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Synthesis and structure–activity relationship study of novel cytotoxic carbamate and *N*-acylheterocyclic bearing derivatives of betulin and betulinic acid

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ABSTRACT

Chemical transformation studies were conducted on betulin and betulinic acid, common plant-derived lupane-type triterpenes. The concise synthesis, via a stepwise approach, of betulin and betulinic acid carbamate and *N*-acylheterocyclic containing derivatives is described. All new compounds, as well as betulinic acid were tested in vitro for their cytotoxic activity. Most of the compounds have shown a better cytotoxic profile than betulinic acid, including the synthesized betulin derivatives. Compounds **25** and **32** were the most promising derivatives, being up to 12-fold more potent than betulinic acid against human PC-3 cell lines (IC_{50} values of 1.1 and 1.8 μM, respectively).

1. Introduction

Natural products have been used to treat human disease for thousands of years and play an increasingly important role in drug discovery and development. In fact, the majority of anticancer and anti-infectious agents are of natural origin.[1,2]

Terpenes are a wide-spread group of natural compounds with considerable practical significance which are synthesized in many plants by rearrangement of squalene epoxide. Almost all terpenes have biological activities in animals including man, and they also play a meaningful role in human medicine.[3] From this point of view, the most important groups of terpenes are triterpenes, triterpenes glycosides, and other triterpenoids. Pentacyclic triterpenoids are a class of pharmacologically active and structurally rich natural products with privileged motifs for further modifications and structure–activity relationship (SAR) analyses.[4] To date several reports have been published demonstrating that either simple or advanced modifications may be performed without loss of the desired biological properties.[5]

The naturally occurring lupane-type triterpenoids betulin **1** and betulinic acid **2** (Fig. 1) have been thoroughly investigated during the past years for their promising medical properties,[6–9] and particularly their chemopreventive and antitumor activities.[10,11]

Compound **2**, derived from birch bark, exhibits a broad spectrum of anticancer activities and potential clinical value as an anti-HIV, antibacterial and antimalarial agent.[6,10] Discovery of **2** as a growth inhibitor of melanoma cell lines and confirmation of its anticancer activity in mice bearing human melanoma xenografts[12] led to considerable interest in **2** as an anticancer drug

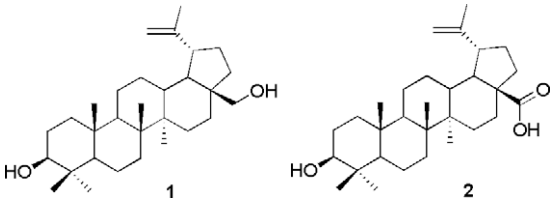

Figure 1. Chemical structure of betulin **1** and betulinic acid **2**.

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candidate, and a significant number of scientific publications in this field has been published. Triterpenoid **2** and its derivatives were shown to be effective against a variety of carcinoma cell lines derived from lung, ovarian, cervical, head and neck carcinomas, as well as from lymphoma, neuroblastoma, medulloblastoma, glioblastoma, and other types of tumors.[6,10,12–14] In addition to tumor cell lines, **2** was also cytotoxic against primary cancer cells isolated from tumor specimens obtained from neuroblastoma, glioblastoma, and leukemia.[15,16] Also, **2** was cytotoxic in different models of drug resistance, for example primary pediatric acute leukemia samples that were refractory to standard chemotherapeutic agents.[15] By comparison, normal cells of different origin have been reported to be much more resistant to **2** than cancer cells, which is indicative of some selectivity for tumor cells.[17,18] This feature makes **2** unique in comparison to compounds that are currently used in cancer therapy, such as taxol, camptothecin (CPT), ellipticine, etoposide, vinblastine or vincristine, among others, which are very toxic and inhibit replication of both cancer and normal cells. Reports have demonstrated that the cytotoxicity induced by **2** involves modulation of Bcl-2 family and cell cycle regulatory proteins,[19–22] regulation of the nuclear factor kappa B (NF-κB),[23,24] as well as inhibition of aminopeptidase N,[25] and growth factor-induced angiogenesis.[26] DNA topoisomerases have been shown to be important targets for several chemotherapeutic agents.[27–30] Compound **2** and some of its derivatives have also been reported as potent inhibitors of eukaryotic topoisomerases I and II.[31–35]

Since natural derivatives of **2** bear anticancer activity, the synthesis of derivatives of this triterpenoic acid, which is commercially available, could be of great value in discovering potential bioactive semisynthetic compounds. In fact, many studies reported that modifications of triterpenoids **1** and **2** at the C-3, C-20, and C-28 positions can produce a number of potentially important derivatives with potent anticancer activity.[6,11,36–38]

Recently, we focused our attention on the synthesis of lupane-type imidazole carbamates and *N*-acylimidazole bearing derivatives. Our results showed that addition of an imidazolyl moiety at the C-3 and/or C-28 positions of **1** and **2** can result in more potent in vitro anticancer agents than **2**, with IC_{50} values between 0.8 and 26.1 μM, in human cancer cell lines of different tumor types. These IC_{50} values were 2–45 times lower than those obtained with compound **2**. The promising results prompted us to extend our study to 2′-methylimidazole and triazole derivatives, in order to establish meaningful SAR. Hence, we report here the synthesis of 22 novel 2′-methylimidazole and triazole carbamates and *N*-acylheterocyclic bearing derivatives of compounds **1** (**3–7**, **9**, **10**, **12**, **13**, **15**, and **17–20**) and **2** (**23–27**, **29**, **31**, and **32**). The in vitro cytotoxic activity of the synthesized compounds was evaluated against human hepatocellular carcinoma (HepG2), leukemia (Jurkat), cervical adenocarcinoma (HeLa), colon adenocarcinoma (HT-29), prostate adenocarcinoma (PC-3), and fibroblasts (BJ) cells. The compounds were also screened for their ability to inhibit topoisomerase I.

2. Results and discussion

2.1. Chemistry

Similar transformations were used to prepare the 2′-methylimidazole and triazole derivatives. The first series of derivatives was synthesized according to the reaction sequence shown in Schemes 1 and 2. Compound **1** was the starting material used directly for the synthesis of derivatives **3–6**, through reaction with 1,1′-carbonylbis(2′-methylimidazole) (CBMI) and 1,1′-carbonyl-di(1,2,4-triazole) (CDT) as previously reported (Scheme 1).[38] In the case of the disubstituted derivatives, the first carbamoylation takes place at carbon C-28 and then at carbon C-3, as determined by ^{1}H NMR. Thus, to obtain the C-3 lupane carbamate **7**, compound **6** was previously prepared according the general procedure with CDT, and was then selectively hydrolyzed at C-28 with silica gel and purified by flash column chromatography (FCC) to afford **7**, in 63% yield (Scheme 1). In order to avoid the formation of multiple derivatives, compound **1** was first acetylated at the C-28 position with acetic anhydride (Ac_2O) and imidazole, in $CHCl_3$, to give betulin 28-acetate **8** (Scheme 1), that was then treated with CBMI or CDT in THF, at reflux, to afford compounds **9** and **10** in 82% and 88% yield, respectively after FCC (Scheme 1). The synthetic pathway employed for the synthesis of 3-acetylbetulin **11** (Scheme 1) was inspired by the procedure developed by Tietze et al.[39] Initially, **1** was acetylated at the C-3 and C-28 positions with acetic anhydride and 4-dimethylaminopyridine (DMAP) in anhydrous THF to afford betulin 3,28-diacetate in quantitative yield, without further purification. Thereafter, the 3,28-diacetate was deacetylated at the C-28 position in the presence of 1 equiv of methanolic potassium hydroxide, in THF, in 89% isolated yield.

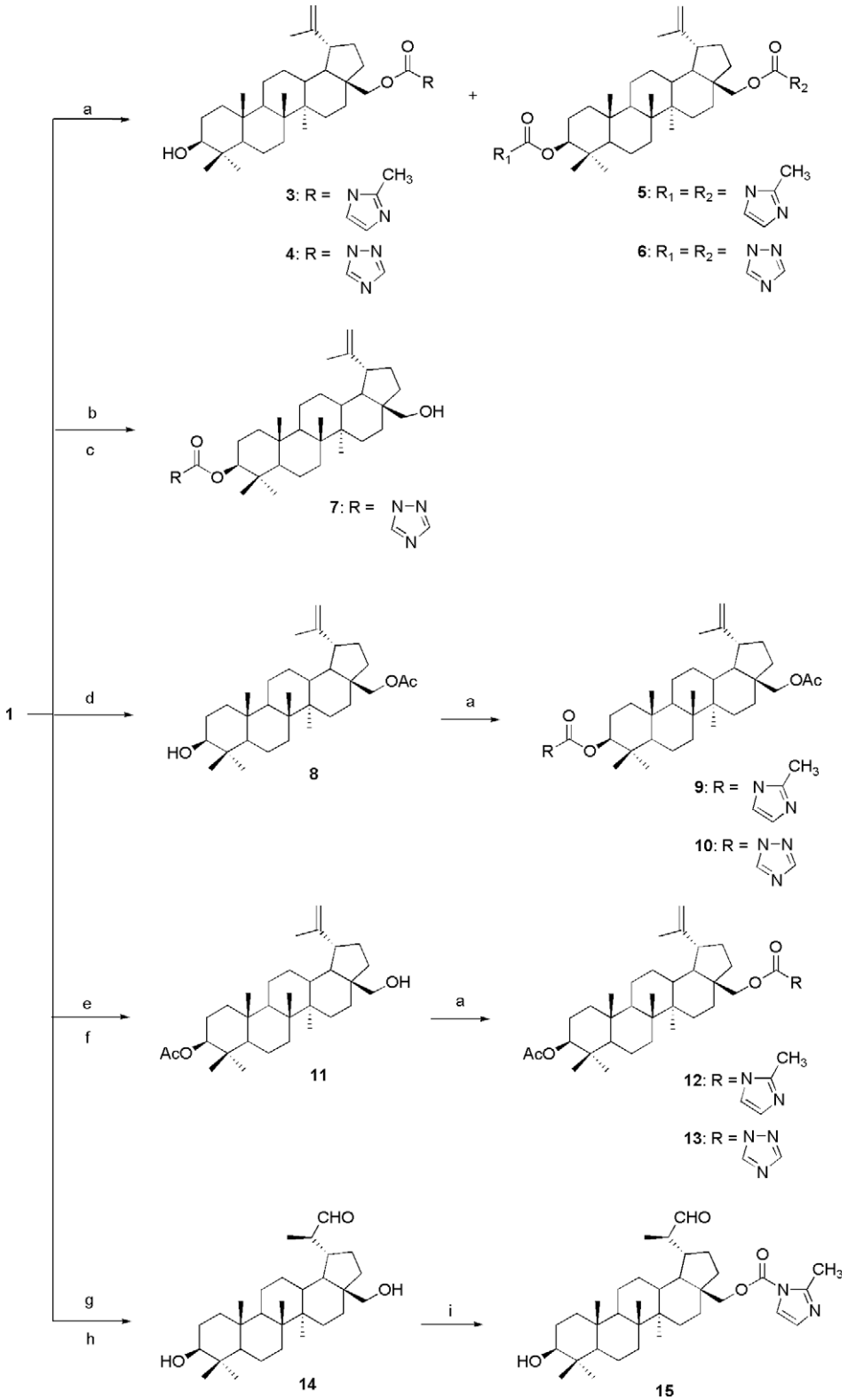
The synthesis of derivative **15** (Scheme 1) began with the epoxidation of the isopropenyl group of the commercially available **1** with *m*-chloroperbenzoic acid (*m*-CPBA), followed by acid catalyzed epoxy-ring opening to afford the epimeric isomer (20*R*-aldehyde) **14** (39%) as the major product.[40] This compound was then treated with CBMI in THF, at reflux, to afford **15** in 75% yield, after FCC (Scheme 1). The intermediate **16** (Scheme 2) was synthesized by methoxylation of compound **1** in two steps, as previously described.[41] The reaction with CBMI and CDT afforded derivatives **17–20** in isolated yields ranging from 17% to 74% (Scheme 2).

The second series of derivatives, originated from compound **2**, was synthesized following the reaction sequence shown in Schemes 3 and 4. Derivatives **23** and **24** (Scheme 3) were prepared directly from the reaction of **2** with 5 equiv of CBMI in anhydrous THF, in 77% and 22% yield, respectively, after FCC. However, only derivative **25** (Scheme 3) was obtained after reaction with CDT, in 82% isolated yield, under the same reaction conditions. This fact could be explained by the similarity of the reaction rate of the hydroxyl and carboxyl groups with CDT. The methyl ester **21** (Scheme 3) was prepared in 83% yield from **2** using CH_3I and K_2CO_3, in dry DMF. Reaction of **21** with CBMI gave the 2′-methylimidazole carbamate derivative **26**, in quantitative yield (Scheme 3). For the synthesis of compound **27** (Scheme 3), prior to the introduction of the 2′-methylimidazole moiety at C-28, betulonic acid **22** was prepared by oxidation of 3β-hydroxyl group under acidic conditions (CrO_3 and sulfuric acid). Derivative **27** was obtained in 84% isolated yield using the same reaction protocol mentioned above.

The new double bond between C1 and C2 was formed by dehydrogenation of **22** with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) in dioxane to afford compound **28** (Scheme 4).[42] This compound was then treated with CBMI to afford **29**, in 87% isolated yield. For reasons that are currently unclear, all attempts to prepare the *N*-acyltriazole derivatives from intermediates **22** and **28** were unsuccessful.

For the synthesis of derivatives **31** and **32**, intermediate **30** (Scheme 4) was prepared according to a previously published procedure using oxygen in the presence of potassium *tert*-butoxide (*t*-BuOK), in 72% yield.[43] Further reaction with CBMI or CDT and purification by FCC led to derivatives **31** and **32**, in 83% and 77% isolated yield, respectively.

The structures of all the newly synthesized compounds (**3–7**, **9**, **10**, **12**, **13**, **15**, **17–20**, **23–27**, **29**, **31**, and **32**) were assigned by 1D and 2D NMR experiments (^{1}H, ^{13}C, DEPT135, COSY, HMQC, and HMBC), IR and MS spectroscopy.

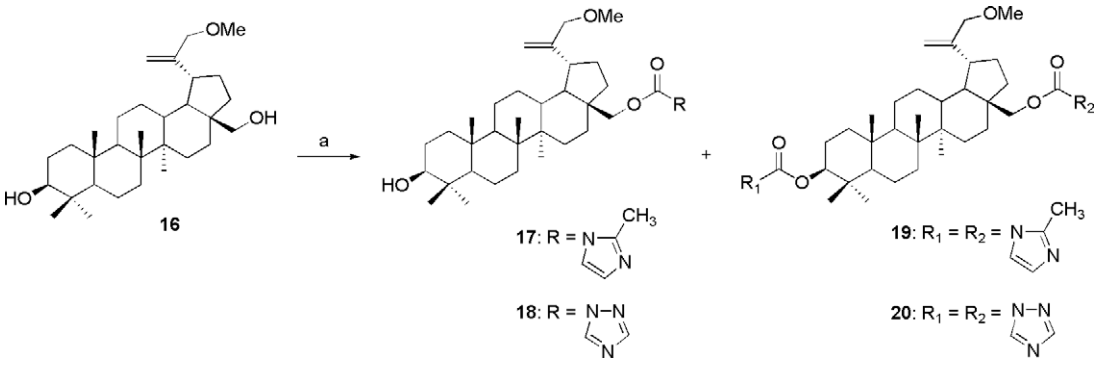


Scheme 1. Synthesis of derivatives **3**–**7**, **9**, **10**, **12**, **13**, and **15**. Reagents and conditions: (a) CBMI or CDT, dry THF, N_2, reflux; (b) CDT, dry THF, N_2, reflux; (c) silica gel, rt; (d) Ac_2O, imidazole, $CHCl_3$, reflux; (e) Ac_2O, DMAP, THF, rt; (f) KOH, MeOH, THF, rt; (g) *m*-CPBA, CH_2Cl_2, 0–5 °C; (h) H_2SO_4 (2 M), 0–5 °C; (i) CBMI, dry THF, N_2, reflux.

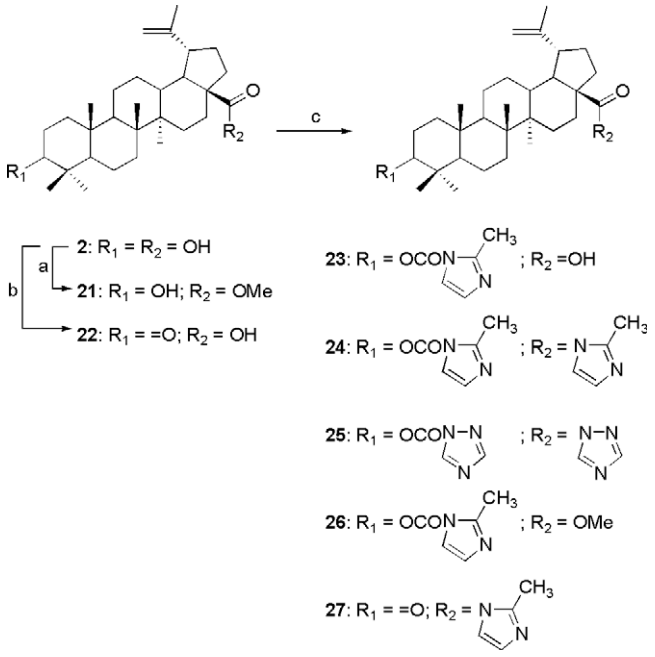
2.2. Antiproliferative activity

2.2.1. Cytotoxicity in human cancer cell lines

Several cancer cell lines were cultured and used in experiments in order to obtain the SAR information of the new lupane-type compounds with respect to their potential cytotoxicity against human cancers. The in vitro inhibition of cell viability mediated by the different synthesized compounds was studied on HepG2 (human hepatocellular carcinoma; adherent epithelial cell line), Jurkat (human acute T cell leukemia; suspended cell line), HeLa (human



Scheme 2. Synthesis of derivatives **17**–**20**. Reagents and conditions: (a) CBMI or CDT, dry THF, N_2, reflux.

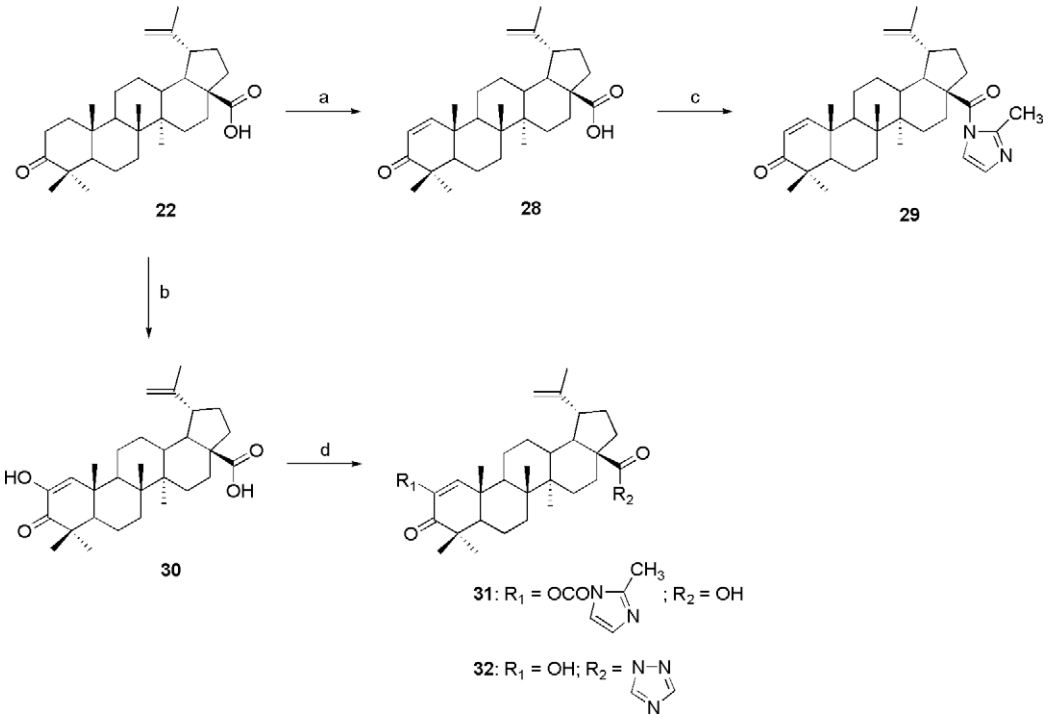


Scheme 3. Synthesis of derivatives **23**–**27**. Reagents and conditions: (a) CH_3I, K_2CO_3, dry DMF, rt; (b) Jones reagent, acetone, 0 °C; (c) CBMI or CDT, dry THF, N_2, reflux.

cervix adenocarcinoma; adherent epithelial cell line), HT-29 (human colon adenocarcinoma; adherent epithelial cell line), and PC-3 (human prostate adenocarcinoma; adherent epithelial cell line) cancer cell lines. These cell lines were chosen because they represent cancer types with great incidence in the human population. All cell lines were exposed to 10 serial threefold dilutions of each drug for 72 h. Cytotoxicity was evaluated using the MTT or XTT assay. Compounds were subdivided into two groups: compound **1** derivatives (**3**–**7**, **9**, **10**, **12**, **13**, **15**, **17**–**20** Group I), and compound **2** derivatives (**23**–**27**, **29**, **31**, and **32**, Group II). Cytotoxicity results are displayed in Tables 1 and 2 and expressed as the concentration inhibiting 50% of cell growth (IC_{50}). The parent triterpenoid **2**, currently in clinical trials,[44,45] was used as a positive control (IC_{50} 12.8–36.4 μM). Compounds with IC_{50} values >30 μM were considered inactive.

As shown in Tables 1 and 2, the synthesized compounds exhibited different degrees of cytotoxicity towards the tested cell lines. Generally, the cytotoxicity results indicate that the introduction of the 2′-methylimidazolyl and triazolyl moieties has a positive impact on the anticancer activity of the lupane-type triterpenoids. These results are in good agreement with previous investigations in which lupane-type derivatives bearing *N*-heterocyclic moieties at C-3 and/or C-28 positions were found to be cytotoxic against human cancer cell lines.[38,46–48] Moreover, the synthesized *N*-acylheterocyclic derivatives induced more potent cytotoxicity in the cancer cell lines than the carbamate derivatives which suggests that the beneficial impact on the cytotoxic activity of the *N*-heterocyclic moieties is highly dependent on the lupane-type triterpenoid used. Compound **2** derivatives (**23**–**27**, **29**, **31**, and **32**) (Table 2) were the most potent compounds showing cytotoxic activity against the tested tumor cells. The *N*-acyltriazole derivatives **25** and **32** (Table 2) were about 19- and 12-fold, more active than **2** against PC-3 cells. Compounds **7**, **17**, **18**, **23**, **24**, and **27**, **29**, and **31** (Tables 1 and 2) also exhibited a better cytotoxic profile than **2**. These preliminary results allowed us to predict which groups are required to obtain a good cytotoxic activity. We first investigated the cytotoxicity of acetyl esters at the C-28 and C-3 positions of 2′-methylimidazole (**9** and **12**) (Table 1) and triazole (**10** and **13**) (Table 1) carbamate derivatives and no cytotoxic activity was found, even at concentrations up to 30 μM. These results were in accordance with a previous work reporting loss of cytotoxicity on acylated derivatives.[49] We also investigated the influence of combining substitution at C-3 and C-28 positions. For compounds of Group I, the introduction of a carbamate moiety at C-3 in the C-28 substituted derivatives (**3**, **4**, **17**, and **18**) (Table 1) afforded compounds **5**, **6**, **19**, and **20** (Table 1) with lower cytotoxic activity against the tested cancer cell lines. In accordance with observations from Kim et al.,[36] this loss of toxic effect suggested a size limitation at the C-3 position. For Group II compounds, introduction of a carbonyl group at C-3 originated compound **27** (Table 2) with a significant cytotoxic activity (IC_{50} values of 4.2, 4.7, 5.3, and 5.5 μM in HeLa, HT-29, Jurkat and HepG2 cells, respectively). This result suggested that more than the size limitation of the moiety used, there is great significance in the electronic density at C-3 position, for the cytotoxic effect.

It has been shown in previous SAR studies that the free C-28 carboxylic acid function is important for the cytotoxicity of **2**,[6,10,11,36] as the corresponding reduced form **1** is devoid of such activity.[36] As shown in Table 2, the lower cytotoxic activity of the 3,28-*N*-acyl-2′-methylimidazole derivative **24** (Table 2) (IC_{50} 7.8–20.1 μM), the 28-*N*-acyl-2′-methylimidazole derivative **29** (Table 2) (IC_{50} 6.8–12.5 μM) and the 28-methyl ester **26** (Table 2) (IC_{50} 12.9–16.4 μM) compared with compounds **23** (Table 2) (IC_{50} 5.7–7.5 μM) and **31** (Table 2) (IC_{50} 4.7–9.4 μM), bearing a free carboxyl group at C-28 position, also suggested the importance of the hydrogen bonding capability and/or acidity in the cytotoxic effect. These observations are consistent with the reported in vitro cytotoxic activities described by Kim et al.,[36] and Chatterjee et al.,[50] Our previous SAR investigation also showed that the addition of an imidazolyl moiety at the C-28 position of **2** significantly decreased cytotoxicity compared to its carboxylic acid derivative.[38] However, this extended study revealed that there are some



Scheme 4. Synthesis of derivatives **29**, **31** and **32**. Reagents and conditions: (a) DDQ, dioxane, N_2, reflux; (b) O_2, *t*-BuOK, *t*-BuOH, 40 °C; (c) CBMI, dry THF, N_2, reflux; (d) CBMI or CDT, dry THF, N_2, reflux.

Table 1
Cytotoxic activity of compound **1** derivatives **3**–**7**, **9**, **10**, **12**, **13**, **15**, and **17**–**20** (Group I), in six human cell lines, after 72 h of exposure

Compound	IC_{50}[a] (μM ± SD)					
	HepG2	Jurkat	HeLa	HT-29	PC-3	BJ
2	36.4 ± 1.5	26.9 ± 2.2	26.0 ± 2.1	12.8 ± 1.7	21.5 ± 2.0	ND
3	8.1 ± 0.4	15.8 ± 2.4	11.0 ± 1.7	16.2 ± 2.1	ND	ND
4	ND	ND	ND	7.6 ± 0.9	4.7 ± 0.4	ND
5	>30	>30	>30	>30	ND	ND
6	ND	ND	ND	17.5 ± 1.8	7.9 ± 1.0	ND
7	7.3 ± 1.2	12.2 ± 2.1	6.9 ± 1.3	4.9 ± 0.6	2.1 ± 0.2	39.4 ± 2.5
9	>30	>30	>30	>30	ND	ND
10	ND	ND	ND	>30	>30	ND
12	>30	>30	>30	>30	ND	ND
13	ND	ND	ND	>30	>30	ND
15	14.3 ± 2.4	21.3 ± 4.1	16.6 ± 1.5	15.8 ± 1.7	ND	ND
17	14.4 ± 1.3	24.8 ± 4.7	8.9 ± 2.1	10.4 ± 1.6	ND	ND
18	9.1 ± 1.8	9.5 ± 2.7	24.3 ± 2.7	5.8 ± 0.3	2.6 ± 0.2	46.6 ± 3.5
19	28.2 ± 2.5	>30	13.4 ± 0.6	>30	ND	ND
20	ND	ND	ND	12.3 ± 0.8	6.4 ± 0.4	ND

Exponentially growing cells were treated with the compounds at different concentrations for 72 h. Cell-growth inhibition was analyzed by the MTT (HepG2, HeLa, HT-29, PC-3, and BJ) or XTT (Jurkat) assay. The assay was done using three replicates and repeated four times.
ND = not determined.
[a] IC_{50} is the concentration of compound that inhibits 50% of cell growth.

exceptions to this general tendency. In fact, compound **25** (Table 2 and Fig. 2 C), a disubstituted derivative of **2**, was found to exhibit a potent cytotoxic profile, with IC_{50} values of 1.9 and 1.1 μM against HT-29 and PC-3 cancer cell lines, respectively.

Although colon cancer cells were reported to be almost completely refractory to treatment with **2**, in our assays the highest cytotoxic activity of this compound was observed in the HT-29 adenocarcinoma cell line. Nevertheless, analogues **7** (Table 1), **25**, **27**, and **32** (Table 2) showed better IC_{50} values (⩽5 μM), in HT-29 cells than compound **2**. PC-3 prostate cancer cells were more sensitive to the tested compounds than the other cancer cell lines. Compounds **7**, **18** (Table 1 and Fig. 2 A and B), **25**, and **32** (Table 2 and Fig. 2 C and D) showed remarkable activities against PC-3 cells, with IC_{50} values of 2.1, 2.6, 1.1, and 1.8 μM, respectively, compared to **2** (IC_{50} 21.5 μM). In fact, compounds **25** and **32** exhibited very similar cytotoxic behavior, as it can be observed in the dose dependent effect curves for cell viability (Fig. 2 C and D), suggesting that both compounds share the same mechanism of cell death in the tested cell lines.

The selectivity was studied by incubating the compounds with a non-tumoral cell line (BJ). All the tested compounds are more cytotoxic towards cancer cell lines than normal cells (Tables 1 and 2) demonstrating a selective cytotoxic activity for malignant cells. Compounds **7**, **18**, **25**, and **32** showed IC_{50} values 15–18 times lower on PC-3 cells than on non-tumoral BJ cells (Tables 1 and 2).

In terms of SAR, these in vitro cytotoxic results suggest that simple modifications of the parent structure either in **1** or **2** can produce new potentially interesting derivatives. Also, derivatives

Table 2
Cytotoxic activity of compound **2** derivatives **23**–**27**, **29**, **31**, and **32** (Group II), in six human cell lines, after 72 h of exposure

Compound	IC_{50}[a] (μM ± SD)					
	HepG2	Jurkat	HeLa	HT-29	PC-3	BJ
2	36.4 ± 1.5	26.9 ± 2.2	26.0 ± 2.1	12.8 ± 1.7	21.5 ± 2.0	ND
23	7.3 ± 1.0	6.1 ± 1.3	7.5 ± 1.5	5.7 ± 0.6	ND	41.3 ± 2.3
24	11.6 ± 0.8	20.1 ± 3.2	12.4 ± 1.7	7.8 ± 1.6	ND	ND
25	6.8 ± 1.1	11.6 ± 2.6	4.1 ± 0.9	1.9 ± 0.7	1.1 ± 0.1	19.4 ± 1.6
26	13.4 ± 1.7	16.4 ± 2.4	13.4 ± 1.5	12.9 ± 0.5	ND	ND
27	5.5 ± 1.1	5.3 ± 0.9	4.2 ± 1.2	4.7 ± 1.1	ND	48.4 ± 3.9
29	6.8 ± 1.5	12.5 ± 1.2	10.6 ± 0.7	9.0 ± 1.7	ND	ND
31	4.7 ± 0.2	9.4 ± 0.8	6.4 ± 0.8	5.9 ± 0.3	ND	37.2 ± 4.3
32	7.4 ± 1.3	10.5 ± 2.6	5.4 ± 0.4	3.3 ± 0.3	1.8 ± 0.1	27.9 ± 2.6

Exponentially growing cells were treated with the compounds at different concentrations for 72 h. Cell-growth inhibition was analyzed by the MTT (HepG2, HeLa, HT-29, PC-3, and BJ) or XTT (Jurkat) assay. The assay was done using three replicates and repeated four times.
ND = not determined.
[a] IC_{50} is the concentration of compound that inhibits 50% of cell growth.

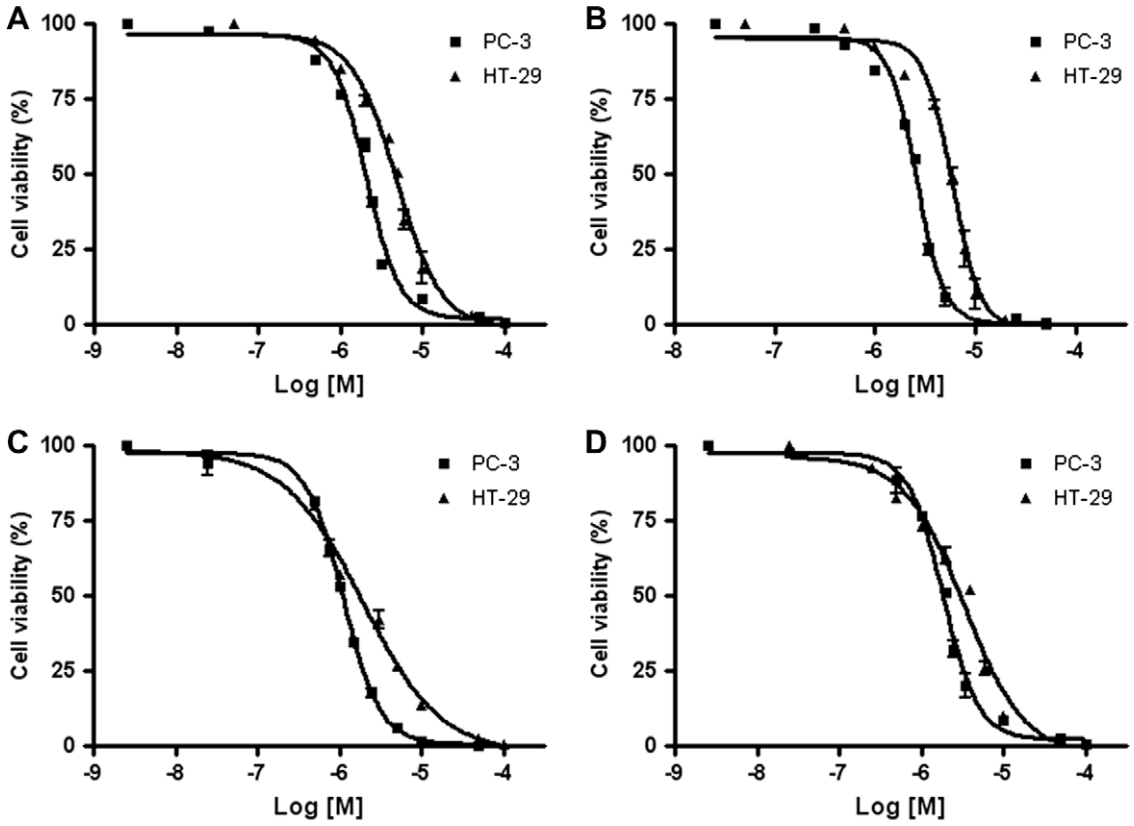


Figure 2. Dose-dependent effect of triazole derivatives (A: **7**, B: **18**, C: **25**, and D: **32**) on cell viability of different cell lines (PC-3, HT-29). Data shown are mean (standard error of at least three independent experiments).

of **2** were found to be generally more potent in vitro cytotoxic agents than those of **1**, with the exception of the triazole derivatives **7** (Table 1 and Fig. 2 A) and **18** (Table 1 and Fig. 2 B). These compounds displayed potent cytotoxic activity against prostate carcinoma (PC-3) and colorectal adenocarcinoma (HT-29) human cell lines. A brief and schematic representation of SAR for cytotoxicity of compound **2** derivatives is presented in Figure 3.

2.2.2. Topoisomerase I inhibitory activity

DNA topoisomerase I is a critical cellular enzyme involved in tumor growth, therefore, inhibition of topoisomerase constitutes a useful strategy for the identification of potential antitumor agents. Considering the promising activity of triterpenoids as inhibitors of topoisomerase I,[31] including **2** and its derivatives,[32–34] we decided to investigate the effect of our synthesized derivatives on topoisomerase I activity, using the topoisomerase I–DNA relaxation assay and CPT as a positive control (Fig. 4).

Figure 4 and Table 3 show the catalytic inhibition of topoisomerase I by derivatives **3**–**7**, and **15**, **17**–**20**, **23**–**27**, **29**, **31**, and **32** at 100 μM, in comparison with 100 μM CPT (Fig. 4, lane 5 and Table 3). In this preliminary study, compounds **23** (Fig. 4, lane 7 and Table 3) and **31** (Fig. 4, lane 9 and Table 3), with a carboxyl group at C-28, showed better topoisomerase I inhibitory activity than CPT, indicating that the free carboxylic group is important for the inhibition of topoisomerase I catalytic activity. A relationship between the topoisomerase I inhibitory effects and the cytotoxicity observed with the synthetic lupane-type triterpenoids could not be clearly established in this study. Although compounds

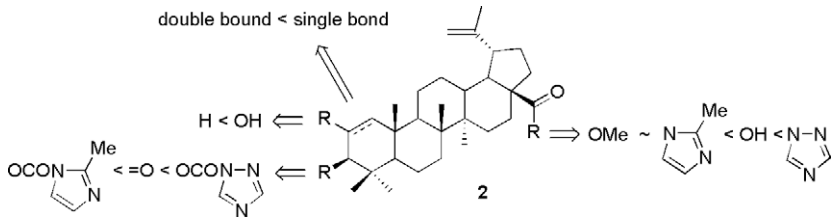


Figure 3. Graphical depiction of SAR for cell cytotoxicity based on the IC_{50} results for compound **2** derivatives.

25 and **32** displayed the best cytotoxicity profile, they were not the most potent inhibitors of topoisomerase I, bearing instead an inhibitory potential similar to CPT for this enzyme. This lack of correlation between the topoisomerase I inhibitory effect and the cytotoxicity of the different tested compounds suggested that there could be multiple mechanisms responsible for the cytostatic activity of these compounds, one of them being the inhibition of the herein assayed enzyme.

3. Conclusions

In conclusion, the cytotoxic activity of semisynthetic lupane-type carbamate and *N*-acylheterocyclic bearing derivatives has been investigated and SAR has been established. The overall results suggest that these new lupane-type derivatives can inhibit the growth of various cancer cell lines at micromolar concentrations and are promising new experimental anticancer agents. Moreover the cytotoxicity of these compounds appears to be selective seeing that non-tumoral BJ cells tolerated substantially higher doses of these compounds than tumor cells. From the library of compounds synthesized, 28-(1*H*-triazol-1-yl)-28-oxo-lup-20(29)-en-3β-yl-1*H*-imidazole-1-carboxylate **25** and 2-hydroxy-28-(1*H*-triazol-1-yl)-lup-1,20(29)-dien-3,28-dione **32** were the most potent towards the tumor cells tested. Compounds **7**, **15**, **18**, **23**, **25**, **31**, and **32** showed topoisomerase I inhibitory activity comparable to or better than CPT.

Work is currently in progress in our laboratory in order to elucidate the exact mechanism of the anticancer action of these lupane-type derivatives and results will be reported in due course.

Table 3
Catalytic inhibition of topoisomerase I by compound **1** (Group I) and **2** (Group II) derivatives

Group	Compound	Topoisomerase I inhibition[a] (100 μM)
I	**3**	0
	4	1+
	5	0
	6	0
	7	2+
	15	2+
	17	0
	18	2+
	19	0
	20	0
II	**23**	3+
	24	1+
	25	2+
	26	0
	27	0
	29	0
	31	3+
	32	2+
	CPT	2+

DNA relaxation assay was carried out as described in Section 4.
[a] Topoisomerase inhibitory activity was scored as 3+ (strong), 2+ (moderate), 1+ (weak), and 0 (none), with the positive control drug CPT scoring 2+.

4. Experimental

4.1. Chemistry

4.1.1. General

Compounds **1** and **2**, CBMI, CDT, AC_2O, imidazole, DMAP, KOH, *m*-CPBA, H_2SO_4, CH_3I, K_2CO_3, DDQ, *t*-BuOK and *tert*-butyl alcohol (*t*-BuOH) were purchased from Sigma–Aldrich Co. (St. Louis, Missouri), whereas solvents were obtained from VWR (Portugal). For thin layer chromatography (TLC) analysis Kieselgel $60HF_{254}$/Kieselgel 60G was used and FCC was performed using Kieselgel 60 (230–400 mesh, Merk). Melting points were determined using a BUCHI melting point B-540 apparatus and are uncorrected. IR spectra were obtained using a JASCO FT/IR-420 spectrophotometer. NMR spectra were recorder on a Bruker Digital NMR-Avance 300 apparatus and on a Bruker Digital NMR-Avance 400 apparatus in $CDCl_3$ with Me_4Si as the internal standard. Elucidation of the chemical structures was based on ^1H, ^{13}C, DEPT135, COSY, HMQC, and HMBC NMR experiments. Chemical shifts values (δ) are given in ppm and the coupling constants (J) are presented in Hz. Mass spectra were obtained using a Finnigan Polaris Q GC/MS Benchtop Ion Trap mass spectrometer with a direct insertion probe.

4.1.2. 3β-Hydroxy-lup-20(29)-en-28-yl-2′-methyl-1*H*-imidazole-1-carboxylate (3) and lup-20(29)-en-3β,28-di-yl-(2′-methyl-1*H*-imidazole-1-carboxylate) (5)

To a solution of compound **1** (200 mg, 0.45 mmol) in anhydrous THF (8 ml), CBMI (238 mg, 1.35 mmol) was added. After 9 h under magnetic stirring at reflux temperature and N_2 atmosphere, the

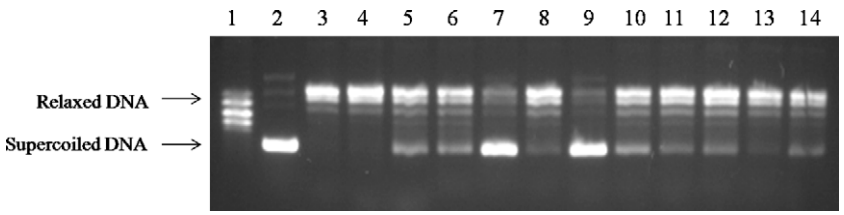


Figure 4. Inhibitory effects of the synthesized derivatives on the catalytic activity of topoisomerase I. Lane 1, relaxed DNA; lane 2, supercoiled plasmid DNA alone (250 ng); lane 3, same as lane 2 + topoisomerase I (5 U); lane 4, same as lane 3 + 0.5% DMSO; lane 5, topoisomerase I (5 U) + 100 μM CPT and DNA. Lanes 6–14, topoisomerase I (5 U) + DNA and 100 μM of the synthesized compounds: lane 6 (**15**); lane 7 (**23**); lane 8 (**24**); lane 9 (**31**); lane 10 (**7**); lane 11 (**25**), lane 12 (**18**); lane 13 (**4**); lane 14 (**32**).

reaction was completed as verified by TLC control. The reaction mixture was poured onto water (30 ml) and extracted with diethyl ether (3 × 30 ml). The combined organic extract was then washed with water (30 ml), and brine (30 ml), dried with anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give a yellowish solid. This solid was submitted to FCC with petroleum ether 40–60 °C/ethyl acetate (4:1) and afforded compound **3** (143 mg, 57%): mp (benzene) 163–165 °C; IR (film) ν_{max} 3313, 3073, 1758, 1642, 884 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.38 (d, J = 1.7 Hz, 1H, H-5′), 6.91 (d, J = 1.7 Hz, 1H, H-4′), 4.72 (br s, 1H, H-29_a), 4.62–4.59 (m, 2H, H-29_b and H-28_a), 4.17 (d, J = 10.9 Hz, 1H, H-28_b), 3.19 (dd, J = 10.8 Hz, J = 5.2 Hz, 1H, H-3α), 2.71 (s, 3H, CH_3-2′), 2.47 (m, 1H, H-19), 1.70 (s, 3H, H-30), 1.06 (s, 3H), 1.01 (s, 3H), 0.97 (s, 3H), 0.83 (s, 3H), 0.77 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 149.6 (C20), 149.5 (OCO), 147.9 (C2′), 126.5 (C4′), 118.1 (C5′), 110.2 (C29), 78.8 (C3), 67.3 (C28); EI-MS m/z (% rel. intensity): 550 (10) M^+, 189 (26), 187 (26), 133 (25), 119 (37), 107 (28), 91 (30), 83 (100), 81 (27), 79 (26). And compound **5** (112 mg, 38%): mp (acetone/*n*-hexane) 127–129 °C; IR (film) ν_{max} 3070, 1753, 1645, 880 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.36 (s, 1H, H-5′), 7.34 (s, 1H, H-5″), 6.86 (s, 1H, H-4′), 6.85 (s, 1H, H-4″), 4.73 (s, 1H, H-29_a), 4.67–4.58 (s, 3H, H-3α, H-28_a, H-29_b), 4.15 (d, J = 10.8 Hz, 1H, H-28_b), 2.66 (s, 3H, CH_3-2′), 2.65 (s, 3H, CH_3-2″), 2.49 (m, 1H, H-19), 1.71 (s, 3H, H-30), 1.08 (s, 3H), 1.02 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.90 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 150.0 (C20), 149.6 (OCO), 149.5 (OCO), 147.9 (C2′ and C2″), 127.9 and 127.8 (C4′, C4″), 118.0 (C5′ and C5″), 110.3 (C29), 85.9 (C3), 66.7 (C28); EI-MS m/z (% rel. intensity): 658 (2) M^+, 127 (13), 119 (12), 105 (10), 95 (19); 93 (13), 91 (16), 83 (100), 81 (19), 79 (11).

4.1.3. 3β-Hydroxy-lup-20(29)-en-28-yl-1*H*-triazole-1-carboxylate (4) and lup-20(29)-en-3β,28-di-yl-(1*H*-triazole-1-carboxylate (6)

The method followed that described for compound **3** but using compound **1** (200 mg, 0.45 mmol) and CDT (295 mg, 1.8 mmol) in anhydrous THF (8 ml) at reflux for 8 h. The resulting yellowish solid was purified by FCC using petroleum ether 40–60 °C/ethyl acetate (3:2) to afford compound **4** (175 mg, 72%): mp (acetone/*n*-hexane) 200–203 °C; IR (film) ν_{max} 3389, 3070, 1791, 1762, 1642, 882 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.83 (br s, 1H, H-5′), 8.09 (br s, 1H, H-3′), 4.74–4.72 (m, 2H, H-29_a and H-28_a), 4.63 (br s, 1H, H-29_b), 4.30 (d, J = 10.8 Hz, 1H, H-28_b), 3.19 (dd, J = 10.8 Hz, J = 5.2 Hz, 1H, H-3α), 2.49 (dt, J = 10.7 Hz, J = 6.0 Hz, 1H, H-19), 1.70 (s, 3H, H-30), 1.06 (s, 3H), 1.01 (s, 3H), 0.97 (s, 3H), 0.84 (s, 3H), 0.77 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 153.6 (C3′), 149.5 (C20), 147.9 (OCO), 145.4 (C5′), 110.3 (C29), 78.9 (C3), 68.4 (C28); EI-MS m/z (% rel. intensity): 537 (4) M^+, 190 (74), 189 (100), 187 (89), 133 (72), 119 (98), 107 (75), 105 (82), 91 (93), 79 (89). And compound **6** (68 mg, 24%): mp (acetone/*n*-hexane) 157–159 °C; IR (film) ν_{max} 3070, 1787, 1763, 1642, 882 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.83 (br s, 1H, H-5′), 8.80 (br s, 1H, H-5″) 8.09 (br s, 1H, H-3′), 8.08 (br s, 1H, H-3″), 4.82–4.73 (m, 3H, H-3α, H-28_a, H-29_a), 4.64 (br s, 1H, H-29_b), 4.30 (d, J = 10.8 Hz, 1H, H-28_b), 2.50 (m, 1H, H-19), 1.72 (s, 3H, H-30), 1.09 (s, 3H), 1.02 (s, 3H), 1.00 (s, 3H), 0.98 (s, 3H), 0.92 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 153.6 and 153.5 (C3′, C3″), 149.4 (C20), 147.9 (OCO), 147.2 (OCO), 145.4 (C5′), 145.3 (C5″), 110.3 (C29), 87.7 (C3), 69.3 (C28); EI-MS m/z (% rel. intensity): 632 (4) M^+, 189 (86), 187 (62), 133 (67), 119 (79), 107 (60), 105 (83), 95 (67), 93 (74), 91 (100).

4.1.4. 28-Hydroxy-lup-20(29)-en-3β-yl-1*H*-triazole-1-carboxylate (7)

A solution of compound **1** (200 mg, 0.45 mmol) and CDT (443 mg, 2.70 mmol) in anhydrous THF (8 ml) was refluxed for 10 h to afford compound **6**. Silica gel (200 mg) was added and this mixture was stirred at room temperature for 15 h. The solid was filtered off and the filtrate was poured onto water (30 ml) and extracted with diethyl ether (3 × 30 ml). The organic phase was then washed with water (30 ml), and brine (30 ml), dried with Na_2SO_4, filtered, and evaporated to dryness. The crude product was purified by FCC eluting with petroleum ether 40–60 °C/ethyl acetate (3:2) to yield compound **7** (152 mg, 63%): mp (acetone/*n*-hexane) 221–224 °C; IR (film) ν_{max} 3406, 3070, 1764, 1642, 886 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.79 (s, 1H, H-5′), 8.08 (s, 1H, H-3′), 4.79 (dd, J = 9.1, 7.5 Hz, 1H, H-3α), 4.69 (br s, 1H, H-29_a), 4.59 (br s, 1H, H-29_b), 3.80 (d, J = 10.8 Hz, 1H, H-28_a), 3.34 (d, J = 10.8 Hz, 1H, H-28_b), 2.40 (dt, J = 10.5 Hz, J = 5.8 Hz, 1H, H-19), 1.69 (s, 3H, H-30), 1.04 (s, 3H), 0.99 (s, 6H), 0.98 (s, 3H), 0.90 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 153.4 (C3′), 150.4 (C20), 147.2 (OCO), 145.3 (C5′), 109.7 (C29), 87.8 (C3), 60.4 (C28); EI-MS m/z (% rel. intensity): 537 (6) M^+, 119 (49), 107 (46), 105 (60), 93 (55), 91 (100), 81 (46), 79 (53), 77 (51), 70 (38).

4.1.5. 3β-Hydroxylup-20(29)-en-28-yl acetate (8)

Details of the synthesis of this compound were reported previously.[39] Compound **8** (323 mg, 84%): IR (film) ν_{max} 3471, 3070, 1734, 1642, 1244, 1102, 880 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.69 (s, 1H, H-29_a), 4.59 (s, 1H, H-29_b), 4.24 (d, J = 10.9 Hz, 1H, H-28_a), 3.86 (d, J = 10.9 Hz, 1H, H-28_b), 3.18 (dd, J = 11.0 Hz, J = 4.7 Hz, 1H, H-3α), 2.45 (dt, J = 10.9 Hz, J = 5.8 Hz, 1H, H-19), 2.07 (s, 3H, $OCOCH_3$), 1.68 (s, 3H, H-30), 1.03 (s, 3H), 0.97 (s, 3H), 0.96 (s, 3H), 0.82 (s, 3H), 0.76 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 171.6 ($OCOCH_3$), 150.2 (C20), 109.9 (C29), 78.9 (C3), 62.8 (C28); EI-MS m/z (% rel. intensity): 484 (12) M^+, 203 (54), 189 (100), 187 (71), 147 (46), 145 (49), 133 (67), 119 (61), 105 (69), 91 (56).

4.1.6. 28-Acetoxy-lup-20(29)-en-3β-yl-2′-methyl-1*H*-imidazole-1-carboxylate (9)

The method followed that described for compound **3** but using compound **8** (242 mg, 0.5 mmol) and CBMI (176 mg, 1 mmol) in anhydrous THF (10 ml) at reflux for 9 h. The resulting white solid was purified by FCC using petroleum ether 40–60 °C/ethyl acetate (4:1) to afford compound **9** (243 mg, 82%): mp (acetone/*n*-hexane) 173–175 °C; IR (film) ν_{max} 3070, 1740, 1642, 882 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.36 (d, J = 1.5 Hz, 1H, H-5′), 6.90 (d, J = 1.5 Hz, 1H, H-4′), 4.69–4.64 (m, 2H, H-3α and H-29_a), 4.60 (br s, 1H, H-29_b), 4.26 (d, J = 11.0 Hz, 1H, H-28_a), 3.85 (d, J = 11.0 Hz, 1H, H-28_b), 2.69 (s, 3H, CH_3-2′), 2.45 (dt, J = 10.7 Hz, J = 5.7 Hz, 1H, H-19), 2.08 (s, 3H, $OCOCH_3$), 1.69 (s, 3H, H-30), 1.05 (s, 3H), 0.99 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.89 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.6 ($OCOCH_3$), 150.0 (C20), 149.1 (OCO), 147.9 (C2′), 126.8 (C4′), 118.1 (C5′), 109.9 (C29), 86.4 (C3), 62.7 (C28); EI-MS m/z (% rel. intensity): 592 (6) M^+, 467 (100), 407 (58), 107 (45), 105 (56), 95 (70), 91 (52), 83 (40), 81 (61), 67 (53).

4.1.7. 28-Acetoxy-lup-20(29)-en-3β-yl-1*H*-triazole-1-carboxylate (10)

The method followed that described for compound **3** but using compound **8** (242 mg, 0.5 mmol) and CDT (246 mg, 1.5 mmol) in anhydrous THF (10 ml) at reflux for 8 h. The resulting white solid was purified by FCC using petroleum ether 40–60 °C/ethyl acetate (4:1) to afford compound **10** (256 mg, 88%): mp (acetone/*n*-hexane) 221–224 °C; IR (film) ν_{max} 3070, 1787, 1766, 1733 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.79 (s, 1H, H-5′), 8.08 (s, 1H, H-3′), 4.79 (dd, J = 9.0 Hz, J = 7.5 Hz, 1H, H-3α), 4.69 (br s, 1H, H-29_a), 4.60 (br s, 1H, H-29_b), 4.26 (d, J = 10.9 Hz, 1H, H-28_a), 3.85 (d, J = 10.9 Hz, 1H, H-28_b), 2.45 (dt, J = 10.9 Hz, J = 5.8 Hz, 1H, H-19), 2.07 (s, 3H, $OCOCH_3$), 1.69 (s, 3H, H-30), 1.05 (s, 3H), 0.99 (s, 3H), 0.98 (s, 3H), 0.97 (s, 3H), 0.90 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.6 ($OCOCH_3$), 153.5 (C3′), 150.1 (C20), 147.3 (OCO), 145.3 (C5′), 109.9 (C29), 87.9 (C3), 62.8 (C28); EI-MS m/z (% rel. inten-

sity): 579 (4) M^+, 203 (51), 189 (84), 187 (69), 159 (47), 119 (56), 107 (57), 105 (75), 91 (100), 79 (54).

4.1.8. 28-Hydroxylup-20(29)-en-3β-yl acetate (11)

Details of the synthesis of this compound were reported previously.[39] Compound **11** (259 mg, 89%): IR (film) ν_{max} 3440, 3070, 1729, 1642, 1246, 978, 882 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.68 (s, 1H, H-29_a), 4.58 (s, 1H, H-29_b), 4.47 (dd, J = 10.3 Hz, J = 5.7 Hz, 1H, H-3α), 3.79 (d, J = 10.6 Hz, 1H, H-28_a), 3.33 (d, J = 10.7 Hz, 1H, H-28_b), 2.39 (dt, J = 10.7 Hz, J = 5.9 Hz, 1H, H-19), 2.04 (s, 3H, $OCOCH_3$), 1.69 (s, 3H, H-30), 1.02 (s, 3H), 0.97 (s, 3H), 0.85 (s, 6H), 0.84 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 171.1 ($OCOCH_3$), 150.5 (C20), 109.7 (C29), 80.9 (C3), 60.5 (C28); EI-MS m/z (% rel. intensity): 484 (9) M^+, 203 (71), 189 (100), 187 (55), 107 (61), 105 (52), 95 (77), 91 (67), 81 (60), 79 (85).

4.1.9. 3β-Acetoxy-lup-20(29)-en-28-yl-2′-methyl-1*H*-imidazole-1-carboxylate (12)

The method followed that described for compound **3** but using compound **11** (242 mg, 0.5 mmol) and CBMI (176 mg, 1 mmol) in anhydrous THF (10 ml) at reflux for 7 h. The resulting white solid was purified by FCC using petroleum ether 40–60 °C/ethyl acetate (4:2) to afford compound **12** (253 mg, 86%): mp (acetone/*n*-hexane) 99–102 °C; IR (film) ν_{max} 3073, 1757, 1731, 1642, 1245, 882 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.38 (d, J = 1.8 Hz, 1H, H-5′), 6.90 (d, J = 1.8 Hz, 1H, H-4′), 4.72 (br s, 1H, H-29_a), 4.62–4.58 (m, 2H, H-28_a and H-29_b), 4.47 (dd, J = 10.2 Hz, J = 5.8 Hz, 1H, H-3α), 4.16 (d, J = 10.8 Hz, 1H, H-28_b), 2.70 (s, 3H, CH_3-2′), 2.48 (dt, J = 10.7 Hz, J = 5.7 Hz, 1H, H-19), 2.05 (s, 3H, $OCOCH_3$), 1.71 (s, 3H, H-30), 1.06 (s, 3H), 0.99 (s, 3H), 0.86 (s, 3H), 0.85 (s, 3H), 0.84 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.9 ($OCOCH_3$), 149.7 (C20), 149.5 (OCO), 147.9 (C2′), 127.1 (C4′), 118.1 (C5′), 110.2 (C29), 80.8 (C3), 67.0 (C28); EI-MS m/z (% rel. intensity): 592 (14) M^+, 189 (20), 187 (16), 145 (15), 119 (22), 107 (19), 105 (25), 91 (28), 83 (100), 79 (16).

4.1.10. 3β-Acetoxy-lup-20(29)-en-28-yl-1*H*-triazole-1-carboxylate (13)

The method followed that described for compound **3** but using compound **11** (242 mg, 0.5 mmol) and CDT (246 mg, 1.5 mmol) in anhydrous THF (10 ml) at reflux for 6 h. The resulting white solid was purified by FCC using petroleum ether 40–60 °C/ethyl acetate (4:2) to afford compound **13** (213 mg, 74%): mp (acetone/*n*-hexane) 111–114 °C; IR (film) ν_{max} 3070, 1795, 1770, 1729, 1642, 1247, 882 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.82 (s, 1H, H-5′), 8.09 (s, 1H, H-3′), 4.75–4.72 (m, 2H, H-28_a and H-29_a), 4.63 (br s, 1H, H-29_b), 4.47 (dd, J = 10.2 Hz, J = 5.8 Hz, 1H, H-3α), 4.30 (d, J = 10.7 Hz, 1H, H-28_b), 2.49 (dt, J = 10.6 Hz, J = 6.0 Hz, 1H, H-19), 2.05 (s, 3H, $OCOCH_3$), 1.71 (s, 3H, H-30), 1.06 (s, 3H), 1.00 (s, 3H), 0.86 (s, 3H), 0.85 (s, 3H), 0.84 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 171.0 ($OCOCH_3$), 153.6 (C3′), 149.5 (C20), 147.9 (OCO), 145.4 (C5′), 110.3 (C29), 80.8 (C3), 68.4 (C28); EI-MS m/z (% rel. intensity): 579 (6) M^+, 202 (58), 189 (88), 187 (78), 145 (59), 119 (78), 107 (62), 105 (77), 91 (100), 79 (58).

4.1.11. 3β,28-Dihydroxy-(20*R*)-lupan-29-al (14)

Details of the synthesis of these compounds were reported previously.[40,51] Compound **14** (354 mg, 39%): IR (film) ν_{max} 3393, 1714 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 9.86 (d, J = 2.0 Hz, 1H, H-29), 3.77 (d, J = 10.8 Hz, 1H, H-28_a), 3.26 (d, J = 10.8 Hz, 1H, H-28_b), 3.20 (dd, J = 10.9 Hz, J = 5.1 Hz, 1H, H-3α) 2.60 (m, 1H, H-20), 1.10 (d, J = 6.9 Hz, 3H, H-30), 1.03 (s, 3H), 0.98 (s, 3H), 0.95 (s, 3H), 0.84 (s, 3H), 0.77 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 206.8 (CHO), 78.9 (C3), 60.2 (C28); EI-MS m/z (% rel. intensity): 458 (2) M^+, 369 (100), 207 (43), 192 (51), 189 (72), 161 (67), 133 (31), 121 (31), 107 (36), 95 (33).

4.1.12. 3β-Hydroxy-(20*R*)-lupan-29-oxo-28-yl-2′-methyl-1*H*-imidazole-1-carboxylate (15)

The method followed that described for compounds **3** but using compound **14** (194 mg, 0.42 mmol) and CBMI (148 mg, 0.84 mmol) in anhydrous THF (8 ml), at reflux for 6 h. The resulting white solid was purified by FCC eluting with petroleum ether 40–60 °C/ethyl acetate (3:2) to afford compound **15** (179 mg, 75%): mp (acetone/*n*-hexane) 132–134 °C; IR (film) ν_{max} 3365, 1759, 1716 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.85 (d, J = 1.8 Hz, 1H, H-29), 7.35 (d, J = 1.7 Hz, 1H, H-5′), 6.87 (d, J = 1.7 Hz, 1H, H-4′), 4.55 (d, J = 10.9 Hz, 1H, H-28_a), 4.09 (d, J = 10.9 Hz, 1H, H-28_b), 3.21 (dd, J = 10.4 Hz, J = 4.6 Hz, 1H, H-3α), 2.66 (s, 3H, CH_3-2′), 1.16 (d, J = 7.0 Hz, 3H, H-30), 1.07 (s, 3H), 0.98 (s, 6H), 0.85 (s, 3H), 0.77 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 206.3 (C29), 149.2 (OCO), 144.3 (C2′), 130.1 (C4′), 118.1 (C5′), 78.9 (C3), 66.0 (C28); EI-MS m/z (% rel. intensity): 566 (8) M^+, 189 (67), 161 (72), 147 (72), 133 (83), 105 (80), 91 (93), 83 (68), 81 (100), 79 (62).

4.1.13. 30-Methoxylup-20-(29)-en-3β,28-diol (16)

Details of the synthesis of these compounds were reported previously.[41] Compound **16** (1.4 g, 64%): IR (film) ν_{max} 3347, 3073, 1645 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.92 (s, 1H, H-29_a), 4.91 (s, 1H, H-29_b), 3.86 (br s, 2H, H-30), 3.78 (d, J = 10.5 Hz, 1H, H-28_a), 3.35 (s, 3H, OCH_3), 3.31 (d, J = 10.5 Hz, 1H, H-28_b), 3.18 (dd, J = 10.8 Hz, J = 5.2 Hz, 1H, H-3α), 2.28 (dt, J = 10.8 Hz, J = 5.4 Hz, 1H, H-19) 1.02 (s, 3H), 0.98 (s, 3H), 0.97 (s, 3H), 0.82 (s, 3H), 0.76 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 150.9 (C20), 109.0 (C29), 78.9 (C3), 74.8 (C30), 60.2 (C28), 58.3 (OCH_3); EI-MS m/z (% rel. intensity): 473 (25) M^+, 201 (93), 189 (86), 187 (100), 145 (75), 131 (66), 121 (71), 119 (73), 95 (66), 81 (69).

4.1.14. 3β-Hydroxy-30-methoxylup-20(29)-en-28-yl-2′-methyl-1*H*-imidazole-1-carboxylate (17) and 30-methoxylup-20(29)-en-3β,28-di-yl-(2′-methyl-1*H*-imidazole-1-carboxylate) (19)

The method followed that described for compound **3** but using compound **16** (213 mg, 0.45 mmol) and CBMI (238 mg, 1.35 mmol) in anhydrous THF (8 ml), at reflux for 7 h. The resulting white solid was purified by FCC eluting with petroleum ether 40–60 °C/ethyl acetate (2:1) to afford compound **17** (193 mg, 74%): mp (acetone/*n*-hexane) 109–112 °C; IR (film) ν_{max} 3389, 3070, 1759, 1645 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.37 (d, J = 1.7 Hz, 1H, H-5′), 6.88 (d, J = 1.7 Hz, 1H, H-4′), 4.96 (br s, 1H, H-29_a), 4.94 (br s, 1H, H-29_b), 4.58 (d, J = 10.4 Hz, 1H, H-28_a), 4.14 (d, J = 10.4 Hz, 1H, H-28_b), 3.88 (s, 2H, H-30), 3.36 (s, 3H, OCH_3), 3.19 (dd, J = 10.8 Hz, J = 5.1 Hz, 1H, H-3α), 2.68 (s, 3H, CH_3-2′), 2.38 (dt, J = 11.1 Hz, J = 5.3 Hz, 1H, H-19), 1.06 (s, 3H), 1.01 (s, 3H), 0.97 (s, 3H), 0.83 (s, 3H), 0.76 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 150.4 (C20), 149.8 (OCO), 147.9 (C2′), 127.4 (C4′), 118.0 (C5′), 109.6 (C29), 78.8 (C3), 74.9 (C30), 66.7 (C28), 58.3 (OCH_3); EI-MS m/z (% rel. intensity): 580 (9) M^+, 189 (25), 187 (28), 119 (27), 107 (23), 105 (25), 91 (24), 83 (100), 81 (27), 79 (25). And compound **19** (65 mg, 21%): mp (acetone/*n*-hexane) 116–118 °C; IR (film) ν_{max} 3070, 1754, 1642 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.36 (br s, 1H, H-5′), 7.34 (br s, 1H, H-5″), 6.87 (br s, 1H, H-4′), 6.86 (s, 1H, H-4″), 4.98 (s, 1H, H-29_a), 4.95 (s, 1H, H-29_b), 4.65 (dd, J = 11.1 Hz, J = 4.8 Hz, 1H, H-3α), 4.58 (d, J = 10.8 Hz, 1H, H-28_a), 4.14 (d, J = 10.8 Hz, 1H, H-28_b), 3.89 (s, 2H, H-30), 3.37 (s, 3H, OCH_3), 2.66 (s, 3H, CH_3-2′), 2.65 (s, 3H, CH_3-2″), 2.39 (dt, J = 11.3 Hz, J = 5.5 Hz, 1H, H-19), 1.08 (s, 3H), 1.03 (s, 3H), 0.95 (s, 6H), 0.90 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 150.4 (C20), 149.9 (OCO), 149.5 (OCO), 147.9 (C2′ and C2″), 127.9 (4″), 127.7 (C4′), 118.0 (C5′ and C5″), 109.8 (C29), 85.9 (C3), 75.1 (C30), 66.5 (C28), 58.4 (OCH_3); EI-MS m/z (% rel. intensity): 688 (11) M^+, 187 (23), 185 (22), 145 (32), 119 (22), 105 (26), 95 (26), 91 (46), 83 (100), 81 (27).

4.1.15. 3β-Hydroxy-30-methoxylup-20(29)-en-28-yl-1*H*-triazole-1-carboxylate (18) and 30-methoxylup-20(29)-en-3β,28-di-yl-(1*H*-triazole-1-carboxylate) (20)

The method followed that described for compound **3** but using compound **16** (213 mg, 0.45 mmol) and CDT (295 mg, 1.8 mmol) in anhydrous THF (8 ml), at reflux for 7 h. The resulting white solid was purified by FCC eluting with petroleum ether 40–60 °C/ethyl acetate (1:1) to afford compound **18** (147 mg, 57%): mp (acetone/*n*-hexane) 137–140 °C; IR (film) ν_{max} 3414, 3070, 1782, 1766, 1645 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.82 (br s, 1H, H-5′), 8.09 (br s, 1H, H-3′), 4.97 (br s, 1H, H-29_a), 4.94 (br s, 1H, H-29_b), 4.72 (d, J = 10.6 Hz, 1H, H-28_a), 4.29 (d, J = 10.6 Hz, 1H, H-28_b), 3.88 (s, 2H, H-30), 3.36 (s, 3H, OCH_3), 3.19 (dd, J = 10.8 Hz, J = 5.0 Hz, H-3α), 2.39 (dt, J = 11.2 Hz, J = 5.3 Hz, 1H, H-19), 1.06 (s, 3H), 1.01 (s, 3H), 0.97 (s, 3H), 0.83 (s, 3H), 0.76 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 153.6 (C3′), 150.3 (C20), 147.9 (OCO), 145.4 (C5′), 109.7 (C29), 78.9 (C3), 74.9 (C30), 68.1 (C28), 58.3 (OCH_3); EI-MS m/z (% rel. intensity): 567 (13) M+, 201 (71), 189 (78), 187 (70), 145 (72), 131 (61), 119 (71), 105 (82), 91 (100), 79 (57). And compound **20** (52 mg, 17%): mp (acetone/*n*-hexane) 172–175 °C; IR (film) ν_{max} 3070, 1782, 1763, 1646 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.83 and 8.79 (both s, each 1H, H-5′, H-5″), 8.09 and 8.08 (both s, each 1H, H-3′, H-3″), 4.98 (br s, 1H, H-29_a), 4.96 (br s, 1H, H-29_b), 4.82–4.71 (m, 2H, H-3α and H-28_a), 4.29 (d, J = 10.8 Hz, 1H, H-28_b), 3.89 (s, 2H, H-30), 3.37 (s, 3H, OCH_3), 2.40 (dt, J = 11.1 Hz, J = 5.4 Hz, 1H, H-19), 1.09 (s, 3H), 1.03 (s, 3H), 0.99 (s, 3H), 0.98 (s, 3H), 0.91 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 153.6 and 153.5 (C3′, C3″), 150.3 (C20), 147.9 (OCO), 147.2 (OCO), 145.4 and 145.3 (C5′, C5″), 109.8 (C29), 87.7 (C3), 74.9 (C30), 68.1 (C28), 58.3 (OCH_3); EI-MS m/z (% rel. intensity): 663 (15) M^+, 201 (70), 119 (81), 107 (66), 105 (80), 95 (75), 91 (100), 81 (94), 79 (82), 67 (74).

4.1.16. Methyl 3β-hydroxylup-20(29)-en-28-oate (21)

Details of the synthesis of these compounds were reported previously.[38] Compound **21** (129 mg, 83%): IR (film) ν_{max} 3320, 3070, 1720, 1643 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.71 (br s, 1H, H-29_a), 4.58 (br s, 1H, H-29_b), 3.67 (s, 3H, $COOCH_3$), 3.18 (dd, 1H, J = 10.9 Hz, J = 4.5 Hz, H-3α) 2.43 (m, 1H, H-19), 1.69 (s, 3H, H-30), 0.96 (s, 3H), 0.94 (3H), 0.92 (s, 3H), 0.82 (s, 3H), 0.75 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 177.2 (C28), 149.7 (C20), 110.1 (C29), 80.6 (C3); EI-MS m/z (% rel. intensity): 470 (25) M^+, 286 (26), 253 (52), 247 (29), 203 (36), 192 (100), 189 (100), 175 (64), 119 (47), 105 (51).

4.1.17. 3-Oxolup-20(29)-en-28-oic acid (22)

Details of the synthesis of this compound were reported previously.[52] Compound **22** (685 mg, 67%): IR (film) ν_{max} 3070, 1703, 1686, 1642 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.77 (br s, 1H, H-29_a), 4.64 (br s, 1H, H-29_b), 3.04 (dt, J = 10.7 Hz, J = 4.3 Hz, 1H, H-19), 1.72 (s, 3H, H-30), 1.09 (s, 3H), 1.04 (s, 3H), 1.02 (s, 3H), 0.99 (s, 3H), 0.95 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 218.2 (C3), 182.2 (C28), 150.3 (C20), 109.7 (C29); EI-MS m/z (% rel. intensity): 454 (19) M^+, 408 (24), 393 (20), 248 (85), 189 (100), 175 (62), 133 (55), 119 (76), 105 (69), 79 (52).

4.1.18. 3β-(2′-Methyl-1*H*-imidazole-1-carbonyloxy)-lup-20(29)-en-28-oic acid (23) and 28-(2′-methyl-1*H*-imidazol-1-yl)-28-oxo-lup-20(29)-en-3β-yl-2′-methyl-1*H*-imidazole-1-carboxylate (24)

The method followed that described for compound **3** but using compound **2** (297 mg, 0.65 mmol) and CBMI (573 mg, 3.25 mmol) in anhydrous THF (12 ml) at reflux for 8 h. The resulting yellowish solid was purified by FCC using petroleum ether 40–60 °C/ethyl acetate (3:2) to afford compound **23** (283 mg, 77%): mp (acetone/*n*-hexane) 170–174 °C; IR (film) ν_{max} 3070, 1756, 1703, 1642, 883 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.35 (d, J = 1.6 Hz, 1H, H-5′), 6.88 (d, J = 1.6 Hz, 1H, H-4′), 4.75 (br s, 1H, H-29_a), 4.68–4.61 (m, 2H, H-3α and H-29_b), 3.05 (dt, J = 10.7 Hz, J = 4.3 Hz, 1H, H-19), 2.67 (s, 3H, CH_3-2′), 1.70 (s, 3H, H-30), 0.99 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.94 (s, 3H), 0.89 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 180.6 (C28), 150.6 (C20), 149.3 (OCO), 147.9 (C2′), 127.3 (C4′), 118.0 (C5′), 109.6 (C29), 86.2 (C3); EI-MS m/z (% rel. intensity): 564 (10) M+, 439 (100), 393 (61), 203 (58), 123 (50), 121 (50), 109 (57), 95 (74), 83 (66), 81 (97). And compound **24** (90 mg, 22%): mp (acetone/*n*-hexane) 164–166 °C; IR (film) ν_{max} 3070, 1752, 1722, 1642 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.39 (br s, 1H, H-5′), 7.34 (br s, 1H, H-5″), 6.86 (br s, 2H, H-4′ and H4″), 4.79 (br s, 1H, H-29_a), 4.66–4.62 (m, 1H, H-3α, H-29_b), 3.06 (dt, J = 11.0 Hz, J = 4.3 Hz, 1H, H-19), 2.65 (s, 3H, CH_3-2′), 2.63 (s, 3H, CH_3-2″), 1.73 (s, 3H, H-30), 1.01 (s, 3H), 0.96 (s, 3H), 0.94 (s, 6H), 0.89 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 175.2 (C28), 150.0 (C20), 149.5 (OCO), 148.9 and 147.9 (C2′, C2″), 127.7 and 127.1 (C4′, C4″), 118.0 (C5′ and C5″), 110.1 (C29), 85.9 (C3); EI-MS m/z (% rel. intensity): 628 (2) M^+, 519 (18), 127 (30), 119 (17), 105 (20), 95 (22), 93 (18), 91 (19), 83 (100), 81 (24).

4.1.19. 28-(1*H*-Triazol-1-yl)-28-oxo-lup-20(29)-en-3β-yl-1*H*-triazole-1-carboxylate (25)

The method followed that described for compound **3** but using compound **2** (297 mg, 0.65 mmol) and CDT (640 mg, 3.9 mmol) in anhydrous THF (12 ml) at reflux for 7 h. The resulting yellowish solid was purified by FCC using petroleum ether 40–60 °C/ethyl acetate (3:2) to afford compound **25** (322 mg, 82%): mp (acetone/*n*-hexane) 253–256 °C; IR (film) ν_{max} 3070, 1787, 1762, 1734, 1642 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.93 (s, 1H, H-5′), 8.79 (s, 1H, H-5″), 8.07 (s, 1H, H-3″), 8.00 (s, 1H, H-3′), 4.81–4.78 (m, 2H, H-3α and H-29_a), 4.66 (br s, 1H, H-29_b), 2.96 (m, 1H, H-19), 1.73 (s, 3H, H-30), 1.02 (s, 3H), 0.99 (s, 3H), 0.97 (s, 3H), 0.95 (s, 3H), 0.92 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.4 (C28), 153.6 (C3″), 152.1 (C3′), 149.9 (C20), 147.3 (OCO), 145.3 (C5″), 145.1 (C5′), 110.1 (C29), 87.8 (C3); EI-MS m/z (% rel. intensity): 602 (10) M^+, 202 (70), 190 (86), 189 (93), 188 (100), 187 (65), 173 (72), 105 (62), 91 (85), 70 (84).

4.1.20. Methyl 3β-(2′-methyl-1*H*-imidazole-1-carbonyloxy)-lup-20(29)-en-28-oate (26)

The method followed that described for compound **3** but using compound **21** (100 mg, 0.2 mmol) and CBMI (70 mg, 0.4 mmol) in anhydrous THF (4 ml), at reflux for 9 h. The crude product was purified by FCC eluting with petroleum ether 40–60 °C/ethyl acetate (3:2) to yield compound **26** (115 mg, 93%): mp (acetone/*n*-hexane) 205–207 °C; IR (film) ν_{max} 3073, 1752, 1728, 1642 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.34 (br s, 1H, H-5′), 6.85 (br s, 1H, H-4′), 4.74 (s, 1H, H-29_a), 4.65 (dd, J = 11.2 Hz, J = 5.1 Hz, 1H, H-3α), 4.61 (s, 1H, H-29_b), 3.67 (s, 3H, $COOCH_3$), 3.00 (dt, J = 10.4 Hz, J = 3.8 Hz, 1H, H-19), 2.65 (s, 3H, CH_3-2′), 1.69 (s, 3H, H-30), 0.98 (s, 3H), 0.95 (s, 3H), 0.94 (s, 3H), 0.93 (s, 3H), 0.88 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 176.6 (C28), 150.5 (C20), 149.5 (OCO), 147.9 (C2′), 127.7 (C4′), 118.0 (C5′), 109.7 (C29), 86.0 (C3); EI-MS m/z (% rel. intensity): 578 (13) M^+, 393 (100), 189 (68), 119 (56), 105 (64), 95 (71), 91 (75), 83 (87), 81 (70), 79 (52).

4.1.21. 28-(2′-Methyl-1*H*-imidazol-1-yl)-lup-20(29)-en-3,28-dione (27)

The method followed that described for compound **3** but using compound **22** (205 mg, 0.45 mmol) and CBMI (238 mg, 1.35 mmol) in anhydrous THF (8 ml), at reflux for 8 h. The crude product was purified by FCC eluting with petroleum ether 40–60 °C/ethyl acetate (3:2) to yield compound **27** (197 mg, 84%): mp (benzene) 196–198 °C; IR (film) ν_{max} 3073, 1721, 1703, 1642 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.40 (d, J = 1.5 Hz, 1H, H-5′), 6.86 (d, J = 1.5 Hz, 1H, H-4′), 4.78 (br s, 1H, H-29_a), 4.65 (br s, 1H, H-29_b),

3.06 (dt, J = 11.1 Hz, J = 4.6 Hz, 1H, H-19), 2.63 (s, 3H, CH_3-2′), 1.72 (s, 3H, H-30), 1.06 (s, 3H), 1.02 (s, 3H), 1.00 (s, 3H), 0.98 (s, 3H), 0.94 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 218.0 (C3), 175.2 (C28), 149.9 (C20), 148.9 (C2′), 126.9 (C4′), 117.9 (C5′), 110.0 (C29); EI-MS m/z (% rel. intensity): 518 (2) M^+, 409 (100), 245 (54), 203 (50), 189 (76), 119 (50), 105 (58), 91 (71), 81 (72), 79 (49).

4.1.22. 3-Oxolup-1,20(29)-dien-28-oic acid (28)

Details of the synthesis of these compounds were reported previously.[38] Compound **28** (179 mg, 45%); IR (film)ν_{max} 3070, 1730, 1689, 1645 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.11 (d, J = 10.3 Hz, 1H, H-1), 5.80 (d, J = 10.3 Hz, 1H, H-2), 4.76 (s, 1H, H-29$_a$), 4.63 (s, 1H, H-29$_b$), 3.03 (m, 1H, H-19), 1.70 (s, 3H, H-30), 1.13 (s, 3H), 1.07 (s, 3H), 1.06 (s, 3H), 1.02 (s, 3H), 0.99 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 205.9 (C3), 181.7 (C28), 160.1 (C1), 150.2 (C20), 125.1 (C2), 109.9 (C29); EI-MS m/z (% rel. intensity): 452 (17) M^+, 213 (100), 150 (39), 137 (34), 95 (31), 91 (42), 81 (36), 79 (41), 77 (29), 67 (34).

4.1.23. 28-(2′-Methyl-1*H*-imidazol-1-yl)-lup-1,20(29)-dien-3,28-dione (29)

The method followed that described for compounds **3** but using compound **28** (204 mg, 0.45 mmol) and CBMI (238 mg, 1.35 mmol) in anhydrous THF (8 ml), at reflux for 9 h. The crude product was purified by FCC eluting with petroleum ether 40–60 °C/ethyl acetate (3:1) to yield compound **29** (202 mg, 87%): mp (acetone/n-hexane) 99–103 °C; IR (film) ν_{max} 3073, 1760, 1721, 1668, 1642 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.42 (br s, 1H, H-5′), 7.12 (d, J = 10.3 Hz, 1H, H-1), 6.90 (br s, 1H, H-4′), 5.80 (d, J = 10.3 Hz, 1H, H-2), 4.80 (s, 1H, H-29$_a$), 4.67 (s, 1H, H-29$_b$) 3.06 (dt, J = 10.8 Hz, J = 4.3 Hz, 1H, H-19), 2.67 (s, 3H, CH_3-2′), 1.73 (s, 3H, H-30), 1.12 (s, 3H), 1.07 (s, 6H), 1.01 (s, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 205.4 (C3), 175.0 (C28), 159.6 (C1), 149.6 (C20), 148.9 (C2′), 126.4 (C4′), 125.1 (C2), 118.0 (C5′), 110.1 (C29); EI-MS m/z (% rel. intensity): 516 (3) M^+, 408 (37), 407 (100), 243 (56), 205 (37), 189 (42), 135 (43), 105 (37), 91 (44), 81 (36).

4.1.24. 2-Hydroxy-3-oxolup-1,20(29)-dien-28-oic acid (30)

Details of the synthesis of these compounds were reported previously.[43] Compound **30** (273 mg, 73%): ν_{max} 3389, 3073, 1730, 1698, 1669, 1645 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.45 (s, 1H, H-1), 4.75 (s, 1H, H-29$_a$), 4.64 (s, 1H, H-29$_b$), 3.02 (m, 1H, H-19), 1.70 (s, 3H, H-30), 1.20 (s, 3H), 1.13 (s, 3H), 1.10 (s, 3H), 1.00 (s, 3H), 0.98 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 201.2 (C3), 182.4 (C28), 150.1 (C20), 143.9 (C2), 128.9 (C1), 109.9 (C29); EI-MS m/z (% rel. intensity): 469 (11) M^+, 321 (43), 213 (100), 189 (32), 150 (45), 136 (29), 91 (63), 80 (34), 75 (54), 69 (65).

4.1.25. 2-(2′-Methyl-1*H*-imidazole-1-carbonyloxy)-3-oxolup-1,20(29)-dien-28-oic acid (31)

The method followed that described for compound **3** but using compound **30** (210 mg, 0.45 mmol) and CBMI (396 mg, 2.25 mmol) in anhydrous THF (8 ml), at reflux for 7 h. The resulting white solid was purified by FCC eluting with petroleum ether 40–60 °C/ethyl acetate (2:3) to afford compound **31** (215 mg, 83%): mp (acetone/n-hexane) 141–143 °C; IR (film) ν_{max} 3394, 3070, 1824, 1770, 1687, 1645 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.41 (d, J = 1.7 Hz, 1H, H-5′), 6.99 (s, 1H, H-1), 6.92 (d, J = 1.7 Hz, 1H, H-4′), 4.75 (s, 1H, H-29$_a$), 4.62 (s, 1H, H-29$_b$), 3.04 (dt, J = 10.9 Hz, J = 4.0 Hz, 1H, H-19), 2.66 (s, 3H, CH_3-2′), 1.69 (s, 3H, H-30), 1.21 (s, 3H), 1.16 (s, 6H), 1.05 (s, 3H), 1.01 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 196.9 (C3), 180.7 (C28), 150.2 (C20), 148.6 (OCO), 147.4 (C2), 145.7 (C2′), 142.0 (C1), 127.6 (C4′), 118.4 (C5′), 109.8 (C29); EI-MS m/z (% rel. intensity): 576 (3) M^+, 215 (100), 213 (62), 107 (35), 105 (46), 93 (35), 91 (62), 81 (34), 79 (40), 67 (34).

4.1.26. 2-Hydroxy-28-(1*H*-triazol-1-yl)-lup-1,20(29)-dien-3,28-dione (32)

The method followed that described for compound **3** but using compound **30** (300 mg, 0.70 mmol) and CDT (500 mg, 3.0 mmol) in anhydrous THF (12 ml), at reflux for 10 h. The resulting white solid was purified by FCC eluting with petroleum ether 40–60 °C/ethyl acetate (1:4) to afford compound **32** (260 mg, 77%): mp (acetone/n-hexane) 149–153 °C; IR (film) ν_{max} 1644, 1667, 1723, 1762, 3073, 3434 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.93 (s, 1H, 3′-H), 7.99 (s, 1H, 5′-H), 6.43 (s, 1H, 1-H), 4.78 (s, 1H, H-29$_b$), 4.67 (s, 1H, H-29$_a$), 2.97 (dt, J = 10,4 Hz e J = 4,0 Hz, 1H, H-19), 1.71 (s, 3H, H-30), 0.99 (3H), 1.05 (6H), 1.14 (3H), 1.19 (3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 201.1 (C3), 172.7 (C28), 152.1 (C5′), 149.5 (C20), 145.3 (C3′), 143.9 (C2), 128.7 (C1), 110.3 (C29); EI-MS m/z (% rel. intensity): 519 (23) M^+, 424 (100), 216 (89), 212 (62), 184 (59), 118 (55), 104 (43), 90 (85), 79 (43), 69 (39).

4.2. Antiproliferative activity

4.2.1. General

All cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD). RPMI 1640 medium, Dulbecco's Modified Eagle Medium (DMEM-D5796), Dulbecco's Phosphate Buffered Saline (DPBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder and dimethylsulfoxide (DMSO) were obtained from Sigma–Aldrich Co. (St. Louis, Missouri). Penicillin/Streptomycin (P/S) and L-glutamine were obtained from Gibco-BRL (Grand Island, New York). Sodium pyruvate and trypsin/EDTA (0.05%/0.02%) were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Fetal Bovine Serum (FBS) were purchased from PAA Laboratories (Pasching, Austria) and XTT kit was obtained from Roche Applied Science (Mannheim, Germany). Topoisomerase I drug screening kit was obtained from TopoGen, Inc. (Port Orange, FL).

HT-29, HeLa, PC-3 and BJ cells were routinely maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% P/S (10,000 units/10,000 μg/ml) solution. HepG2 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, 1% P/S and 1 mM of sodium pyruvate. Jurkat cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1% P/S and 2 mM L-glutamine. All cell cultures were incubated at 37 °C in an atmosphere of 5% CO_2.

4.2.2. Cell viability assay

The cell viability of HeLa, HepG2, HT-29, PC-3, and BJ cells in the presence of the synthesized compounds was determined by the MTT assay. Briefly, exponentially growing cells were plated in 96-well plates at a density of 1–8 $\times$ 10^3 cells/well, for 24 h before treatment. The growth medium was replaced with one containing either the test compounds dissolved in DMSO (final DMSO concentration <0.1%) at different concentrations or only DMSO, in triplicate wells, and cells were incubated for 72 h. After incubation with the compounds, the medium was removed, and cell viability was assessed by the MTT colorimetric assay. MTT solution (0.5 mg/ml, 100 μl) was added to each well and the plates were incubated again at 37 °C for 1 h. MTT solution was removed, formazan crystals were dissolved in DMSO (100 μl) and the absorbance was immediately read at 550 nm on an ELISA plate reader (Tecan Sunrise MR20-301, TECAN, Austria).

For Jurkat cells, the cell viability was determined by the XTT assay. Briefly, exponentially growing cells were plated in 96-well plates at a density of 5.5 $\times$ 10^3 cells/well, treated with different concentrations of compounds or vehicle (medium with DMSO) in triplicate and incubated for 72 h. After incubation the XTT labeling mixture (100 μl) was added to each of the wells and after a 4 h incubation period at 37 °C the absorbance was read at 450 nm on an ELISA plate reader.

Concentrations that inhibit cell growth by 50% (IC_{50}) compared to non-treated cells were determined by non-linear regression with GRAPHPAD PRISM software version 4.0 (GRAPHPAD Software, Inc., San Diego, CA). All the IC_{50} results represent an average of a minimum of three experiments and were expressed as means ± standard deviation (S.D.). Compound **2** was used as a positive control.

4.3. DNA relaxation assay

Topoisomerase I drug screening kit (TopoGen, Inc., Port Orange, FL) was used to determine the activity of compounds **3–7** and **15**, **17–20**, **23–27**, **29**, **31**, and **32** to block or reduce topoisomerase I DNA relaxation activity. For this assay, 250 ng of supercoiled plasmid DNA was added to the assay buffer (10 mM Tris–HCl, pH 7.9, 1 mM EDTA, 150 mM NaCl, 0.1% BSA, 0.1 mM spermidine, and 5% glycerol), followed by test compounds or CPT at a final concentration of 100 μM, or vehicle alone. Equal concentrations of DMSO (0.5%) were maintained in each reaction mixture so as not to produce solvent-mediated inhibition of topoisomerase I activity. The reaction was started by the addition of topoisomerase I (5 U) to the assay mixture, and allowed to proceed at 37 °C for 30 min and then terminated by addition of 1% sodium dodecyl sulfate (SDS). The reaction mixtures were digested with proteinase K (50 μg/ml) for 30 min at 37 °C followed by chloroform–isoamyl alcohol (20 μl) extraction. The aqueous phase was collected, and DNA was separated by electrophoresis in a 1% agarose gel in TAE buffer (40 mM Tris–acetate, 1 mM EDTA) at 80 V for 3 h at room temperature. The agarose gels were stained with ethidium bromide (0.5 μg/ml) and extensively destained in water, and the DNA bands were visualized by transillumination with UV light (320 nm) and photographed. DNA bands were quantified and calculated from gel photographs using the molecular imaging software IMAGEQUANT 5.0 (Molecular Dynamics, Inc., Sunnyvale, CA). The ability of each drug to inhibit topoisomerase I activity was scored as 3+ (strong), 2+ (moderate), 1+ (weak), and 0 (none), with the positive control drug CPT scoring 2+.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.04.085.

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