), 2.85 (m, 1H, 18β-H), 3.06 (m, 1H), 3.68 (s, 3H, 28-OCH_3), 6.37 (m, 1H, 2-H); ^{13}C NMR δ 82.7 (pinacol-[$C(CH_3)_2]_2$), 138.3 (C2), 160.9 (C12), 178.7 (C28). EI-MS *m/z* (%) 609 (5) M^+, 174 (80), 132 (84), 118 (100), 106 (93), 104 (85), 100 (75), 90 (65). Anal. Calcd. for $C_{37}H_{60}BNO_5$: C, 72.89; H, 9.92; N, 2.30. Found: C, 73.03; H, 10.32; N, 2.23.

4.15. 2-Oxoolean-12-en-28-oyl chloride (**16**)

The method followed that of compound **6** using oleanonic acid **15** (100 mg; 0.22 mmol), oxalyl chloride (0.5 mL; 5.72 mmol), and chloroform (1.5 mL), at room temperature. After 4 h the reaction was complete and worked up to afford compound **16** (102 mg; 98%). IR 1786, 1703 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.83 (s, 3H), 0.91 (s, 6H), 1.04 (s, 3H), 1.05 (s, 3H), 1.08 (s, 3H), 1.15 (s, 3H), 2.84 (m, 1H, 18β-H), 5.34 (m, 1H, 12-H); ^{13}C NMR δ 123.5 (C12), 142.0 (C13), 179.7 (COCl), 217.6 (C3). EI-MS *m/z* (%) 472 (4) M^+, 409 (6), 265 (12), 204 (24), 201 (100), 187 (15), 131 (15), 90 (16).

4.16. 2-Oxoolean-12-en-28-amide (**17**)

The method followed that of compound **7** using compound **16** (500 mg, 1.1 mmol), THF (20 mL), and ammonia (0.5 mL), cooled in an ice bath. After 1.5 h the reaction was complete and worked up to afford compound **17** (476 mg; 99%). IR 3459, 1700, 1669 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.87 (s, 3H), 0.89 (s, 6H), 1.03 (s, 6H), 1.07 (s, 3H), 1.16 (s, 3H), 5.37 (m, 1H, 12-H), 5.91 (br s, 2H, $CONH_2$); ^{13}C NMR δ 122.5 (C12), 144.8 (C13), 181.3 ($CONH_2$), 217.4 (C3). EI-MS *m/z* (%) 453 (15) M^+, 233 (69), 202 (100), 190 (56), 186 (40), 132 (84), 118 (43), 104 (39).

4.17. 28-Cyanooleana-2,12-diene 3-trifluoromethanesulfonate (**18**)

The method followed that of compound **3** using compound **17** (340 mg, 0.75 mmol), dichloromethane (7 mL), Tf_2O (0.23 mL, 1.4 mmol) and DTBMP (310 mg, 1.51 mmol), at reflux. After 1.5 h the reaction was complete and worked up to afford compound **18** (386 mg, 91%). Mp (ethyl acetate:petroleum ether 40–60): 169–170 °C. IR 2227, 1407, 1244, 1207, 1140 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.91 (s, 3H), 0.95 (s, 3H), 1.03 (s, 3H), 1.05 (s, 3H), 1.08 (s, 3H), 1.15 (s, 6H), 2.59 (m, 1H, 18β-H), 5.41 (m, 1H, 12-H), 5.57 (dd, 1H, 2-H, $J_1 = 1.76$ Hz and $J_2 = 6.60$ Hz); ^{13}C NMR δ 113.5 (C2), 116.8 and 120 ($J = 319$ Hz, CF_3), 124.3 (C12), 125.5 (CN), 141.6 (C13), 155.4 (C3); EI-MS *m/z* (%) 418 (80) M^+ – OTf, 390 (32), 213 (40), 186 (85), 172 (100), 156 (70), 144 (65), 130 (55). Anal. Calcd. for $C_{31}H_{44}F_3NO_3S$: C, 65.58; H, 7.81; N, 2.47. Found: C, 65.69; H, 8.11; N, 2.52.

4.18. 3-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)-oleana-2,12-diene-28-nitrile (**19**)

The method followed that of compound **4** using compound **18** (100 mg; 0.18 mmol), $PdCl_2(PPh_3)_2$ (9 mg, 0.012 mmol), THF (2 mL), B_2pin_2 (67 mg, 0.26 mmol), and a 0.8 M solution of Na_2CO_3 (0.84 mL; 0.68 mmol), at reflux. After 1.5 h additional $PdCl_2(PPh_3)_2$ (4.5 mg, 0.006 mmol), B_2pin_2 (34 mg, 0.13 mmol), and THF (0.5 mL) were added and after 2 h the reaction was complete and worked up to afford compound **19** (87 mg, 91%). Mp (THF:methanol): 292–294 °C. IR 2227, 1367 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.90 (s, 3H), 0.93 (s, 3H), 0.94 (s, 3H), 1.04 (s, 3H), 1.06 (s, 3H), 1.09 (s, 3H), 1.14 (s, 3H), 1.25 (s, 12H, pinacol-[$C(CH_3)_2]_2$), 2.57 (m, 1H, 18β-H), 5.39 (m, 1H, 12-H), 6.37 (m, 1H, 2-H); ^{13}C NMR δ 82.7 (pinacol-[$C(CH_3)_2]_2$), 124.9 (C12), 125.6 (CN), 138.5 (C2), 141.4 (C13). EI-MS *m/z* (%) 545 (22) M^+, 315 (15), 228 (100), 213 (80), 172 (70), 146 (55), 92 (60), 90 (74). Anal. Calcd. for $C_{36}H_{56}BNO_2$: C, 79.24; H, 10.34; N, 2.57. Found: C, 79.02; H, 10.03; N, 2.74.

4.19. Biology

Human Jurkat, K562, Jijoye, Hela, SW480, SKOV-3 cells and fibroblasts were obtained from the American Type Culture Collection (USA). Jurkat, K562, Jijoye, SW480 and SKOV-3 cells were routinely grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 5% CO_2. Hela cells and human fibroblasts were routinely grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 5% CO_2. PBMC were obtained from healthy individuals after Ficoll-Hypaque density separation. MG132 and epoxomycin were purchased from Enzo Life Sciences AG, Lausen, Switzerland.

4.20. Cell viability and proliferation assays

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For the MTT assay cells were seeded at a concentration of 25 × 10^3 cells/well on 96-well plates and treated with the compounds at the specified concentrations. After the indicated periods of time, the cells were incubated with a 5 mg/mL solution of MTT in phosphate saline buffer (PBS) for 2 h. Following this incubation lysis buffer (20% w/v SDS in 1:1 DMF:H_2O at pH 4.7) was added to the cells and after 4 h the plates were read at 570 nm (Sunrise, TECAN, Austria GmbH).

4.21. Proteasome inhibition assays – cell pellets [43]

Jurkat cells (5 × 10^8) or PBMC (80 × 10^6) were washed with cold PBS and resuspended in buffer (400 μL) containing 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM DTT (Sigma–Aldrich, Co), 2 mM ATP, and 250 mM sucrose. An amount equivalent to half the volume of cell suspension was added in glass beads and the mixture was vortexed for 2 min at 4 °C. Beads and cell debris were removed by 7 min centrifugation at 1000 *g*, followed by 20 min of centrifugation

at 10,000 *g*, at 4 °C. The lysates were cleared by ultracentrifugation for 1 h at 100,000 *g* followed by another ultracentrifugation for 5 h at the same rpm, at 4 °C. Proteasome-containing pellets were resuspended in homogenization buffer (200 μL) [50 mM Tris–HCl (pH 7.4), 100 mM KCl, 15% glycerol]. Protein concentration was determined using the BCA protocol (Pierce, Rockford, IL). The fluorogenic substrates Suc-LLVY-AMC and Boc-LRR-AMC (Enzo Life Sciences AG, Lausen, Switzerland) were used to measure the ChT-L and T-L activities of the proteasome, respectively. Semipurified proteasomes were pretreated with vehicle (DMSO) or the compounds for 30 min in a buffer containing 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 500 μM EDTA, 1 mM DTT, and 2 mM ATP, at 37 °C, and after this time the different peptide substrates were added to the mixture. Fluorescence (Spectrafluor plus, Tecan, Salzburg, Austria) was read at 360 nm excitation wavelength and 465 nm emission wavelength for 2 h, at 37 °C.

4.22. Proteasome inhibition assays – Jurkat cells [43]

Jurkat cells (1.6×10^6 cells/well) were plated on 24-well plates and treated with the compounds at the specified concentrations or with the vehicle for 30 min after which the pellets were collected, washed with PBS and the lysates were prepared and assayed according to the procedure reported above.

4.23. Western blotting

Equal amounts of proteins were loaded onto a 4–12% SDS-PAGE and electroblotted onto Protran nitrocellulose membranes (Schleicher & Schuell Microscience, Keene, NH). Blots were probed with antibodies specific for Ubiquitin (DAKO, Glostrup, Denmark), GAPDH (Thermo Scientific, Rockford, IL) and developed by ECL (Amersham Biosciences, Uppsala).

4.24. Flow cytometry

Jurkat cells (1.6×10^6 cells/well) were plated on 24-well plates and treated with the compounds at the specified concentrations or with the vehicle for the indicated time periods after which the pellets were collected, and to each pellet 500 μL of Krishan buffer (0.1% sodium citrate, 0.3% NP-40, 0.05 mg/mL PI, 0.02 mg/mL RNase) was added. The mixtures were kept at 4 °C, in the dark, for 15 min and after this time analyzed by flow cytometry (FACScan; Becton Dickson, San Jose, CA). For the annexin-V-FITC/PI double staining assay, after the collection of the pellets, 150 μL of binding buffer was added together with 5 μL of annexin V-FITC (20 μg/mL in Tris–NaCl) per sample and after 10 min, 10 μL of PI (50 μg/mL in 1× binding buffer) and an extra 350 μL of binding buffer was added, according to the manufacturer's protocol (ApoAlert® Annexin V, Clontech Laboratories, Inc.) The samples were analyzed by flow cytometry (FACScan; Becton Dickson, San Jose, CA).

4.25. Caspases 3/7 assay

Apoptosis assays were performed by using Caspase Glo® 3/7 assay (Promega, Milan, Italy) according to the manufacturer's instructions. Briefly, Jurkat cells were seeded at a concentration of 25×10^3 cells/well on 96-well plates and treated with the compounds at the specified concentrations. After overnight treatment, the cells were incubated with 100 μl/well of reconstituted Caspase-Glo® 3/7 reagent for 1 h in the dark. Following this incubation the plates were read by Wallac EnVision 2104 Multilabel Reader (PerkinElmer, Monza, Italy), and luminescence was expressed as RLU. Samples were run in triplicate. Viability assays were performed by using ATPlite 1 step assay kit (PerkinElmer, Monza, Italy). Jurkat cells were seeded at a concentration of 25×10^3 cells/well on 96-well plates and treated with the compounds at the specified concentrations. After overnight treatment, the cells were incubated with 100 μl/well of substrate solution and then the luminescence was immediately read by Wallac EnVision 2104 Multilabel Reader (PerkinElmer, Monza, Italy). Samples were run in triplicate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2013.01.040.

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