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Synthesis and structure–activity relationship study of cytotoxic lupane-type 3β-*O*-monodesmosidic saponins with an extended C-28 side chain

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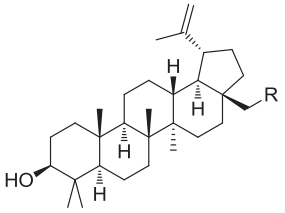
ABSTRACT

A concise synthesis of lupane triterpenes with an elongated carbon chain at the C-28 position, as well as saponins containing D-mannose, L-arabinose, and L-rhamnose moieties at the C-3 position is described. The overall synthesis of the new triterpenes involved seven linear steps starting from natural betulin: selective protection of a hydroxyl group, oxidation, elongation of the carbon chain by Grignard reaction, and deoxygenation. *O*-Glycosides were obtained by glycosylation of triterpenes with classical Schmidt's donors. Additionally, all new compounds were evaluated in vitro for their cytotoxic activities. Several triterpenes and the corresponding saponins exhibited an interesting cytotoxic activity profile against human cancer cell lines. The therapeutical index of active triterpenes is very high, since almost none of them were cytotoxic for normal BJ fibroblasts. These results open the way to the synthesis of various lupane-type saponin derivatives as potentially bioactive compounds.

1. Introduction

Saponins are steroid or triterpenoid glycosides widely distributed in plants and in some marine organisms.[1,2] They possess interesting biological properties including antitumor, antiviral, antifungal, and antiinflammatory activities, which have been extensively studied and reviewed in the last years.[3–10] Their biological effects were correlated to both sugar residues and aglycones.[3,11–13] Usually, mono-, di-, tri- or tetrasaccharides are attached to the sapogenin backbone, they constitutes a hydrophilic part, while sapogenin is a hydrophobic fragment.

Natural saponins based on the betulin scaffold occur less frequently than those having other triterpene-type aglycones, although a growing interest in their synthesis is noticeable.[14–23] However, little information is available regarding the effect of the non-sugar substitution at the lupane C-28 position and cytotoxicity of the resulting saponins bearing sugar moiety at the C-3 position.[24–28] In addition, there are certain gaps on the structure–activity relationships (SAR) studies concerning other types of branching at the C-28 position. Herein we report on the synthesis of novel lupane-type triterpenes and saponins, and studies derived from the cytotoxicity evaluation of these compounds. The synthetic strategy is directed toward an elongation of the carbon chain at the lupeol C-28 position and introduction of different sugar moieties at the O-3 position. Fig. 1 depicts the structural features of the lupanes **1**–**3** to be synthesized. Those lupanes were further transformed into saponins whose cytotoxicity against a series of cell lines have been tested.



1: R = Me
2: R = Et
3: R = Pr

Fig. 1. Target chain-elongated derivatives of lupeol.

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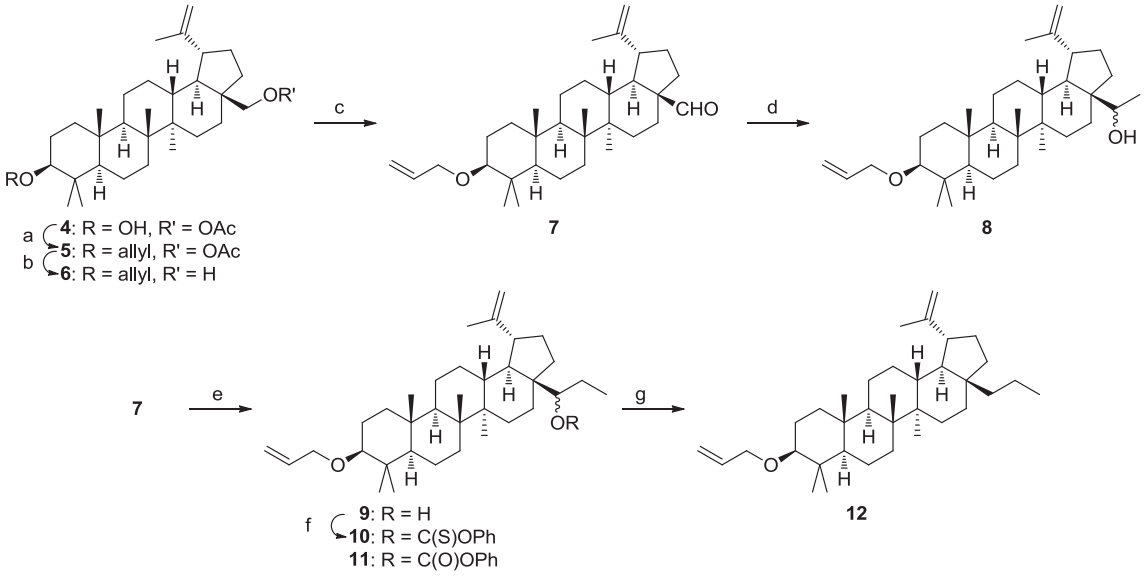
2. Results and discussion

2.1. Chemistry

2.1.1. Synthesis of lupanes with carbon chain elongated at the C-28 position. For the elongation of the carbon chain at the C-28 position of lupeol, we chose the Grignard reaction of betulinal derivatives followed by removal of the newly formed hydroxyl group. First we used 3-*O*-allylbetulinal (**7**), because of the flexibility of the allyl group, which may be selectively removed under very mild conditions, or used for linker construction. Compound **7** was easily available by acidic allylation of 28-*O*-acetylbetulin[29] (**4**) with allyl trichloroacetimidate[30] to yield derivative **5**, followed by subsequent hydrolysis of the acetyl group and oxidation of the hydroxyl group at C-28–OH with PCC. The reaction of aldehyde **7** with methyl- or ethylmagnesium bromide afforded corresponding alcohols **8** and **9**. In both cases a mixture of diastereoisomers (partially separated by column chromatography) was obtained. Full separation was not necessary due to the subsequent deoxygenation, which destroys this newly created stereogenic center (Scheme 1).

Due to problems with the purification of **12**, we decided to use 3-*O*-acetylbetulinal[13] (**13**) instead of 3-*O*-allylbetulinal (**7**) as starting material for the synthesis of the target triterpenes. Its reaction with methylmagnesium bromide gave elongated alcohols **14** in 82% yield. Reaction with ethylmagnesium bromide afforded alcohols **15** in 86% yield. In this case, partial deacetylation was also observed and small amounts of the corresponding diols **16** were isolated as dibenzoates **17** in 2–11% yield (as inseparable mixture), depending on batch. The composition of the main products was additionally confirmed by acetylation of the analytical samples of pure diastereoisomers **15a** and **15b** (to **18**). Similarly, reaction with propylmagnesium chloride gave elongated alcohols **19** in 73% yield. A small sample of a **15ab** mixture was further acylated with benzoyl chloride providing benzoate **20** used for studying the influence of substitution on cytotoxicity. Then, alcohols **14**, **15**, and **19** were acylated with phenyl chlorothionoformate yielding the expected thiocarbonates **21**–**23**. In all cases, minute percentages of the inseparable corresponding carbonates **24**–**26** were also detected. Composition of the carbonates **24**–**26** was confirmed by acylation of analytical samples of the starting alcohols with phenyl chlor-



Scheme 1. Synthesis of 3-*O*-allyl triterpenes and their elongation at C-28 position. Reagents and conditions: (a) CH_2=$CHCH_2OC(NH)CCl_3$, TMSOTf, DCM, 0 °C, 1 h, 75%; (b) KOH, EtOH, reflux, 2 h, 82%; (c) PCC, DCM, 0 °C, 90 min, 75%; (d) MeMgBr, THF, −40 °C, 94%; (e) EtMgBr, THF, −40 °C, 61%; (f) PhOC(S)Cl, DCM, Py, rt, 24 h; (g) $(Me_3Si)_3SiH$, AIBN, toluene, 80 °C, 5 h.

We expected that the Robins–Wilson[31] modification of the Barton–McCombie[32] deoxygenation would provide efficient and convenient ways to the target triterpenes. The reaction of **9a** with phenyl chlorothionoformate yielded expected the thiocarbonate **10** in high yield. However, this product contained a significant amount of a byproduct in which the H-28 protons appeared at higher field (5.34 ppm) than for thiocarbonate (6.00 ppm). In the ^{13}C NMR spectrum the thiocarbonyl group of **10** was observed at δ ~195 ppm, whereas the carbonyl carbon of the contamination appeared at δ 154 ppm. The molecular mass of the contaminant was 16 units lower than for thiocarbonate. Such data suggests that thiocarbonate **10** was contaminated with the corresponding carbonate **11**. Its origin is unclear,[33] but this hypothesis was confirmed during further studies by independent synthesis of similar compounds. Free-radical deoxygenation of **10** with tris(trimethylsilyl)silane in the presence of AIBN afforded the expected product **12**, although badly contaminated with several inseparable byproducts.

oformate. The obtained derivatives had identical NMR spectra as the above additives. In the next step the thiocarbonate group was removed by reduction with tris(trimethylsilyl)silane in the presence of AIBN to afford acetates **27**–**29**. At this stage, unreacted carbonates were readily removed by column chromatography. Structures of the acetylated target triterpenes **27**–**29** were unequivocally confirmed by X-ray analysis (Fig. 2). Superimposition of X-ray structures shows only minimal differences in the space arrangement around the aliphatic chain at C-28 position (Fig. 3).

Acetyl groups present in betulin derivatives are known to be hydrolytically stable, and classical methods for their deprotection (basic treatment) usually do not work.[34,35] Therefore, deacetylation at the O-3 position was performed by reduction with $LiAlH_4$. All target compounds (**1**–**3**) were obtained in 96% yield (Scheme 2).

It is clearly visible that changing the 3-*O*-protective group from allyl to acetyl was crucial for the successful synthesis of required triterpenes. A similar influence of a substituent distant to the reacting center of lupane-type triterpenes was observed earlier by

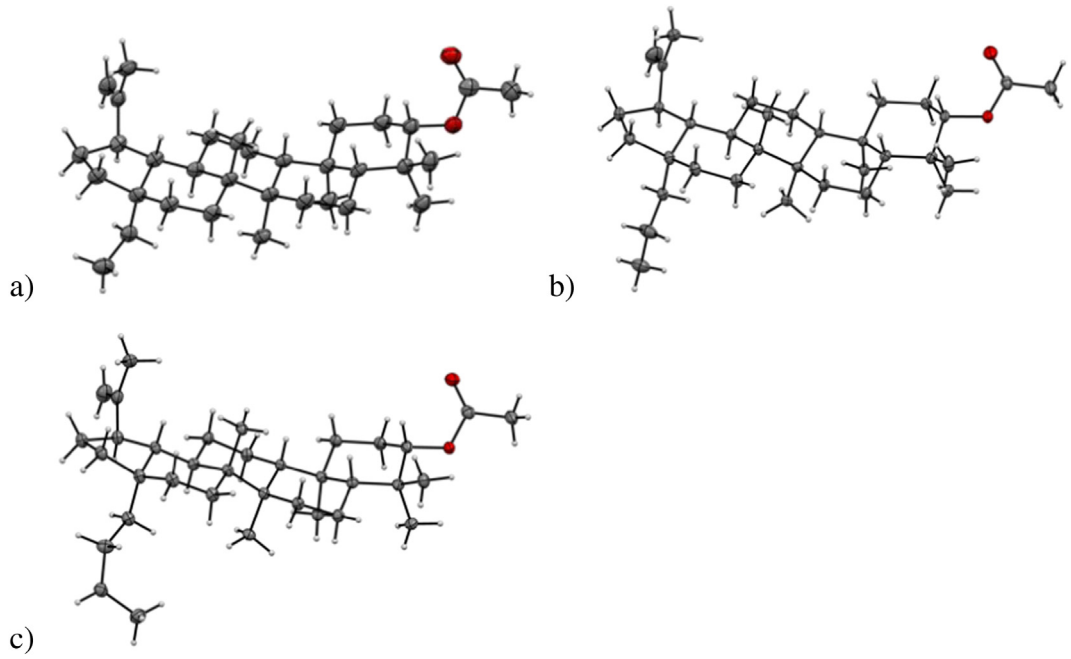


Fig. 2. Crystal structures of compounds **27** (a), **28** (b), and **29** (c). View along the C17–C18 bond.

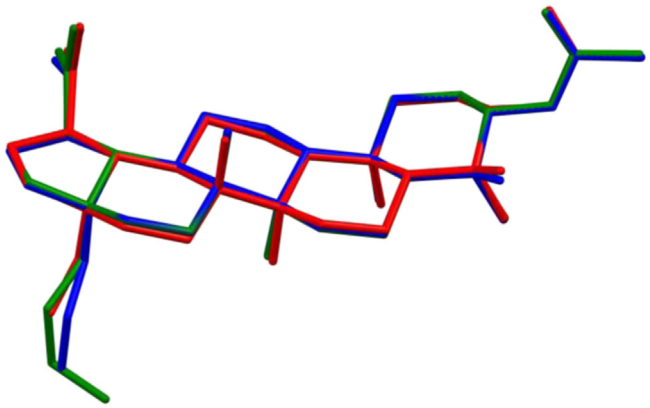


Fig. 3. Overlaid molecules of **27** (—, only one of two symmetrically independent molecules was used), **28** (—), and **29** (—).

Pichette.[15] Its origin is unclear, but it must be considered as an important factor affecting synthesis of the particular lupane derivatives, and as an explanation for some unsuccessful trials.

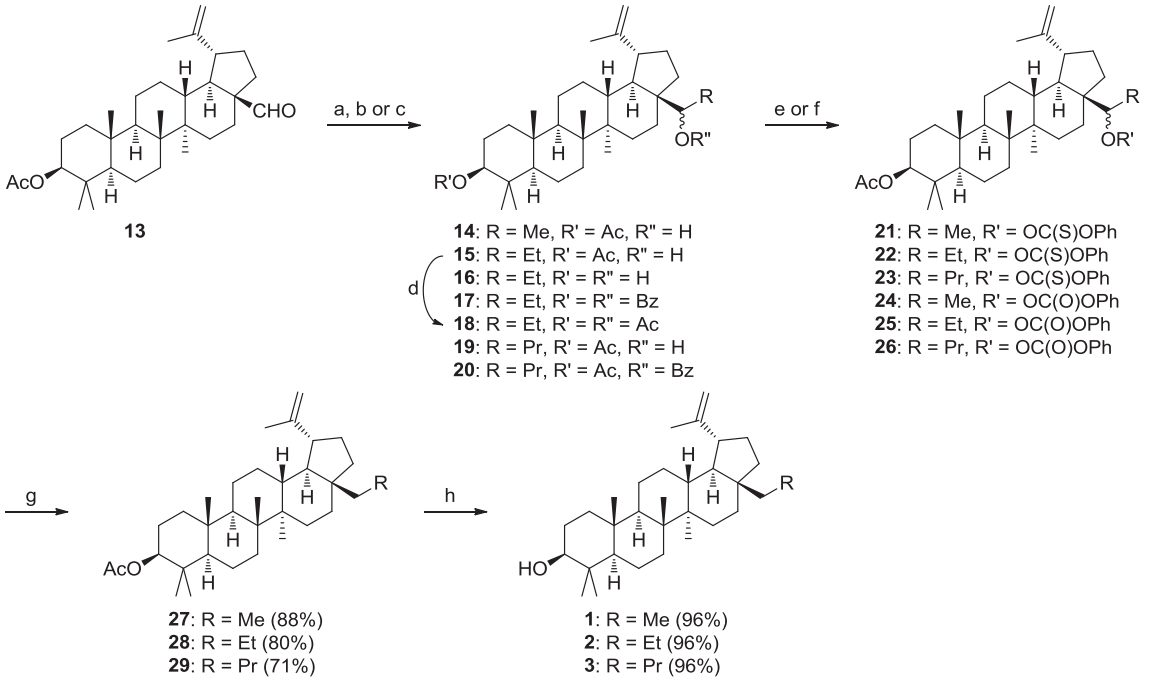
2.1.2. Synthesis of saponins. Glycosylation of triterpenes **1**–**3** was performed by treatment of glycosyl acceptors with perbenzoylated donors **30**,[36] **31**,[37] and **32**[37] in the presence of TMSOTf under standard conditions (Scheme 3).[35] Protected saponins were obtained in good to high yields (61–90%). As expected, the presence of benzoyl protecting groups in position 2 of the sugar donors directed the anomeric selectivity of the glycosidation reaction.[38] In all cases 1,2-*trans*-monodesmosidic saponins (α-D-mannopyranosides, α-L-arabinopyranosides, and α-L-rhamnopyranosides) were obtained exclusively, which was confirmed by the chemical shifts and the vicinal coupling constants of the anomeric protons. Final deprotection of the hydroxyl groups was performed by treatment of benzoates with potassium carbonate in methanol. With one exception (60% for **33d**) all saponins were obtained in very good yields (87–98%). The structures of all synthesized saponins were confirmed by extended 1D and 2D NMR experiments, as well as elemental analysis and HRMS (Tables 1 and 2, Experimental section).

2.2. In vitro results

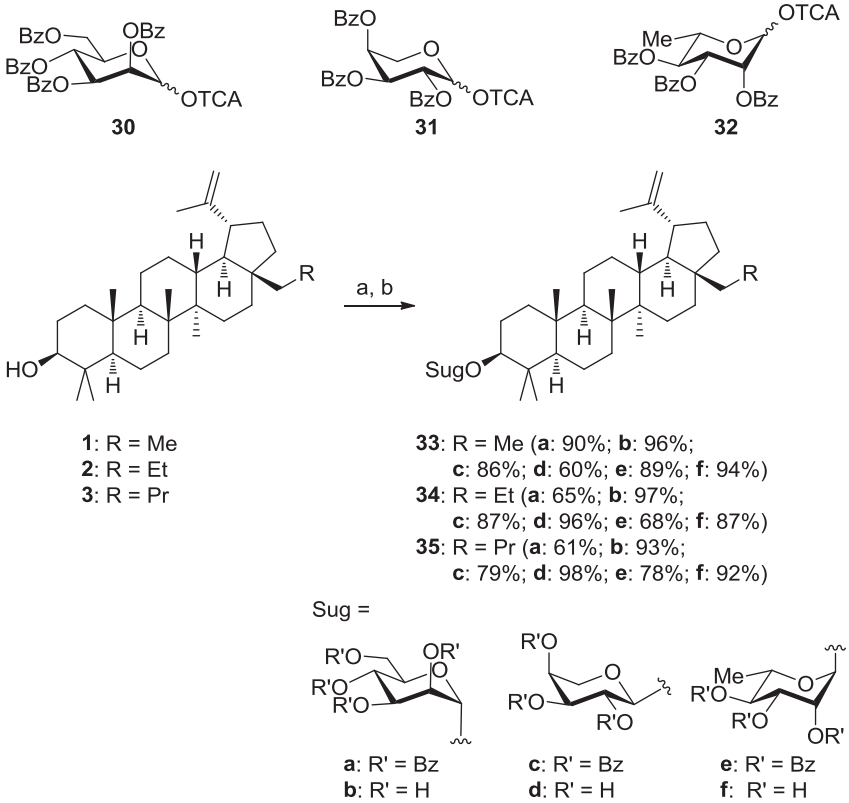
Cytotoxic activity of the parent triterpenoid lupanes with different carbon chains at the C-28 position, as well as their 3β-*O*-monodesmosidic saponin derivatives was tested. Several normal and cancer cell lines were cultured and used in experiments to examine the structure–activity relationships of this lupane-type derivative, with respect to their activities against human cancers. We compared the in vitro cytotoxic activity of selected analogues against human BJ-H-*tert* fibroblasts and cancer cell lines of various histopathological origins, including T-lymphoblastic leukemia CEM, breast carcinoma MCF-7, and cervical carcinoma HeLa lines. Cells of all of these lines were exposed to six serial four-fold dilutions of each drug for 72 h, the proportions of surviving cells were then estimated and IC_{50} values (50% inhibitory concentrations) were calculated. The results obtained from Calcein AM assays are presented in Table 3.

The most potent triterpene derivative was compound **1**, which showed cytotoxic activity against all of the tumor cell lines. However, this compound had also significant toxicity towards normal cells (BJ fibroblasts). Triterpenoid derivatives (**1**, **6**, **9a**, **13**, **15a**, **19a**, **19b**) were more active against cervical carcinoma HeLa line (IC_{50} 12.3–43.4 μM) than the other types of cancer cells.

The therapeutical index of these type of compounds is very high, as none of the terpenoids (except **1**) were cytotoxic for normal BJ fibroblasts. Furthermore, compounds **1** (IC_{50} 30.8 μM), **10a** (IC_{50} 27.8 μM), **13** (IC_{50} 38.3 μM), and **15a** (IC_{50} 41.9 μM) were also active against T-lymphoblastic leukemia CEM cells. A striking observation from this data was that much lower lupane-mediated loss of viability was observed in the BJ fibroblasts, suggesting that the lupane derivative induces different responses in cancer and normal cells. At present, only a few natural agents are known to possess the potential ability for selective/preferential elimination of cancer cells without affecting the growth of normal cells.[39–41] This was also observed in our previous study.[35] There were two unique compounds **14ab** and **19ab** exhibiting selective cytotoxic activity



Scheme 2. Synthesis of triterpenes elongated at the C-28 position. Reagents and conditions: (a) **13**→**14**, MeMgBr, THF, −40 °C, 82%; (b) **13**→**15**, EtMgBr, THF, −40 °C, 86%+**16** (2–11%); (c) **13**→**19**, PrMgCl, THF, −40 °C, 73%; (d) Ac_2O, Py; (e) PhOC(S)Cl, DCM, Py, rt, 24 h, **14**→**21**, **15**→**22**, **19**→**23**; (f) PhOC(O)Cl, DCM, Py, rt, 24 h, **14**→**24**, **15**→**25**, **19**→**26**; (g) $(Me_3Si)_3SiH$, AIBN, toluene, 80 °C, 5 h; (h) $LiAlH_4$, THF, rt, 30 min.



Scheme 3. Synthesis of saponins from triterpenes elongated at the C-28 position. Reagents and conditions: (a) **30**, **31**, or **32**, TMSOTf, DCM, molecular sieves 4 Å; (b) K_2CO_3, MeOH.

for breast carcinoma MCF-7. On the other hand, several of these derivatives (**1**, **15b**, **20**, **24a**, and **25a**) were cytotoxic to normal fibroblasts, thus demonstrating unusable the substitutions of the lupane scaffold for cancer treatment.

The majority of the lupane-type 3β-*O*-monodesmosidic saponins (**33f**, **34f**, **35b**,**d**,**f**) showed zero cytotoxicity towards the cancer and normal cell lines used, even when tested at concentrations up to 50 μM. The potent compounds were saponin

Table 1
^{1}H NMR spectroscopic data of compounds **1**–**3**, **6**, **18b**, and **33**–**35bdf**

Atom no.	**1**[a]	**2**[a]	**3**[a]	**6**[a,c]	**18b**[d]	**33b**[a]	**34b**[a]
Triterpene unit							
1	0.90, 1.66	0.90, 1.66	0.90, 1.66	0.82, 1.68	0.98, 1.66	0.81, 1.67	0.81, 1.66
2	1.57	1.57	1.58	1.47, 1.71	1.60	1.39, 1.70	1.39, 1.70
3	3.18	3.18	3.18	2.80	4.46	3.14	3.14
5	0.68	0.68	0.68	0.68	0.78	0.67	0.67
6	1.39, 1.52	1.40, 1.52	1.39, 1.52	1.39, 1.51	1.40, 1.49	1.37, 1.49	1.37, 1.49
7	1.38	1.38	1.38	1.39		1.37	1.37
9	1.28	1.27	1.27	1.25	1.27	1.25	1.24
11	1.22, 1.42	1.23, 1.42	1.23, 1.42	1.21, 1.41	1.25, 1.41	1.21, 1.40	1.22, 1.40
12	1.06, 1.64	1.06, 1.64	1.06, 1.64	1.04, 1.63	1.11, 1.61	1.06, 1.64	1.06, 1.65
13	1.77	1.79	1.79	1.64	1.86	1.77	1.80
15	0.95, 1.55	0.95, 1.57	0.95, 1.57	1.05, 1.70	0.94, 1.93	0.95, 1.54	0.95, 1.57
16	1.12, 1.72	1.14, 1.71	1.13, 1.72	1.21, 1.92		1.12, 1.72	1.14, 1.72
18	1.46	1.42	1.43	1.59	1.72	1.46	1.42
19	2.40	2.40	2.40	2.38	2.62	2.39	2.43
21	1.31, 1.85	1.32, 1.87	1.31, 1.86	1.41, 1.96	1.42, 1.90	1.31, 1.85	1.32, 1.87
22	0.92, 1.63	0.95, 1.63	0.95, 1.63	1.04, 1.86	1.10, 1.90	0.93, 1.64	0.96, 1.64
23	0.76	0.76	0.76	0.78	0.83	0.74	0.74
24	0.97	0.97	0.97	0.97	0.84	0.92	0.92
25	0.83	0.83	0.83	0.83	0.85	0.82	0.82
26	1.02	1.03	1.03	1.02	1.09	1.02	1.03
27	0.96	0.96	0.96	0.97	0.97	0.95	0.95
28	0.98, 1.49	0.90, 1.42	0.90, 1.46	3.33, 3.80	5.28	0.99, 1.49	0.90, 1.42
28a[e]	0.77	1.19	1.30	—	1.54, 1.72	0.77	1.17, 1.22
28b[e]	—	0.91	1.15	—	0.87	—	0.92
28c[e]	—	—	0.92	—	—	—	—
29	4.56, 4.67	4.55, 4.67	4.56, 4.68	4.58, 4.68	4.69, 4.59	4.57, 4.68	4.58, 4.69
30	1.68	1.68	1.68	1.68	1.68	1.68	1.68
Monosaccharide unit							
1	—	—	—	—	—	4.96	4.96
2	—	—	—	—	—	3.82	3.81
3	—	—	—	—	—	3.80	3.82
4	—	—	—	—	—	3.94	3.94
5	—	—	—	—	—	3.63	3.63
6	—	—	—	—	—	3.72, 3.94	3.72, 3.94

Atom no.	**35b**[a]	**33d**[a]	**34d**[b]	**35d**[a]	**33f**[b]	**34f**[a]	**35f**[b]
Triterpene unit							
1	0.81, 1.66	0.89, 1.66	0.90, 1.66	0.88, 1.65	0.92, 1.68	0.88, 1.65	0.90, 1.66
2	1.39, 1.70	1.69, 1.83	1.68, 1.82	1.68, 1.81	1.66, 1.76	1.64, 1.74	1.66, 1.76
3	3.14	3.13	3.13	3.11	3.07	3.05	3.06
5	0.67	0.70	0.71	0.69	0.72	0.68	0.71
6	1.37, 1.49	1.38, 1.50	1.39, 1.51	1.37, 1.49	1.40, 1.51	1.37, 1.48	1.39, 1.50
7	1.37	1.38	1.38	1.37	1.40	1.37	1.38
9	1.25	1.25	1.27	1.25	1.30	1.25	1.28
11	1.22, 1.40	1.22, 1.41	1.24, 1.42	1.22, 1.41	1.24, 1.43	1.22, 1.41	1.24, 1.42
12	1.06, 1.65	1.05, 1.64	1.06, 1.65	1.05, 164	1.07, 1.66	1.06, 1.64	1.07, 1.65
13	1.80	1.77	1.81	1.80	1.80	1.79	1.80
15	0.95, 1.56	0.95, 1.54	0.96, 1.58	0.95, 1.56	0.97, 1.57	0.95, 1.56	0.96, 1.58
16	1.14, 1.72	1.12, 1.72	1.14, 1.72	1.13, 1.71	1.14, 1.74	1.14, 1.71	1.14, 1.72
18	1.43	1.46	1.44	1.43	1.48	1.42	1.44
19	2.42	2.40	2.42	2.41	2.41	2.43	2.42
21	1.32, 1.87	1.31, 1.85	1.32, 1.87	1.31, 1.86	1.32, 1.86	1.31, 1.87	1.32, 1.87
22	0.96, 1.64	0.89, 1.66	0.96, 1.64	0.95, 1.63	0.94, 1.65	0.96, 1.63	0.96, 1.64
23	0.74	0.80	0.81	0.79	0.76	0.74	0.75
24	0.91	0.97	1.00	0.97	0.92	0.89	0.91
25	0.82	0.83	0.84	0.82	0.85	0.83	0.84
26	1.02	1.02	1.04	1.02	1.04	1.02	1.04
27	0.95	0.95	0.96	0.94	0.98	0.95	0.96
28	0.90, 1.46	0.98, 1.48	0.91, 1.44	0.90, 1.46	1.01, 1.51	0.90, 1.42	0.91, 1.47
28a[e]	1.31	0.77	1.17, 1.22	1.31	0.78	1.16, 1.22	1.32
28b[e]	1.15	—	0.93	1.15	—	0.92	1.16
28c[e]	0.92	—	—	0.92	—	—	0.93
29	4.58, 4.69	4.56, 4.68	4.56, 4.68	4.57, 4.68	4.56, 4.68	4.57, 4.68	4.56, 4.68
30	1.69	1.68	1.69	1.68	1.69	1.68	1.69
Monosaccharide unit							
1	4.96	4.39	4.33	4.32	4.76	4.80	4.76
2	3.81	3.77	3.65	3.75	3.88	3.93	3.88
3	3.82	3.71	3.60	3.67	3.69	3.75	3.70
4	3.94	3.92	3.87	3.92	3.38	3.44	3.38
5	3.63	3.56, 3.90	3.52, 3.88	3.52, 3.90	3.75	3.79	3.75
6	3.72, 3.94	—	—	—	1.26	1.27	1.26

[a] Recorded in $CDCl_3$ at 600 MHz, δ in ppm.
[b] Recorded in $CDCl_3/CD_3OD$ mixture (3:1) at 600 MHz, δ in ppm.
[c] 3-*O*-Allyl group: OCH_2 3.89–3.85 (m) and 4.12–4.08 (m) ppm; =*CH* 5.95–5.88 (m) ppm; =CH_2 5.12–5.09 (m) and 5.27–5.22 (m) ppm.
[d] 3-*O*-Acetyl group: 2.03 (s) ppm; 28-*O*-acetyl group: 2.07 (s) ppm.
[e] Atom numbering according to IUPAC rules (Fig. 4).[42,43]

Table 2
^{13}C NMR spectroscopic data of compounds **1**–**3**, **6**, **18b**, and **33**–**35bdf**

Atom no.	**1**[a]	**2**[a]	**3**[a]	**6**[a,c]	**18b**[d]	**33b**[a]	**34b**[a]	**35b**[a]	**33d**[a]	**34d**[b]	**35d**[a]	**33f**[b]	**34f**[a]	**35f**[b]
Triterpene unit														
1	38.7	38.7	38.7	38.6	38.3	38.3	38.3	38.3	38.7	38.9	38.7	38.7	38.6	38.9
2	27.4	27.4	27.4	23.1	23.7	22.1	22.1	22.1	26.0	26.2	26.0	25.5	25.5	25.7
3	79.0	79.0	79.0	86.3	80.9	82.9	82.9	82.9	90.0	90.0	89.8	89.2	89.6	89.5
4	38.9	38.9	38.9	38.9	37.8	38.4	38.4	38.4	39.1	39.3	39.1	39.0	39.0	39.2
5	55.3	55.3	55.3	55.9	55.4	55.6	55.6	55.6	55.6	55.8	55.6	55.5	55.4	55.7
6	18.3	18.3	18.3	18.3	18.1	18.3	18.3	18.2	18.2	18.4	18.2	18.3	18.3	18.5
7	34.2	34.2	34.2	34.3	33.9	34.2	34.2	34.2	34.2	34.4	34.2	34.2	34.2	34.4
8	40.8	40.9	40.9	41.0	41.0	40.9	40.9	40.9	40.9	41.0	40.9	40.9	40.9	41.1
9	50.5	50.5	50.5	50.4	50.2	50.4	50.4	50.4	50.5	50.6	50.4	50.5	50.4	50.7
10	37.2	37.2	37.2	37.1	37.0	37.1	37.1	37.1	36.9	37.1	36.9	36.9	36.9	37.1
11	21.0	21.0	21.0	20.9	20.8	21.0	21.0	21.0	21.0	21.2	21.0	21.0	21.0	21.2
12	25.1	25.1	25.1	25.3	25.2	25.1	25.1	25.1	25.2	25.4	25.1	25.2	25.1	25.4
13	37.0	37.0	37.0	37.3	36.9	37.0	37.0	37.0	37.0	37.1	37.0	37.0	37.0	37.2
14	42.5	42.5	42.5	42.7	43.0	42.5	42.5	42.5	42.5	42.7	42.5	42.5	42.5	42.7
15	27.1	27.2	27.2	27.0	28.1	27.1	27.2	27.2	27.1	27.4	27.2	27.1	27.2	27.4
16	30.3	31.2	31.2	29.2	33.9	30.3	31.2	31.1	30.4	31.4	31.1	20.3	31.2	31.3
17	45.8	45.7	45.6		50.2	45.8	45.7	45.6	45.8	45.8	45.6	45.8	45.7	45.8
18	49.7	49.8	49.8	48.8	49.9	49.7	49.8	49.8	49.7	50.2	49.8	49.8	49.8	50.0
19	47.4	47.4	47.4	47.8	48.2	47.4	47.4	47.4	47.4	47.6	47.4	47.5	47.4	47.6
20	151.1	151.2	151.2	150.5	150.4	151.1	151.1	151.1	151.2	151.3	151.1	151.0	151.1	151.4
21	30.0	30.1	30.1	29.8	31.6	30.0	30.1	30.1	30.0	30.3	30.1	30.0	30.1	30.3
22	35.0	35.8	35.7	34.0	34.2	35.0	35.8	35.7	35.0	36.0	35.7	35.0	35.8	35.9
23	15.3	15.3	15.3	16.3	16.5	16.4	16.4	16.4	16.4	16.3	16.4	16.0	16.2	16.3
24	28.0	28.0	28.0	28.1	27.9	28.6	28.6	28.6	28.2	28.0	28.0	27.9	28.2	28.2
25	16.1	16.1	16.1	16.1	16.0	16.1	16.1	16.1	16.1	16.2	16.1	16.0	16.1	16.3
26	16.0	16.0	16.0	16.0	15.9	16.0	16.0	16.0	16.0	16.1	16.0	15.9	16.0	16.2
27	14.8	14.9	14.9	14.8	14.9	14.9	14.9	14.9	14.8	15.0	14.8	14.7	14.9	15.0
28	19.3	30.0	26.9	60.6	76.5	19.3	30.0	27.0	19.3	30.1	26.9	19.3	30.0	27.2
28a[e]	8.1	17.0	23.8	—	24.7	8.1	17.0	23.9	8.1	17.2	23.8	7.8	17.0	24.0
28b[e]	—	15.4	26.0	—	11.5	—	15.4	26.0	—	15.5	26.0	—	15.4	26.2
28c[e]	—	—	14.2	—	—	—	—	14.2	—	—	14.2	—	—	14.3
29	109.2	109.2	109.2	109.7	110.1	109.2	109.3	109.3	109.2	109.3	109.2	109.1	109.2	109.4
30	19.3	19.3	19.3	19.1	18.7	19.3	19.3	19.4	19.3	19.4	19.4	19.1	19.3	19.4
Monosaccharide unit														
1	—	—	—	—	—	96.5	96.5	96.5	104.3	105.2	104.8	102.8	102.2	102.8
2	—	—	—	—	—	71.9	71.7	71.7	71.6	71.4	71.5	71.1	71.3	71.3
3	—	—	—	—	—	71.8	71.9	71.9	72.3	72.8	72.6	71.5	72.0	71.7
4	—	—	—	—	—	66.1	66.1	66.1	66.9	67.6	67.5	72.9	73.6	73.2
5	—	—	—	—	—	72.7	72.8	72.8	64.0	64.6	64.6	68.3	67.8	68.4
6	—	—	—	—	—	60.9	60.9	60.9	—	—	—	17.0	17.3	17.4

[a] Recorded in $CDCl_3$ at 150 MHz, δ in ppm.
[b] Recorded in $CDCl_3/CD_3OD$ mixture (3:1) at 150 MHz, δ in ppm.
[c] 3-*O*-Allyl group: OCH_2 70.7 ppm; =*C*H 135.9 ppm; =*C*H_2 115.9 ppm.
[d] 3-*O*- and 28-*O*-Acetyl groups: 171.0 and 170.9 ppm.
[e] Atom numbering according to IUPAC rules (Fig. 4).[42,43]

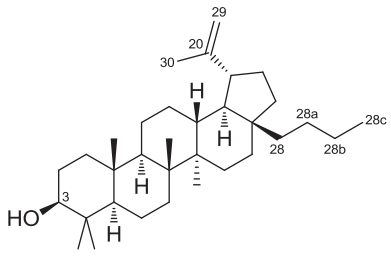


Fig. 4. Atom numbering.

derivatives **33b** and **33d** that showed cytotoxic activity against almost all of the tumor cell lines, but also much stronger toxicity towards normal BJ-H-*tert* fibroblasts. Monosaccharide analogue **34d** containing L-arabinose fragment (IC_{50} 36.1 μM) was slightly potent to CEM leukemia with comparable cytotoxicity to the saponin analogue **34b**. The results show several new unique terpenoid lupane structures exhibiting cytotoxic activity in low micromolar range with higher tolerated doses for nonmalignant cells (BJ-H-*tert* fibroblasts), demonstrating their potential for further development as anticancer drugs.

2.2.1. Cell cycle and apoptosis. We have tested whether the two most active compounds on HeLa cells, **13** and **15a**, could influence their cell cycle. Flow cytometric analysis was used to quantify the distribution of HeLa cells in cell cycle phases, and the subG$_1$ fraction as a marker of the proportion of apoptotic cells, following incubation with **13** and **15a**. The results show that treatment with 50 μM **13** increased the proportions of S-phase and G$_2$/M cells, with concomitant reductions in the proportions of G$_0$/G$_1$ cells. The proportion of cells with subG$_1$ amounts of DNA (apoptotic cells) increased after two treatments with **13**, from 3.7% in control cells to 5.8% in cells treated with 50 μM **13**. Compound **15a** had no effect on the cell cycle of HeLa cells (Fig. 5).

3. Conclusions

In conclusion, a series of the lupane-type triterpenes elongated at the C-28 position, and saponins bearing the D-mannose, L-arabinose, and L-rhamnose moieties at the C-3 position obtained from the above triterpenes were synthesized and evaluated for their cytotoxic activities. Several triterpenes, as well as the corresponding saponins show an interesting cytotoxic activity profile against human cancer cell lines.

Table 3
IC_{50} (μM) values obtained from the Calcein AM assays with the tested cancer and normal cell lines; means±SD obtained from three independent experiments performed in triplicate. Betulinic acid was used as a positive control

Compound	Cell lines (IC_{50} μM)			
	CEM	MCF7	HeLa	BJ
Triterpenes				
Betulinic acid	40±2.8	>50	47.6±1.9	>50
1	30.8±0.6	46.0±5.7	28.5±5.0	45.0±1.3
2	>50	>50	>50	>50
3	>50	>50	>50	>50
5	>50	>50	>50	>50
6	>50	>50	29.0±3.2	>50
8	>50	>50	>50	>50
9a	>50	>50	43.4±1.0	>50
10a	27.8±4.3	>50	>50	>50
13	38.3±15.6	>50	12.3±0.8	>50
14a	>50	>50	>50	>50
14ab	>50	32.1±3.3	>50	>50
15a	41.9±11.5	>50	16.3±8.7	>50
15b	>50	>50	>50	44.7
19a	>50	>50	19.8±11.6	>50
19b	>50	>50	31.3±5.9	>50
19ab	>50	36.3	20.5±11.3	>50
20ab	>50	>50	>50	28.3±6.9
21a	>50	>50	>50	>50
22a	>50	>50	>50	>50
22b	>50	>50	>50	>50
23a	>50	>50	>50	>50
23b	>50	>50	>50	>50
24a	>50	>50	>50	45.9±5.8
25a	>50	>50	>50	47.5
26a	>50	>50	>50	>50
27	>50	>50	>50	>50
28	>50	>50	>50	>50
29	>50	>50	>50	>50
Saponins				
33b	33.3±1.6	44.4±0.6	39.7±2.1	14.8±1.3
33d	32.0±1.3	>50	33.0±0.6	14.9±2.3
33f	>50	>50	>50	>50
34b	>50	>50	47.1±3.3	43.3±2.7
34d	36.1±1.7	>50	>50	44.0±1.2
34f	>50	>50	>50	>50
35b	>50	>50	>50	>50
35d	>50	>50	>50	>50
35f	>50	>50	>50	>50

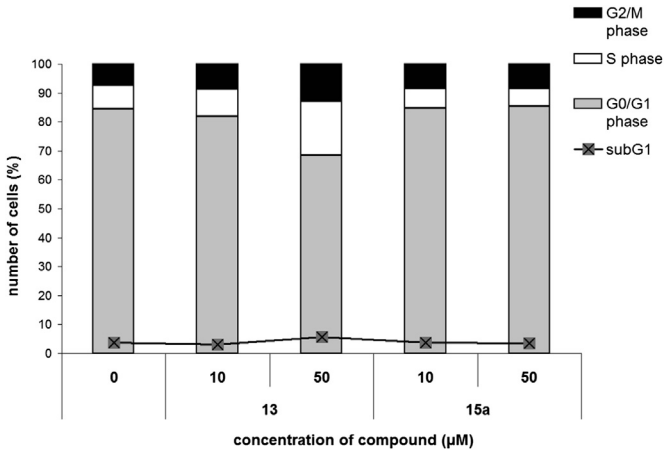


Fig. 5. Cell cycle and apoptosis.

4. Experimental section

4.1. General notes

Silica gel HF_{254} and Silica gel 230–400 mesh (E. Merck) were used for TLC and column chromatography, respectively. ^1H and ^{13}C NMR spectra were recorded at 298 K with a Varian NMR-VNMRS600 or VNMRS500 spectrometers. Standard experimental conditions and standard Varian programs (ChemPack 4.1) were used. Configurational assignments were based on the NMR measurements including two-dimensional techniques like COSY, and $^1H–^{13}C$ gradient selected HSQC (*g*-HSQC), as well as $^1H–^{13}C$ gradient selected HMBC (*g*-HMBC) experiments, which were employed in several cases. Internal TMS was used as the ^1H and ^{13}C NMR chemical shift standard. *J* values are given in Hertz. High resolution mass spectra (HRMS ESI) were acquired with a MARINER and MaldiSYNAPT G2-S HDMS (Waters) mass spectrometers. Optical rotations were measured with a JASCO P-2000 automatic polarimeter. IR spectra were recorded on Jasco 6200 FT-IR spectrophotometer.

Diffraction data for **27**–**29** were collected at 100 K using an Agilent SuperNova diffractometer with Cu Kα radiation. All structures were solved by direct methods (SHELXS-97), and refined on F^2 by full-matrix least-squares method (SHELXL-97). In all cases crystals suitable for X-ray analysis were obtained by cooling of a hot methanol solution.

Data for **27**: a=15.3096(6), b=10.3795(5), c=18.0492(7), β=97.943(4), monoclinic, $P2_1$, $R1$=0.0499, $wR2$=0.1325, S=1.037. Structure contains two symmetrically independent molecules of **27** in the unit cell.

Data for **28**: a=12.4455(2), b=14.5119(2), c=16.1425(3), orthorhombic, $P2_12_12_1$, $R1$=0.0322, $wR2$=0.0845, S=1.055.

Data for **29**: a=12.5606(3), b=14.1350(3), c=16.7391(4), orthorhombic, $P2_12_12_1$, $R1$=0.0373, $wR2$=0.0989, S=1.061.

Crystallographic data (excluding structure factors) for the structures in this paper have been deposited with the Cambridge Crystallographic Data Centre as Supplementary data (deposition numbers: CCDC 927960–927962). Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK, (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

The absolute configuration of the diastereoisomeric alcohols obtained during Grignard reaction and their esters was not determined. For analytical reasons, individual diastereoisomers were described as **a** and **b** in order of appearance during column chromatography, provided that at least partial separation took place.

4.2. Chemistry

*4.2.1. 28-O-Acetyl-3-O-allylbetulin (**5**).* To a solution of **4** (2.46 g, 5.08 mmol) in DCM (50 mL) cooled in an ice-bath, allyl trichloroacetimidate (2.91 g, 15.27 mmol) and TMSOTf (1 mL) were added. The reaction mixture was stirred at 0 °C for 1 h, then water (50 mL) was added, the organic phase was washed with aq satd $NaHCO_3$, water, and dried with Na_2SO_4. Column chromatography (hexane/ethyl acetate, 50:1→30:1) of the residue gave 2.00 g (75%) of the title compound as colorless crystals, mp: 180–182 °C; $[\alpha]_D^{20}$ 27.8 (*c* 0.3, $CHCl_3$); ν_{max} (film) 2943, 2870, 1740, 1643, 1233, 757 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 5.97–5.89 (m, 1H, CH–allyl), 5.28–5.24 (m, 1H, CH–allyl), 5.13–5.11 (m, 1H, CH–allyl), 4.69 (br d, 1H, *J* 2.3 Hz, H-29), 4.59–4.58 (m, 1H, H-29), 4.24 (dd, 1H, *J* 1.6 and 11.0 Hz, H-28), 4.10–4.14 (m, 1H, CH–allyl), 3.90–3.85 (m, 2H, H-28, CH–allyl), 2.79 (dd, 1H, *J* 4.4, 11.8 Hz, H-3), 2.48–2.41 (m, 1H, H-19), 2.07 (s, 3H, OAc), 1.69 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 0.78 (s, 3H, CH_3), 2.00–0.66 (m, 26H, lupane protons); δ_C (125 MHz, $CDCl_3$) 171.6 (C), 150.2 (C), 135.9, 115.9 (CH_2), 109.8 (CH_2), 86.3, 70.6 (CH_2), 62.8 (CH_2), 55.8, 50.4, 48.8, 47.7, 46.3 (C), 42.7 (C), 40.9 (C), 38.9 (C), 38.6 (CH_2), 37.6, 37.1, 34.6 (CH_2), 34.2 (CH_2), 29.8 (CH_2), 29.6 (CH_2), 28.1, 27.1 (CH_2), 25.2 (CH_2), 23.1 (CH_2), 21.0, 20.8 (CH_2), 19.1, 18.2 (CH_2), 16.3, 16.1,

16.0, 14.7; HRMS (ESI): MNa^+, found 547.4122. $C_{35}H_{56}NaO_3$ requires 547.4127.

*4.2.2. 3-O-Allylbetulin (**6**).* A solution of **5** (500 mg, 0.95 mmol) and KOH (110 mg, 2.0 mmol) in ethanol (15 mL) was refluxed for 2 h, the solvent was evaporated under reduced pressure, and the solid residue dissolved in ethyl acetate (50 mL) and water (30 mL). The organic phase was dried with Na_2SO_4. Column chromatography (hexane/ethyl acetate, 20:1→10:1) of the residue gave 378 mg (82%) of the title compound as colorless crystals, mp: 190–192 °C; [found: C, 82.03; H, 11.22. $C_{33}H_{54}O_2$ requires C, 82.10; H, 11.27%]; $[\alpha]_D^{20}$ 38.8 (*c* 0.3, $CHCl_3$); ν_{max} (film) 2942, 2869, 1642, 1454, 1026, 758 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2.

*4.2.3. 3-O-Allylbetulinal (**7**).* To a solution of **6** (4.71 g, 9.77 mmol) in DCM (100 mL) cooled in an ice-bath PCC (12.71 g, 59.0 mmol) was added in one portion and stirred at rt for 1.5 h. The solution was decanted from the gummy residue, which was washed with DCM twice, and the collected organic layers were evaporated to dryness. Column chromatography (hexane/ethyl acetate, 50:1) of the residue gave 3.50 g (75%) of the title compound as colorless crystals, mp: 150–152 °C; [found: C, 82.03; H, 10.97. $C_{33}H_{52}O_2$ requires C, 82.44; H, 10.90%]; $[\alpha]_D^{20}$ 43.1 (*c* 0.35, $CHCl_3$); ν_{max} (film) 2943, 2866, 1726, 1643, 1453, 1376, 1084, 920, 885, 739 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 9.68 (d, 1H, *J* 1.4 Hz, CHO), 5.96–5.89 (m, 1H, CH–allyl), 5.28–5.24 (m, 1H, CH–allyl), 5.13–5.10 (m, 1H, CH–allyl), 4.76 (br d, 1H, *J* 1.6 Hz, H-29), 4.63–4.62 (m, 1H, H-29), 4.14–4.10 (m, 1H, CH–allyl), 3.90–3.86 (m, 1H, CH–allyl), 2.89–2.83 (m, 1H), 2.79 (dd, 1H, *J* 4.3, 11.8 Hz, H-3), 1.70 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.82 (s, 3H, CH_3), 0.77 (s, 3H, CH_3), 2.10–0.65 (m, 24H, lupane protons); δ_C (150 MHz, $CDCl_3$) 206.7 (CHO), 149.7 (C), 135.9, 115.9 (CH_2), 110.1 (CH_2), 86.3, 70.6 (CH_2), 59.3 (C), 55.8, 50.5, 48.1, 47.5, 42.5 (C), 40.9 (C), 38.7, 38.6 (CH_2), 36.6 (C), 37.1 (C), 34.3 (CH_2), 33.2 (CH_2), 29.8 (CH_2), 29.3 (CH_2), 28.8 (CH_2), 28.1, 25.5 (CH_2), 23.1 (CH_2), 20.8 (CH_2), 19.0, 18.2 (CH_2), 16.3, 16.1, 15.9, 14.2.

4.2.4. Grignard reaction. General procedure

*4.2.4.1. 3-O-Acetyl-28-C-methylbetulin (**14**).* To a solution of 3-*O*-acetylbetulinal (**13**, 2.900 g, 6.00 mmol) in THF (60 mL) cooled to −40 °C, a 3 M solution of methylmagnesium bromide in Et_2O (10 mL, 30.0 mmol) was slowly added. Stirring was continued for 30 min at −40 °C. The reaction was quenched by the addition of satd aq NH_4Cl (2 mL). Silica gel (10–15 g) was poured into the mixture and the solvent was evaporated under reduced pressure to dryness. The residue was used for purification by flash chromatography (hexane/ethyl acetate, 40:1→10:1). Products were partially separated and **14a** (1.272 g, 42%) and **14ab** (1.195 g, 40%, **a**:**b**≈4:1) were obtained as white foams. *Data for* **14a**: [found: C, 79.50; H, 11.07. $C_{33}H_{54}O_3$ requires C, 79.46; H, 10.91%]; $[\alpha]_D^{20}$ 28.1 (*c* 0.4, $CHCl_3$); ν_{max} (film) 2945, 2873, 1718, 1374, 1028, 979, 882, 758 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 4.71 (d, 1H, *J* 2.4 Hz, H-29), 4.58–4.57 (m, 1H, H-29), 4.47 (dd, 1H, *J* 5.6, 10.9 Hz, H-3), 4.32 (q, 1H, *J* 6.3 Hz, H-28), 2.89–2.84 (m, 1H), 2.04 (s, 3H, CH_3CO), 1.69 (s, 3H, CH_3), 1.17 (d, 3H, *J* 6.3 Hz, CH_3), 1.03 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.85 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 2.10–0.76 (m, 25H, lupane protons); δ_C (150 MHz, $CDCl_3$) 171.0 (C), 151.3 (C), 109.5 (CH_2), 80.9, 68.3, 55.4, 50.3, 50.1, 50.1 (C), 49.0, 42.9 (C), 40.9 (C), 38.3 (CH_2), 37.8 (C), 37.0 (C), 36.8, 34.6 (CH_2), 34.1 (CH_2), 32.8 (CH_2), 32.2 (CH_2), 27.9, 27.7 (CH_2), 25.1 (CH_2), 23.7 (CH_2), 21.3, 20.9 (CH_2), 19.8, 18.8, 18.1 (CH_2), 16.5, 16.1, 16.1, 15.2. *Data for* **14b**: δ_H (600 MHz, $CDCl_3$)—selected signals 4.60–4.59 (m, 1H, H-29), 4.11 (q, 1H, *J* 6.2 Hz), 2.65–2.62 (m, 1H), 2.04 (s, 3H, OAc); δ_C (150 MHz, $CDCl_3$)—selected signals 150.6 (C), 110.0 (CH_2), 80.9, 67.7, 50.3 (C), 49.9, 48.2, 41.0 (C), 36.8, 34.2 (CH_2), 33.2 (CH_2), 33.1 (CH_2), 28.1 (CH_2), 25.2 (CH_2), 20.9, 20.8 (CH_2), 16.0, 14.9.

*4.2.4.2. 3-O-Allyl-28-C-methylbetulin (**8**).* Starting from 3-*O*-allylbetulinal (**7**, 1.20 g, 2.5 mmol) and 3 M methylmagnesium bromide in Et_2O (4.2 mL, 12.6 mmol), **8a** (745 mg, 60%), and **8ab** (420 mg, 34%, **a**:**b**≈3:1) were obtained as white foams (column chromatography, hexane/ethyl acetate, 20:1). *Data for* major isomer (**8a**): [found: C, 82.08; H, 11.23. $C_{34}H_{56}O_2$ requires C, 82.20; H, 11.36%]; $[\alpha]_D^{20}$ 36.2 (*c* 0.3, $CHCl_3$); ν_{max} (film) 3474 (br), 2942, 2871, 1455, 1375, 1091, 1068, 919, 883, 758 cm^{-1}; δ_H (500 MHz, $CDCl_3$) 5.97–5.89 (m, 1H, CH–allyl), 5.29–5.24 (m, 1H, CH–allyl), 5.14–5.10 (m, 1H, CH–allyl), 4.71 (d, 1H, *J* 2.7 Hz, H-29), 4.58–4.57 (m, 1H, H-29), 4.33 (q, 1H, *J* 6.4 Hz, H-28), 4.15–4.10 (m, 1H, CH–allyl), 3.91–3.86 (m, 1H, CH–allyl), 2.90–2.84 (m, 1H), 2.80 (dd, 1H, *J* 4.2, 11.5 Hz, H-3), 1.69 (s, 3H, CH_3), 1.17 (d, 3H, *J* 6.4 Hz, H-28a), 1.03 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.78 (s, 3H, CH_3), 2.10–0.65 (m, 25H, lupane protons); δ_C (125 MHz, $CDCl_3$) 151.4 (C), 135.9, 115.9 (CH_2), 109.4 (CH_2), 86.3, 70.6 (CH_2), 68.3, 55.9, 50.4, 50.2, 50.1 (C), 49.0, 43.0 (C), 41.0 (C), 38.9 (C), 38.6 (CH_2), 37.1 (C), 36.9, 34.6 (CH_2), 34.3 (CH_2), 32.8 (CH_2), 32.3 (CH_2), 28.1, 27.7 (CH_2), 25.2 (CH_2), 23.1 (CH_2), 21.0 (CH_2), 19.8, 18.9, 18.2 (CH_2), 16.3, 16.1, 16.1, 15.2.

*4.2.4.3. 3-O-Acetyl-28-C-ethylbetulin (**15**).* Starting from 3-*O*-acetylbetulinal (**13**, 560 mg, 1.16 mmol) and 3 M ethylmagnesium bromide in Et_2O (1.9 mL, 5.7 mmol), **15a** (351 mg, 59%), **15ab** (77 mg, 13%), and **15b** (86 mg, 14%) were obtained as white foams. In some experiments deacetylation on O-3 position was observed. The corresponding diols **16** were isolated as the dibenzoates **17** (white foam) after acylation (pyridine, benzoyl chloride) of slowest moving fraction in 2–11% yield calculated on starting betulinal. *Data for* **15a**: [found: C, 79.17; H, 11.12. $C_{34}H_{56}O_3$ requires C, 79.63; H, 11.01%]; $[\alpha]_D^{20}$ 27.2 (*c* 0.3, $CHCl_3$); δ_H (500 MHz, $CDCl_3$) 4.71 (d, 1H, *J* 2.3 Hz, H-29), 4.57–4.56 (m, 1H, H-29), 4.47 (dd, 1H, *J* 5.8, 10.7 Hz, H-3), 3.96–3.91 (m, 1H, H-28), 2.95–2.89 (m, 1H), 2.04 (s, 3H, OAc), 1.69 (s, 3H, CH_3), 1.04 (s, 3H, CH_3), 1.02 (t, 3H, *J* 7.4 Hz, CH_3), 1.00 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.85 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 2.10–0.76 (m, 27H, lupane protons); δ_C (125 MHz, $CDCl_3$) 171.0 (C), 151.5 (C), 109.4 (CH_2), 80.9, 74.4, 55.4, 50.5, 50.3, 50.1 (C), 49.0, 43.0 (C), 41.0 (C), 38.4 (CH_2), 37.8 (C), 37.1 (C), 36.9, 34.3 (CH_2), 34.1 (CH_2), 33.2 (CH_2), 32.9 (CH_2), 27.9, 27.8 (CH_2), 25.2 (CH_2), 23.7 (CH_2), 21.3, 20.9 (CH_2), 18.9, 18.1, 16.5, 16.1, 15.2, 11.4. *Data for* **15b**: [found: C, 79.30; H, 10.75. $C_{34}H_{56}O_3$ requires C, 79.63; H, 11.01%]; $[\alpha]_D^{20}$ 24.1 (*c* 0.25, $CHCl_3$); δ_H (600 MHz, $CDCl_3$) 4.69 (d, 1H, *J* 2.3 Hz, H-29), 4.58 (s, 1H, H-29), 4.45 (dd, 1H, *J* 5.5, 11.0 Hz, H-3), 3.70 (br d, 1H, *J* 9.7 Hz, H-28), 2.66–2.61 (m, 1H), 2.02 (s, 3H, CH_3CO), 1.68 (s, 3H, CH_3), 1.02 (t, 3H, *J* 7.1 Hz, CH_3), 1.00 (s, 3H, CH_3), 0.98 (s, 3H, CH_3), 0.83 (s, 6H, 2× CH_3), 0.82 (s, 3H, CH_3), 2.10–0.75 (m, 27H, lupane protons); δ_C (150 MHz, $CDCl_3$) 171.0 (C), 150.7 (C), 109.9 (CH_2), 80.9, 74.5, 55.4, 50.4 (C), 50.3, 50.1, 48.2, 42.9 (C), 41.0 (C), 38.3 (CH_2), 37.8 (C), 37.0 (C), 36.8, 34.2 (CH_2), 34.0 (CH_2), 33.2 (CH_2), 31.4 (CH_2), 28.2 (CH_2), 27.9, 26.5 (CH_2), 25.3 (CH_2), 23.7 (CH_2), 21.3, 20.9 (CH_2), 18.7, 18.2 (CH_2), 16.5, 16.1, 15.9, 14.9, 12.1.

*4.2.4.4. 3,28-Di-O-benzoyl-28-C-ethylbetulin (**17**).* White foam. [Found: C, 81.41; H, 9.25. $C_{46}H_{62}O_4$ requires C, 81.37; H, 9.20%]; δ_H (500 MHz, $CDCl_3$)—major diastereoisomer 8.08–8.04 (m, 4H, Ar), 7.57–7.42 (m, 6H, Ar), 5.79 (dd, 1H, *J* 2.4, 10.1 Hz, H-28), 4.72 (dd, 1H, *J* 5.1, 11.0 Hz, H-3), 4.44–4.43 (m, 1H, H-29), 4.31–4.30 (m, 1H, H-29), 2.77–2.72 (m, 1H), 1.60 (s, 3H, CH_3), 1.23 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 0.93 (s, 6H, 2× CH_3), 0.91 (s, 3H, CH_3), 2.40–0.85 (m, 26H, lupane protons); δ_C (125 MHz, $CDCl_3$) 166.6 (C), 166.3 (C), 150.7 (C), 132.8, 132.7, 131.0, 130.9, 129.5, 129.4, 128.4, 128.3, 128.3, 109.4 (CH_2), 81.6, 76.2, 55.5, 50.4, 50.3, 50.2 (C), 48.4, 43.0 (C), 41.1 (C), 38.4 (CH_2), 38.2 (C), 37.1 (C), 36.5, 34.7 (CH_2), 34.3 (CH_2), 33.6 (CH_2), 32.3 (CH_2), 28.1, 27.9 (CH_2), 25.1 (CH_2), 24.0 (CH_2), 23.8 (CH_2), 21.0 (CH_2), 18.6, 18.2 (CH_2), 16.8, 16.2, 16.2, 15.2, 10.9.

*4.2.4.5. 3,28-Di-O-acetyl-28-C-ethylbetulin (**18**).* Analytical samples of the corresponding diacetates were obtained as white foams by acylation of **15a** and **15b** with acetic anhydride in pyridine under standard conditions. *Data for* **18a**: [found: C, 77.43; H, 10.66. $C_{36}H_{58}O_4$ requires C, 77.93; H, 10.54%]; $[\alpha]_D^{20}$ 21.2 (*c* 0.3, $CHCl_3$); ν_{max} (film) 2938, 2872, 1733, 1367, 1244, 1017, 760 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 5.46 (dd, 1H, *J* 2.1, 10.2 Hz, H-28), 4.60 (d, 1H, *J* 1.5 Hz, H-29), 4.56 (br s, 1H, H-29), 4.46 (dd, 1H, *J* 6.6, 9.9 Hz, H-3), 2.33–2.28 (m, 1H), 2.07 (s, 3H, CH_3CO), 2.03 (s, 3H, CH_3CO), 1.66 (s, 3H, CH_3), 1.11 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.86 (t, 3H, *J* 7.4 Hz, CH_3), 0.85 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 2.12–0.76 (m, 26H, lupane protons); δ_C (150 MHz, $CDCl_3$) 171.0 (C), 170.8 (C), 150.9 (C), 109.4 (CH_2), 80.9, 75.4, 55.4, 50.4, 50.2, 50.1 (C), 48.9, 42.8 (C), 41.0 (C), 38.4 (CH_2), 37.8 (C), 37.1 (C), 36.5, 34.6 (CH_2), 34.3 (CH_2), 33.7 (CH_2), 32.4 (CH_2), 27.9, 27.8 (CH_2), 25.1 (CH_2), 23.7 (CH_2), 23.5 (CH_2), 21.7, 21.3, 21.0 (CH_2), 18.7, 18.1 (CH_2), 16.5, 16.1, 16.1, 15.1, 10.7. *Data for* **18b**: [found: C, 77.45; H, 10.69. $C_{36}H_{58}O_4$ requires C, 77.93; H, 10.54%]; $[\alpha]_D^{20}$ 23.1 (*c* 0.4, $CHCl_3$); ν_{max} (film) 2938, 2870, 1728, 1366, 1247, 1017, 760 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2.

*4.2.4.6. 3-O-Allyl-28-C-ethylbetulin (**9**).* Starting from 3-*O*-allylbetulinal (**7**, 1.086 g, 2.26 mmol) and 3 M ethylmagnesium bromide in Et_2O (3.8 mL, 11.4 mmol) **9a** (437 mg, 38%), and **9ab** (270 mg, 23%, **a**:**b**≈3:2), were obtained as white foams (column chromatography, hexane/ethyl acetate, 20:1). *Data for* major isomer (**9a**): [found: C, 82.23; H, 11.38. $C_{35}H_{58}O_2$ requires C, 82.29; H, 11.44%]; $[\alpha]_D^{20}$ 38.2 (*c* 0.3, $CHCl_3$); ν_{max} (film) 2943, 2871, 1458, 1375, 1216, 1091, 1070, 975, 921, 882, 758 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 5.95–5.88 (m, 1H, CH–allyl), 5.27–5.23 (m, 1H, CH–allyl), 5.12–5.08 (m, 1H, CH–allyl), 4.71 (br d, 1H, *J* 2.3 Hz, H-29), 4.57–4.56 (m, 1H, H-29), 4.13–4.09 (m, 1H, CH–allyl), 3.92 (br d, 1H, H-28), 3.89–3.85 (m, 1H, CH–allyl), 2.93–2.88 (m, 1H, H-19), 2.79 (dd, 1H, *J* 4.4, 11.9 Hz, H-3), 1.67 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.00 (t, 3H, *J* 7.3 Hz, CH_3), 0.99 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.82 (s, 3H, CH_3), 0.77 (s, 3H, CH_3), 2.10–0.65 (m, 27H, lupane protons); δ_C (150 MHz, $CDCl_3$) 151.5 (C), 135.9, 115.9 (CH_2), 109.3 (CH_2), 86.3, 74.4, 70.6 (CH_2), 55.8, 50.5, 50.4, 50.1 (C), 49.0, 43.0 (C), 41.0 (C), 38.8 (C), 38.6 (CH_2), 37.1 (C), 36.9, 34.3 (CH_2), 34.2 (CH_2), 32.2 (CH_2), 32.9 (CH_2), 28.1, 27.8 (CH_2), 25.3 (CH_2), 25.2 (CH_2), 23.1 (CH_2), 20.9 (CH_2), 19.0, 18.2 (CH_2), 16.3, 16.1, 16.0, 15.2, 11.4.

*4.2.4.7. 3-O-Acetyl-28-C-propylbetulin (**19**).* Starting from 3-*O*-acetylbetulinal (**13**, 560 mg, 1.16 mmol) and 2 M propylmagnesium chloride in Et_2O (2.9 mL, 5.8 mmol), **19a** (355 mg, 58%), **19ab** (38 mg, 6%, **a**:**b**≈8:1), and **19b** (53 mg, 9%) were obtained as white foams. *Data for* **19a**: [found: C, 80.02; H, 11.18. $C_{35}H_{58}O_3$ requires C, 79.79; H, 11.10%]; $[\alpha]_D^{20}$ 34.3 (*c* 0.3, $CHCl_3$); ν_{max} (film) 2953, 2872, 1733, 1718, 1639, 1249, 757 cm^{-1}; δ_H (500 MHz, $CDCl_3$) 4.70 (d, 1H, *J* 2.4 Hz, H-29), 4.58–4.57 (m, 1H, H-29), 4.47 (dd, 1H, *J* 5.7, 10.7 Hz, H-3), 4.06 (br d, 1H, *J* 8.2 Hz, H-28), 2.94–2.88 (m, 1H), 2.04 (s, 3H, CH_3CO), 1.69 (s, 3H, CH_3), 1.04 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.96 (t, 3H, *J* 7.2 Hz, CH_3), 0.85 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 2.10–0.76 (m, 29H, lupane protons); δ_C (125 MHz, $CDCl_3$) 171.0 (C), 151.5 (C), 109.4 (CH_2), 80.9, 72.0, 55.4, 50.5, 50.3, 50.0 (C), 49.0, 43.0 (C), 41.0 (C), 38.4 (CH_2), 37.8 (C), 37.1 (C), 36.9, 34.6 (CH_2), 34.3 (CH_2), 34.1 (CH_2), 33.2 (CH_2), 32.7 (CH_2), 27.9, 27.8 (CH_2), 25.2 (CH_2), 23.7 (CH_2), 21.3, 20.9 (CH_2), 19.6 (CH_2), 18.9, 18.1 (CH_2), 16.5, 16.1, 16.0, 15.2, 14.0. *Data for* **19b**: [found: C, 79.86; H, 10.99. $C_{35}H_{58}O_3$ requires C, 79.79; H, 11.10%]; $[\alpha]_D^{20}$ 18.6 (*c* 0.2, $CHCl_3$); ν_{max} (film) 2951, 2870, 1735, 1719, 1640, 1249, 758 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 4.72 (d, 1H, *J* 2.0 Hz, H-29), 4.60 (s, 1H, H-29), 4.47 (dd, 1H, *J* 5.5, 11.0 Hz, H-3), 3.85 (br d, 1H, *J* 10.3 Hz, H-28), 2.68–2.63 (m, 1H), 2.04 (s, 3H, CH_3CO), 1.70 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 0.85 (s, 6H, 2× CH_3), 0.83 (s, 3H, CH_3), 2.15–0.76 (m, 29H, lupane protons); δ_C (150 MHz, $CDCl_3$) 171.0 (C), 150.7 (C), 109.9 (CH_2), 80.9, 72.1, 55.4, 50.3, 50.2, 50.2 (C), 48.2, 42.9 (C), 41.0 (C), 38.3 (CH_2), 37.8 (C), 37.0 (C), 36.8, 35.8 (CH_2), 34.2 (CH_2), 34.1 (CH_2), 33.1 (CH_2), 31.3 (CH_2), 28.3 (CH_2), 27.9, 25.3 (CH_2), 23.7 (CH_2), 21.3, 20.9 (CH_2), 20.3 (CH_2), 18.7, 18.2 (CH_2), 16.5, 16.1, 15.9, 14.9, 14.0.

*4.2.4.8. 3-O-Acetyl-28-O-benzoyl-28-C-propylbetulin (**20**).* Analytical sample of the title compound was obtained as white foam by acylation of **19ab** with benzoyl chloride in pyridine. [Found: C, 79.92; H, 9.98. $C_{42}H_{62}O_4$ requires C, 79.95; H, 9.90%]; δ_H (600 MHz, $CDCl_3$)—major diastereoisomer 8.07–7.26 (m, 5H, Ar), 5.84 (dd, 1H, *J* 1.8, 9.9 Hz, H-28), 4.49–4.46 (m, 1H, H-3), 4.43–4.41 (m, 1H, H-29), 4.30 (br d, 1H, *J* 1.9 Hz, H-29), 2.04 (s, 3H, CH_3CO), 1.58 (s, 3H, CH_3), 1.21 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.92 (t, 3H, *J* 7.4 Hz, CH_3), 0.88 (s, 3H, CH_3), 0.85 (s, 6H, 2× CH_3), 2.40–0.76 (m, 29H, lupane protons); δ_C (150 MHz, $CDCl_3$)—major diastereoisomer (selected peaks) 171.0 (C), 166.4 (C), 150.8 (C), 109.4 (CH_2), 81.0, 74.5.

4.2.5. Acylation with phenyl chlorothionoformate. General procedure

*4.2.5.1. Phenyl (3-O-acetyl-28-C-methyl)betulinyl thiocarbonate (**21**).* To a solution of **14** (1.206 g, 2.42 mmol) in DCM (20 mL), pyridine (1.22 mL, 6.2 mmol), and phenyl chlorothionoformate (0.86 mL, 6.2 mmol) were added, and the reaction stirred at rt for 24 h. Solvents were evaporated, column chromatography (hexane/ethyl acetate, 40:1) of the residue gave 1.510 g of **21** as a pale yellow foam contaminated by a small amount of phenyl (3-*O*-acetyl-28-*C*-methyl)betulinyl carbonate (**24**). *Data for* **21**: [found: C, 75.47; H, 9.05; S, 5.03. $C_{40}H_{58}O_4S$ requires C, 75.66; H, 9.21; S, 5.05%]; δ_H (600 MHz, $CDCl_3$)—major diastereoisomer 7.42–7.08 (m, 5H, Ar), 5.96 (q, 1H, *J* 6.3 Hz, H-28), 4.68 (br d, 1H, H-29), 4.59–4.58 (m, 1H, H-29), 4.46 (dd, 1H, *J* 5.8, 10.6 Hz, H-3), 2.50–2.44 (m, 1H), 2.03 (s, 3H, OAc), 1.68 (s, 3H, CH_3), 1.34 (d, 3H, *J* 6.3 Hz, CH_3), 1.07 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 0.82 (s, 6H, CH_3), 0.82 (s, 3H, CH_3), 2.10–0.75 (m, 24H, lupane protons); δ_C (150 MHz, $CDCl_3$) 194.0 (C=S), 171.0 (C=O), 153.3 (C), 150.8 (C), 129.5, 126.4, 122.0, 109.7 (CH_2), 83.3, 80.9, 55.4, 50.4, 50.3 (C), 49.9, 48.9, 43.0 (C), 41.0 (C), 38.4 (CH_2), 37.8 (C), 37.2, 37.1 (C), 34.3 (CH_2), 34.2 (CH_2), 33.0 (CH_2), 31.9 (CH_2), 27.9, 27.7 (CH_2), 25.1 (CH_2), 23.7 (CH_2), 21.3, 21.0 (CH_2), 18.1 (CH_2), 16.5, 16.5, 16.1, 15.2, 15.1.

*4.2.5.2. Phenyl (3-O-acetyl-28-C-ethyl)betulinyl thiocarbonate (**22**).* Starting from **15** (1.314 g, 2.56 mmol) 1.495 g of **22** contaminated by small amount of phenyl (3-*O*-acetyl-28-*C*-ethyl)betulinyl carbonate (**25**) was obtained as white foam. *Data for* major isomer (**22a**)—[found: C, 75.96; H, 9.48; S, 4.74. $C_{41}H_{60}O_4S$ requires C, 75.88; H, 9.32; S, 4.94%]; δ_H (600 MHz, $CDCl_3$) 7.42–7.08 (m, 5H, Ar), 5.99 (dd, 1H, *J* 3.0, 9.7 Hz, H-28), 4.70 (br d, 1H, *J* 1.8 Hz, H-29), 4.60 (br s, 1H, H-29), 4.46 (dd, 1H, *J* 5.7, 10.6 Hz, H-3), 2.50–2.44 (m, 1H), 2.03 (s, 3H, CH_3CO), 1.69 (s, 3H, CH_3), 1.09 (s, 3H, CH_3), 1.06 (t, 3H, *J* 7.4 Hz, CH_3), 1.00 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 0.81 (s, 3H, CH_3), 0.81 (s, 3H, CH_3), 2.40–0.75 (m, 26H, lupane protons); δ_C (150 MHz, $CDCl_3$) 195.0 (C), 171.0 (C), 153.3 (C), 150.8 (C), 129.5, 126.4, 122.0, 109.6 (CH_2), 87.6, 80.9, 55.4, 51.1 (C), 50.4, 50.4, 48.6, 42.9 (C), 41.1 (C), 38.4 (CH_2), 37.8 (C), 37.1, 37.0 (C), 34.6 (CH_2), 34.3 (CH_2), 33.7 (CH_2), 32.2 (CH_2), 27.9, 27.8 (CH_2), 25.1 (CH_2), 24.2 (CH_2), 23.7 (CH_2), 21.3, 21.0 (CH_2), 18.1 (CH_2), 16.6, 16.5, 16.1, 15.3, 10.7; HRMS (ESI): $2MNa^+$, found 1319.8317. $C_{82}H_{120}NaO_2S_2$ requires 1319.8322. *Data for* minor isomer (**22b**)—[found: C, 75.86; H, 9.38; S, 4.85. $C_{41}H_{60}O_4S$ requires C, 75.88; H, 9.32; S, 4.94%]; δ_H (600 MHz, $CDCl_3$) 7.40–7.07 (m, 5H, Ar), 5.74 (dd, 1H, *J* 1.7, 10.4 Hz, H-28), 4.70 (br d, 1H, H-29), 4.60 (s, 1H, H-29), 4.46 (dd, 1H, *J* 5.4, 11.0 Hz, H-3), 2.81–2.76 (m, 1H), 2.03 (s, 3H, CH_3CO), 1.69 (s, 3H, CH_3), 1.10 (s, 3H, CH_3), 1.07 (t, 3H, *J* 7.3 Hz, CH_3), 1.00 (s, 3H, CH_3), 0.83 (s, 6H, 2× CH_3), 0.81 (s, 3H, CH_3), 2.18–0.75 (m, 26H, lupane protons); δ_C (150 MHz, $CDCl_3$) 195.8 (C), 171.0 (C), 153.5 (C), 150.2 (C), 129.4, 126.3, 122.0, 110.3 (CH_2), 89.3, 80.9, 55.4, 50.9 (C), 50.3, 50.2, 47.9, 43.1 (C), 41.1

(C), 38.4 (CH_2), 37.8 (C), 37.0, 37.0 (C), 34.9 (CH_2), 34.1 (CH_2), 33.2 (CH_2), 31.3 (CH_2), 28.8 (CH_2), 27.9, 25.6 (CH_2), 25.3 (CH_2), 23.7 (CH_2), 21.3, 20.9 (CH_2), 18.1 (CH_2), 16.5, 16.1, 15.0, 11.7.

*4.2.5.3. Phenyl (3-O-acetyl-28-C-propyl)betulinyl thiocarbonate (**23**).* Starting from **19** (1.496 g, 2.84 mmol) 1.614 g of **23** contaminated by a small amount of phenyl (3-*O*-acetyl-28-*C*-propyl) betulinyl carbonate (**26**) was obtained as a white foam. *Data for* major isomer (**23a**). [Found: C, 75.98; H, 9.33; S, 4.81. $C_{42}H_{62}O_4S$ requires C, 76.09; H, 9.43; S, 4.84%]; δ_H (600 MHz, $CDCl_3$) 7.43–7.09 (m, 5H, Ar), 6.03 (dd, 1H, *J* 2.5, 9.7 Hz, H-28), 4.71 (br s, 1H, H-29), 4.61 (br s, 1H, H-29), 4.47 (dd, 1H, *J* 5.7, 10.6 Hz, H-3), 2.04 (s, 3H, CH_3CO), 1.71 (s, 3H, CH_3), 1.10 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 1.00 (t, 3H, *J* 7.3 Hz, CH_3), 0.84 (s, 3H, CH_3), 0.82 (s, 3H, CH_3), 0.82 (s, 3H, CH_3), 2.52–0.75 (m, 29H, lupane protons); δ_C (150 MHz, $CDCl_3$) 194.9 (C), 171.0 (C), 153.5 (C), 150.9 (C), 129.5, 126.4, 122.2, 109.6 (CH_2), 86.3, 81.0, 55.4, 51.1 (C), 50.5, 50.4, 48.6, 43.0 (C), 41.1 (C), 38.4 (CH_2), 37.8 (C), 37.1, 37.1 (C), 34.7 (CH_2), 34.4 (CH_2), 33.8 (CH_2), 33.4 (CH_2), 32.3 (CH_2), 28.0, 27.8 (CH_2), 25.1 (CH_2), 23.7 (CH_2), 21.3, 21.1 (CH_2), 19.1 (CH_2), 18.1 (CH_2), 16.6, 16.5, 16.2, 15.3, 14.5. *Data for* minor isomer (**23b**)—[found: C, 76.04; H, 9.62; S, 4.70. $C_{42}H_{62}O_4S$ requires C, 76.09; H, 9.43; S, 4.84%]; δ_H (600 MHz, $CDCl_3$) 7.43–7.07 (m, 5H, Ar), 5.78 (br d, 1H, *J* 9.9 Hz, H-28), 4.70 (br s, 1H, H-29), 4.61 (br s, 1H, H-29), 4.47 (dd, 1H, *J* 5.4, 11.0 Hz, H-3), 2.84–2.79 (m, 1H), 2.04 (s, 3H, CH_3CO), 1.71 (s, 3H, CH_3), 1.11 (s, 3H, CH_3), 1.01 (m, 6H, 2× CH_3), 0.84 (s, 6H, 2× CH_3), 0.82 (s, 3H, CH_3), 2.18–0.75 (m, 28H, lupane protons); δ_C (150 MHz, $CDCl_3$) 195.7 (C), 171.0 (C), 153.5 (C), 150.2 (C), 129.5, 126.3, 122.0, 110.3 (CH_2), 87.7, 80.9, 55.4, 50.8 (C), 50.3, 50.3, 47.8, 43.1 (C), 41.1 (C), 38.4 (CH_2), 37.8 (C), 37.0, 37.0 (C), 35.2 (CH_2), 34.7 (CH_2), 34.1 (CH_2), 32.9 (CH_2), 31.2 (CH_2), 28.8 (CH_2), 27.9, 25.3 (CH_2), 23.7 (CH_2), 21.3, 20.9 (CH_2), 19.8 (CH_2), 18.7, 18.1 (CH_2), 16.5, 16.1, 14.9, 14.4.

*4.2.5.4. Phenyl (3-O-allyl-28-C-ethyl)betulinyl thiocarbonate (**10**) and phenyl (3-O-allyl-28-C-ethyl)betulinyl carbonate (**11**).* Compounds (**10**) and (**11**) were prepared from stereoisomer **9a**. Column chromatography (hexane/ethyl acetate, 40:1) of the residue gave **10** (390 mg) as white foam contaminated by approx. 8% of **11**. An analytical sample was separated on preparative TLC to afford pure samples of the title compounds. *Data for* **10**: [found: C, 77.85; H, 9.72. $C_{42}H_{62}O_3S$ requires C, 77.97; H, 9.66%]; $[\alpha]_D^{20}$ 62.5 (*c* 0.25, $CHCl_3$); δ_H (600 MHz, $CDCl_3$) 7.47–7.21 (m, 5H, Ar), 6.00 (dd, 1H, *J* 3.1, 9.7 Hz, H-28), 5.96–5.89 (m, 1H, CH–allyl), 5.27–5.23 (m, 1H, CH–allyl), 5.13–5.10 (m, 1H, CH–allyl), 4.71 (br d, 1H, *J* 1.9 Hz, H-29), 4.61–4.60 (m, 1H, H-29), 4.13–4.09 (m, 1H, CH–allyl), 3.88–3.85 (m, 1H, CH–allyl), 2.79 (dd, 1H, *J* 4.3, 11.8 Hz, H-3), 1.70 (s, 3H, CH_3), 1.09 (s, 3H, CH_3), 1.07 (t, 3H, *J* 7.4 Hz, CH_3), 1.00 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 0.80 (s, 3H, CH_3), 0.77 (s, 3H, CH_3), 2.50–0.60 (m, 27H, lupane protons); δ_C (150 MHz, $CDCl_3$)—selected signals 194.8 (C=S), 153.6 (C), 115.9 (CH_2), 87.6, 86.3, 70.6 (CH_2), 55.9, 50.6, 50.4, 38.7 (CH_2), 37.1, 34.6 (CH_2), 34.5 (CH_2), 33.7 (CH_2), 28.1, 27.8 (CH_2), 24.2 (CH_2), 23.1 (CH_2), 21.0 (CH_2), 18.2 (CH_2), 16.6, 16.3, 16.1, 15.3, 10.7; *m*/*z* (ESI) 669.4 (100, MNa^+), 1317.1 (36, $2MNa^+$). *Data for* **11**: [found: C, 80.01; H, 10.11. $C_{42}H_{62}O_4$ requires C, 79.95; H, 9.90%]; $[\alpha]_D^{20}$ 53.6 (*c* 0.6, $CHCl_3$); δ_H (600 MHz, $CDCl_3$) 7.42–7.24 (m, 5H, Ar), 5.96–5.89 (m, 1H, CH–allyl), 5.34 (dd, 1H, *J* 2.5, 10.3 Hz, H-28), 5.27–5.23 (m, 1H, CH–allyl), 5.12–5.10 (m, 1H, CH–allyl), 4.70 (br d, 1H, *J* 2.1 Hz, H-29), 4.60–4.59 (m, 1H, H-29), 4.13–4.09 (m, 1H, CH–allyl), 3.89–3.85 (m, 1H, CH–allyl), 2.78 (dd, 1H, *J* 4.3, 11.8 Hz, H-3), 2.51–2.46 (m, 1H, H-19), 1.70 (s, 3H, CH_3), 1.07 (s, 3H, CH_3), 1.03 (t, 3H, *J* 7.3 Hz, CH_3), 1.00 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.80 (s, 3H, CH_3), 0.77 (s, 3H, CH_3), 2.20–0.65 (m, 26H, lupane protons); δ_C (150 MHz, $CDCl_3$)—selected signals 154.0 (C=O), 151.3 (C), 135.9, 115.8 (CH_2), 109.6 (CH_2), 86.3, 81.6, 70.6 (CH_2), 55.9, 50.5, 50.3, 48.9, 42.9 (C), 41.0 (C), 38.8 (C), 38.6 (CH_2), 37.1 (C), 36.7, 34.5 (CH_2), 34.4 (CH_2), 33.5 (CH_2), 32.3 (CH_2), 28.1, 27.8 (CH_2), 25.2 (CH_2), 23.5 (CH_2), 23.1 (CH_2), 21.0, 18.9, 18.2 (CH_2), 16.3, 16.1, 16.1, 15.2, 10.5; *m*/*z* (ESI) 653.6 (100, MNa^+), 1284.0 (37, $2MNa^+$).

*4.2.6. Phenyl (3-O-acetyl-28-C-alkyl)betulinyl carbonate (**24**–**26**).* Analytical samples of the corresponding carbonates were obtained by acylation of **14a**, **15a**, and **19a** with phenyl chloroformate under standard conditions (DCM, pyridine).

*4.2.6.1. Phenyl (3-O-acetyl-28-C-methyl)betulinyl carbonate (**24**).* $[\alpha]_D^{20}$ 23.1 (*c* 0.3, $CHCl_3$); ν_{max} (film) 2946, 2873, 1782, 1755, 1732, 1593, 1493, 1247, 1181, 1160, 1022, 790, 768, 752, 688 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 7.42–7.26 (m, 5H, Ar), 5.45 (q, 1H, *J* 6.4 Hz, H-28), 4.69 (br d, 1H, *J* 2.1 Hz, H-29), 4.60–4.59 (m, 1H, H-29), 4.47 (dd, 1H, *J* 5.6, 10.8 Hz, H-3), 2.54–2.49 (m, 1H), 2.04 (s, 3H, CH_3CO), 1.70 (s, 3H, CH_3), 1.31 (d, 3H, *J* 6.3 Hz, CH_3), 1.06 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 2.06–0.75 (m, 24H, lupane protons); δ_C (150 MHz, $CDCl_3$) 171.0 (C), 153.2 (C), 151.2 (C), 151.0 (C), 150.8 (C), 129.6, 126.3, 120.9, 109.7 (CH_2), 80.9, 76.6, 55.4, 50.3, 49.8, 48.9, 42.9 (C), 40.9 (C), 38.4 (CH_2), 37.8 (C), 37.1 (C), 36.9, 34.2 (CH_2), 32.8 (CH_2), 32.0 (CH_2), 27.9, 27.7 (CH_2), 25.1 (CH_2), 23.7 (CH_2), 21.3, 20.9 (CH_2), 18.8 (CH_2), 18.1 (CH_2), 16.5, 16.2, 16.1, 15.2; HRMS (ESI): MNa^+, found 641.4172. $C_{40}H_{58}NaO_5$ requires 641.4182.

*4.2.6.2. Phenyl (3-O-acetyl-28-C-ethyl)betulinyl carbonate (**25**).* Compound (**25**): [found: C, 77.89; H, 9.70. $C_{41}H_{60}O_5$ requires C, 77.80; H, 9.56%]; $[\alpha]_D^{20}$ 32.4 (*c* 0.3, $CHCl_3$); ν_{max} (film) 2946, 2874, 1756, 1733, 1640, 1251, 1211, 758 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 7.40–7.15 (m, 5H, Ar), 5.33 (dd, 1H, *J* 2.5, 10.4 Hz, H-28), 4.69 (br d, 1H, *J* 2.0 Hz, H-29), 4.60 (br s, 1H, H-29), 4.46 (dd, 1H, *J* 5.7, 10.7 Hz, H-3), 2.51–2.45 (m, 1H), 2.02 (s, 3H, CH_3CO), 1.69 (s, 3H, CH_3), 1.07 (s, 3H, CH_3), 1.02 (t, 3H, *J* 7.3 Hz, CH_3), 1.01 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 0.81 (s, 6H, 2× CH_3), 2.18–0.75 (m, 26H, lupane protons); δ_C (150 MHz, $CDCl_3$) 171.0 (C), 154.0 (C), 151.3 (C), 150.8 (C), 129.5, 125.9, 121.2, 109.6 (CH_2), 81.7, 80.9, 55.4, 50.4, 50.2, 48.9, 42.9 (C), 41.0 (C), 38.4 (CH_2), 37.8 (C), 37.1 (C), 36.7, 34.4 (CH_2), 34.3 (CH_2), 33.5 (CH_2), 32.3 (CH_2), 27.9, 27.8 (CH_2), 25.2 (CH_2), 23.7 (CH_2), 23.5 (CH_2), 21.3, 21.0 (CH_2), 18.8, 18.1 (CH_2), 16.5, 16.1, 15.2, 10.5; HRMS (ESI): MNa^+, found 655.4340. $C_{41}H_{60}NaO_5$ requires 655.4333.

*4.2.6.3. Phenyl (3-O-acetyl-28-C-propyl)betulinyl carbonate (**26**).* $[\alpha]_D^{20}$ 25.5 (*c* 0.3, $CHCl_3$); ν_{max} (film) 2951, 2873, 1782, 1755, 1732, 1593, 1493, 1246, 1181, 1160, 752, 688 cm^{-1}; δ_H (500 MHz, $CDCl_3$) 7.36–7.09 (m, 5H, Ar), 5.36 (br d, 1H, *J* 9.5 Hz, H-28), 4.62 (br d, 1H, *J* 2.0 Hz, H-29), 4.54–4.52 (m, 1H, H-29), 4.40 (dd, 1H, *J* 5.9, 10.5 Hz, H-3), 2.46–2.40 (m, 1H), 1.96 (s, 3H, CH_3CO), 1.63 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.92 (t, 3H, *J* 7.1 Hz, CH_3), 0.77 (s, 3H, CH_3), 0.75 (s, 6H, 2× CH_3), 2.18–0.75 (m, 28H, lupane protons); δ_C (125 MHz, $CDCl_3$) 171.0 (C), 153.9 (C), 151.3 (C), 151.0 (C), 129.6, 126.3, 120.9, 109.6 (CH_2), 80.9, 79.9, 55.4, 50.4, 50.2, 48.9, 42.9 (C), 41.0 (C), 38.4 (CH_2), 37.8 (C), 37.1 (C), 36.7, 34.5 (CH_2), 34.3 (CH_2), 33.6 (CH_2), 32.7 (CH_2), 32.3 (CH_2), 27.9, 27.7 (CH_2), 25.2 (CH_2), 23.7 (CH_2), 21.0 (CH_2), 19.0 (CH_2), 18.1 (CH_2), 16.5, 16.1, 15.2, 14.0; HRMS (ESI): MNa^+, found 669.4495. $C_{42}H_{62}NaO_5$ requires 669.4495.

4.2.7. Reduction of thiocarbonates. General procedure. A solution of thiocarbonate (**21**, **22**, or **23**; 0.59 mmol) in toluene was degassed, then AIBN (20 mg) and tris(trimethylsilyl)silane (0.97 mmol) were added and the mixture was heated under an argon atmosphere at 80 °C for 5 h. Silica gel (5–10 g) was poured into the mixture and the solvent was evaporated to dryness. The residue was used for purification by flash chromatography (hexane/ethyl acetate, 40:1). Crude product was crystallized from methanol yielded expected product as colorless crystals.

*4.2.7.1. 3-O-Acetyl-28-C-methyl-lup-20(29)-en-3-ol (**27**).* Yield 88%; mp 198–200 °C; [found: C, 82.11; H, 11.26. $C_{33}H_{54}O_2$ requires

C, 82.10; H, 11.27%]; $[\alpha]_D^{20}$ 29.6 (*c* 0.2, $CHCl_3$); ν_{max} (film) 2946, 2872, 1732, 1641, 1456, 1376, 1246, 1038, 979, 882, 758 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 4.66 (br d, 1H, *J* 2.3 Hz, H-29), 4.55–4.54 (m, 1H, H-29), 4.46 (dd, 1H, *J* 5.5, 10.9 Hz, H-3), 2.38 (m, 1H), 2.03 (s, 3H, OAc), 1.68 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 0.82 (s, 3H, CH_3), 0.76 (t, 3H, *J* 7.4 Hz), 1.88–0.75 (m, 26H, lupane protons); δ_C (150 MHz, $CDCl_3$) 171.0 (C), 151.1 (C), 109.2 (CH_2), 80.9, 55.4, 50.4, 49.7, 47.4, 45.7 (C), 42.5 (C), 40.9 (C), 38.3 (CH_2), 37.8 (C), 37.1 (C), 36.9, 35.0 (CH_2), 34.1 (CH_2), 30.3 (CH_2), 30.0 (CH_2), 27.9, 27.1 (CH_2), 25.1 (CH_2), 23.7 (CH_2), 21.3, 21.0 (CH_2), 19.3 (CH_2), 18.2 (CH_2), 16.5, 16.1, 16.0, 14.8, 8.1.

4.2.7.2. 3-O-Acetyl-28-C-ethyl-lup-20(29)-en-3-ol (***28***). Yield 80%; mp 247–249 °C; [found: C, 80.76; H, 11.23. 2× $C_{34}H_{56}O_2$×-CH_3OH requires C, 80.80; H, 11.40%]; $[\alpha]_D^{20}$ 14.4 (*c* 0.3, $CHCl_3$); ν_{max} (film) 2949, 2870, 1733, 1640, 1245, 758 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 4.72 (d, 1H, *J* 2.4 Hz, H-29), 4.60–4.59 (m, 1H, H-29), 4.51 (dd, 1H, *J* 5.6, 10.9 Hz, H-3), 2.48–2.43 (m, 1H), 2.08 (s, 3H, CH_3CO), 1.73 (s, 3H, CH_3), 1.07 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 0.96 (t, 3H, *J* 7.3 Hz, CH_3), 0.90 (s, 3H, CH_3), 0.89 (s, 3H, CH_3), 0.88 (s, 3H, CH_3), 1.95–0.80 (m, 28H, lupane protons); δ_C (150 MHz, $CDCl_3$) 171.0 (C), 151.1 (C), 109.2 (CH_2), 80.9, 55.4, 50.4, 49.8, 47.4, 45.7 (C), 42.5 (C), 40.9 (C), 38.4 (CH_2), 37.8 (C), 37.1 (C), 37.0, 35.8 (CH_2), 34.1 (CH_2), 31.2 (CH_2), 30.1 (CH_2), 30.0 (CH_2), 27.9, 27.2 (CH_2), 25.1 (CH_2), 23.7 (CH_2), 21.3, 21.0 (CH_2), 19.3, 18.2 (CH_2), 17.0 (CH_2), 16.5, 16.1, 16.0, 15.4, 14.8; HRMS (ESI): M^+, found 496.4274. $C_{34}H_{56}O_2$ requires 496.4280.

4.2.7.3. 3-O-Acetyl-28-C-propyl-lup-20(29)-en-3-ol (***29***). Yield 71%; mp 213–214 °C; [found: C, 82.08; H, 11.66. $C_{35}H_{58}O_2$ requires C, 82.29; H, 11.44%]; $[\alpha]_D^{20}$ 21.6 (*c* 0.2, $CHCl_3$); ν_{max} (film) 2948, 2870, 1733, 1640, 1455, 1375, 1246, 1027, 979, 758 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 4.68 (d, 1H, *J* 2.2 Hz, H-29), 4.57–4.56 (m, 1H, H-29), 4.47 (dd, 1H, *J* 5.5, 10.9 Hz), 2.44–2.39 (m, 1H), 2.04 (s, 3H, CH_3CO), 1.68 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.92 (t, 3H, *J* 7.3 Hz, CH_3), 0.85 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 1.90–0.75 (m, 30H, lupane protons); δ_C (150 MHz, $CDCl_3$) 171.0 (C), 151.1 (C), 109.2 (CH_2), 80.9, 55.4, 50.4, 49.8, 47.4, 45.6 (C), 42.5 (C), 40.9 (C), 38.4 (CH_2), 37.8 (C), 37.1 (C), 37.0, 35.7 (CH_2), 34.1 (CH_2), 31.1 (CH_2), 30.1 (CH_2), 27.9, 27.2 (CH_2), 26.9 (CH_2), 26.0 (CH_2), 25.1 (CH_2), 23.8, 23.7 (CH_2), 21.3, 21.0 (CH_2), 19.3, 18.2 (CH_2), 16.5, 16.1, 15.9, 14.8, 14.2.

4.2.8. Reduction of acetyl group. General procedure. To a suspension of $LiAlH_4$ (575 mg, 15.1 mmol) in THF (20 mL) the corresponding acetate (**27**, **28**, or **29**; 3.64 mmol) was slowly added. Stirring was continued at rt for 30 min, then ethyl acetate (5 mL) was slowly added and the whole mixture was filtered through a short silica column (hexane/ethyl acetate, 1:1). Crude product was purified by column chromatography (hexane/ethyl acetate, 40:1→5:1) to afford product as foam.

4.2.8.1. 28-C-Methyl-lup-20(29)-en-3-ol (***1***). Yield 96%; [found: C, 84.63; H, 12.02. $C_{31}H_{52}O$ requires C, 84.48; H, 11.89%]; $[\alpha]_D^{20}$ 15.3 (*c* 0.25, $CHCl_3$); ν_{max} (film) 2943, 2871, 1457, 1041, 881, 766 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2.

4.2.8.2. 28-C-Ethyl-lup-20(29)-en-3-ol (***2***). Yield 96%; [found: C, 84.56; H, 12.11. $C_{32}H_{54}O$ requires C, 84.51; H, 11.97%]; $[\alpha]_D^{20}$ 6.1 (*c* 0.4, $CHCl_3$); ν_{max} (film) 2951, 2869, 1640, 1455, 1215, 1042, 882, 759 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2.

4.2.8.3. 28-C-Propyl-lup-20(29)-en-3-ol (***3***). Yield 96%; [found: C, 84.50; H, 12.25. $C_{33}H_{56}O$ requires C, 84.55; H, 12.04%]; $[\alpha]_D^{20}$ 6.3 (*c* 0.2, $CHCl_3$); ν_{max} (film) 2942, 2869, 1455, 1376, 1042, 882, 759 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2.

4.2.9. Synthesis of saponins (glycosylation). General procedure. A solution of trichloroacetimidate (**30**, **31** or **32**, 0.25 mmol) and a suitable acceptor (**1**, **2** or **3**, 0.20 mmol) in DCM (7 mL) was stirred for 20–30 min at rt over molecular sieves (4 Å, 300 mg, finely ground), then cooled to −40 °C and TMSOTf (20 μL, 0.1 mmol) was added. After 20 min, the reaction was quenched with Et_3N (0.2 mL), and the solvents were evaporated under diminished pressure. Column chromatography (hexane/ethyl acetate, 10:1) gave the protected saponins as foams.

4.2.9.1. 28-C-Methyllupeol 3β-O-(2,3,4,6-tetra-O-benzoyl-α-D-mannopyranoside) (***33a***). Yield 90%; $[\alpha]_D^{20}$ −5.8 (*c* 0.3, $CHCl_3$); ν_{max} (film) 2946, 2871, 1730, 1451, 1266, 1109, 1068, 757, 710 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 8.11–7.25 (m, 20H, Ar), 6.09 (t, 1H, *J* 10.1 Hz, H-4′), 5.91 (dd, 1H, *J* 3.2, 10.1 Hz, H-3′), 5.62 (dd, 1H, *J* 1.7, 3.2 Hz, H-2′), 5.29 (br d, 1H, *J* 1.7 Hz, H-1′), 4.71–4.66 (m, 2H, H-29, H-6′), 4.58–4.53 (m, 2H, H-29, H-5′), 4.48 (dd, 1H, *J* 5.1, 12.0 Hz, H-6′), 3.35 (dd, 1H, *J* 4.4, 11.8 Hz, H-3), 2.44–2.38 (m, 1H), 1.70 (s, 3H, CH_3), 1.11 (s, 3H, CH_3), 1.04 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.88 (s, 3H, CH_3), 0.78 (t, 3H, *J* 7.4 Hz, CH_3), 1.90–0.68 (m, 26H, lupane protons); δ_C (150 MHz, $CDCl_3$) 166.1 (C), 165.6 (C), 165.5 (C), 151.1 (C), 133.4, 133.1, 133.0, 129.8–128.3 (aromatic carbons), 109.3 (CH_2), 94.3 (C-1′), 84.2, 71.6, 70.3, 69.4, 67.0, 63.1 (CH_2), 55.7, 50.5, 49.7, 47.4, 45.8 (C), 42.5 (C), 40.9 (C), 38.6 (C), 38.2 (CH_2), 37.1 (C), 37.0, 35.1 (CH_2), 34.2 (CH_2), 30.4 (CH_2), 30.0 (CH_2), 28.8, 27.1 (CH_2), 25.1 (CH_2), 22.2 (CH_2), 21.0 (CH_2), 19.3 (CH_2), 16.5 (CH_2), 16.1, 16.0, 14.9, 14.2, 8.1; HRMS (ESI): MNa^+, found 1041.5486. $C_{65}H_{78}NaO_{10}$ requires 1041.5493.

4.2.9.2. 28-C-Methyllupeol 3β-O-(2,3,4-tri-O-benzoyl-α-L-arabinopyranoside) (***33c***). Yield 86%; [found: C, 76.00; H, 8.02. $C_{57}H_{72}O_8$×H_2O requires C, 75.80; H, 8.26%]; $[\alpha]_D^{20}$ 114.2 (*c* 0.3, $CHCl_3$); ν_{max} (film) 2945, 2870, 1729, 1452, 1282, 1263, 1093, 1069, 1028, 758, 710 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 8.07–7.26 (m, 15H, Ar), 5.76 (dd, 1H, *J* 6.4, 8.8 Hz, H-2′), 5.69–5.66 (m, 1H, H-4′), 5.59 (dd, 1H, *J* 3.6, 8.8 Hz, H-3′), 4.78 (d, 1H, *J* 6.4 Hz, H-1′), 4.67 (br d, 1H, *J* 2.1 Hz, H-29), 4.57–4.55 (m, 1H, H-29), 4.32 (dd, 1H, *J* 3.9, 12.9 Hz, H-5′), 3.87 (dd, 1H, *J* 2.1, 12.9 Hz, H-5′), 3.13 (dd, 1H, *J* 4.7, 11.7 Hz, H-3), 2.42–2.36 (m, 1H), 1.68 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.81 (s, 3H, CH_3), 0.77 (s, 3H, CH_3), 0.76 (t, 3H, *J* 7.5 Hz, CH_3), 0.64 (s, 3H, CH_3), 1.90–0.60 (m, 26H, lupane protons); δ_C (150 MHz, $CDCl_3$) 165.8 (C), 165.6 (C), 165.2 (C), 151.1 (C), 133.3, 133.2, 133.1, 129.9–128.3 (aromatic carbons), 109.2 (CH_2), 103.0 (C-1′), 90.1, 70.7, 70.3, 68.7, 62.5 (CH_2), 55.6, 50.4, 49.7, 47.4, 45.7 (C), 42.5 (C), 40.8 (C), 39.0 (C), 38.8 (CH_2), 38.7 (C), 36.9, 36.9 (CH_2), 35.0 (CH_2), 34.2 (CH_2), 30.3 (CH_2), 30.0, 27.7, 27.1 (CH_2), 26.1 (CH_2), 25.1 (CH_2), 21.0 (CH_2), 19.3 (CH_2), 18.1 (CH_2), 16.1, 16.0, 14.8, 8.1; HRMS (ESI): MNa^+, found 907.5120. $C_{57}H_{72}NaO_8$ requires 907.5125.

4.2.9.3. 28-C-Methyllupeol 3β-O-(2,3,4-tri-O-benzoyl-α-L-rhamnopyranoside) (***33e***). Yield 89%; [found: C, 77.61; H, 8.33. $C_{58}H_{74}O_8$ requires C, 77.47; H, 8.30%]; $[\alpha]_D^{20}$ 82.0 (*c* 0.3, $CHCl_3$); ν_{max} (film) 2944, 2871, 1731, 1451, 1278, 1263, 1108, 1069, 1028, 758, 711 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 8.11–7.26 (m, 15H, Ar), 5.83 (dd, 1H, *J* 3.3, 10.1 Hz, H-3′), 5.67 (t, 1H, *J* 9.9, 10.1 Hz, H-4′), 5.64 (dd, 1H, *J* 1.8, 3.3 Hz, H-2′), 5.08 (br d, 1H, H-1′), 4.68 (br d, 1H, *J* 2.1 Hz, H-29), 4.57–4.56 (m, 1H, H-29), 4.30 (dq, 1H, *J* 9.9, 6.2 Hz, H-5′), 3.21 (dd, 1H, *J* 7.4, 9.1 Hz, H-3), 2.43–2.38 (m, 1H), 1.68 (s, 3H, CH_3), 1.33 (d, 3H, *J* 6.2 Hz, H-6′), 1.05 (s, 3H, CH_3), 1.04 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.78 (t, 3H, *J* 7.4 Hz, CH_3), 1.90–0.70 (m, 26H, lupane protons); δ_C (150 MHz, $CDCl_3$) 165.8 (C), 165.7 (C), 165.6 (C), 151.2 (C), 133.4, 133.2, 133.0, 129.9–128.2 (aromatic carbons), 109.2 (CH_2), 99.7 (C-1′), 90.1, 72.0, 71.2, 70.2, 66.7, 55.5, 50.4, 49.7, 47.4, 45.8 (C), 42.5 (C), 40.9 (C), 39.1 (C), 38.6 (CH_2), 37.0, 36.9 (C), 35.0 (CH_2), 34.2 (CH_2), 30.3 (CH_2), 30.0 (CH_2), 28.2,

27.1 (CH_2), 25.7 (CH_2), 25.1 (CH_2), 21.0 (CH_2), 19.3 (CH_2), 18.3 (CH_2), 17.6, 16.4, 16.2, 16.0, 14.8, 8.1.

*4.2.9.4. 28-C-Ethyllupeol 3β-O-(2,3,4,6-tetra-O-benzoyl-α-D-mannopyranoside) (**34a**).* Yield 65%; $[\alpha]_D^{20}$ −9.2 (*c* 0.3, $CHCl_3$); ν_{max} (film) 2952, 2869, 1730, 1451, 1266, 1109, 1068, 1027, 758, 710 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 8.11–7.25 (m, 20H, Ar), 6.10 (t, 1H, *J* 10.1 Hz, H-4′), 5.91 (dd, 1H, *J* 3.2, 10.1 Hz, H-3′), 5.62 (dd, 1H, *J* 1.7, 3.2 Hz, H-2′), 5.29 (br d, 1H, *J* 1.7 Hz, H-1′), 4.71–4.66 (m, 2H, H-29, H-6′), 4.59–4.57 (m, 1H, H-29), 4.57–4.54 (m, 1H, H-5′), 4.48 (dd, 1H, *J* 5.1, 12.0 Hz, H-6′), 3.35 (dd, 1H, *J* 4.3, 11.7 Hz, H-3), 2.46–2.40 (m, 1H), 1.70 (s, 3H, CH_3), 1.11 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 0.93 (t, 3H, *J* 7.3 Hz, CH_3), 0.89 (s, 3H, CH_3), 1.90–0.65 (m, 28H, lupane protons); δ_C (150 MHz, $CDCl_3$) 166.1 (C), 165.6 (C), 165.5 (C), 151.1 (C), 133.4, 133.1, 133.0, 129.8–128.3 (aromatic carbons), 109.3 (CH_2), 94.3 (C-1′), 84.2, 71.6, 70.3, 69.4, 67.0, 63.1 (CH_2), 55.7, 50.5, 49.9, 47.4, 45.7 (C), 42.5 (C), 40.9 (C), 38.6 (C), 38.2 (CH_2), 37.1 (C), 37.0, 35.8 (CH_2), 34.2 (CH_2), 31.2 (CH_2), 30.1 (CH_2), 30.0 (CH_2), 28.8, 27.3 (CH_2), 25.1 (CH_2), 22.2 (CH_2), 21.0 (CH_2), 19.3, 18.3 (CH_2), 17.0 (CH_2), 16.5, 16.1, 16.0, 15.4, 14.9; HRMS (ESI): MNa^+, found 1055.5645. $C_{66}H_{80}NaO_{10}$ requires 1055.5649.

*4.2.9.5. 28-C-Ethyllupeol 3β-O-(2,3,4-tri-O-benzoyl-α-L-arabinopyranoside) (**34c**).* Yield 87%; [found: C, 76.46; H, 8.05. $C_{58}H_{74}O_8 \times ½H_2O$ requires C, 76.70; H, 8.32%]; $[\alpha]_D^{20}$ 110.3 (*c* 0.25, $CHCl_3$); ν_{max} (film) 2950, 2869, 1730, 1281, 1262, 1093, 709 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 8.07–7.26 (m, 15H, Ar), 5.76 (dd, 1H, *J* 6.3, 8.8 Hz, H-2′), 5.69–5.66 (m, 1H, H-4′), 5.59 (dd, 1H, *J* 3.6, 8.8 Hz, H-3′), 4.78 (d, 1H, *J* 6.3 Hz, H-1′), 4.68 (br d, 1H, *J* 2.2 Hz, H-29), 4.55–4.57 (m, 1H, H-29), 4.32 (dd, 1H, *J* 3.9, 12.9 Hz, H-5′), 3.88 (dd, 1H, *J* 2.1, 12.9 Hz, H-5′), 3.13 (dd, 1H, *J* 4.7, 11.6 Hz, H-3), 2.44–2.38 (m, 1H), 1.68 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.91 (t, 3H, *J* 7.3 Hz, CH_3), 0.81 (s, 3H, CH_3), 0.77 (s, 3H, CH_3), 0.65 (s, 3H, CH_3), 1.90–0.60 (m, 28H, lupane protons); δ_C (150 MHz, $CDCl_3$) 165.8 (C), 165.6 (C), 165.2 (C), 151.1 (C), 133.3, 133.2, 133.1, 129.9–128.3 (aromatic carbons), 109.3 (CH_2), 103.0 (C-1′), 90.1, 70.7, 70.3, 68.7, 62.6 (CH_2), 55.6, 50.4, 49.8, 47.4, 45.7 (C), 42.5 (C), 40.9 (C), 39.0 (C), 38.7 (CH_2), 37.0, 36.9 (C), 35.8 (CH_2), 34.2 (CH_2), 31.2 (CH_2), 30.1 (CH_2), 30.0 (CH_2), 27.7, 27.2 (CH_2), 26.1 (CH_2), 25.1 (CH_2), 21.0 (CH_2), 19.3, 18.1 (CH_2), 17.0 (CH_2), 16.1, 16.0, 15.4, 14.8; HRMS (ESI): MNa^+, found 921.5277. $C_{58}H_{74}NaO_8$ requires 921.5281.

*4.2.9.6. 28-C-Ethyllupeol 3β-O-(2,3,4-tri-O-benzoyl-α-L-rhamnopyranoside) (**34e**).* Yield 68%; [found: C, 77.39; H, 8.53. $C_{59}H_{76}O_8$ requires C, 77.60; H, 8.39%]; $[\alpha]_D^{20}$ 75.9 (*c* 0.3, $CHCl_3$); ν_{max} (film) 2944, 2868, 1730, 1451, 1263, 1110, 758, 712 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 8.10–7.23 (m, 15H, Ar), 5.82 (dd, 1H, *J* 3.3, 10.1 Hz, H-3′), 5.67 (dd, 1H, *J* 9.9, 10.1 Hz, H-4′), 5.64 (dd, 1H, *J* 1.8, 3.3 Hz, H-2′), 5.07 (br d, 1H, H-1′), 4.67 (br d, 1H, *J* 2.1 Hz, H-29), 4.56–4.55 (m, 1H, H-29), 4.30 (dq, 1H, *J* 9.9, 6.2 Hz, H-5′), 3.20 (dd, 1H, *J* 7.3, 9.1 Hz, H-3), 2.44–2.38 (m, 1H), 1.67 (s, 3H, CH_3), 1.32 (d, 3H, *J* 6.2 Hz, H-6′), 1.04 (s, 6H, 2× CH_3), 0.95 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.91 (t, 3H, *J* 7.3 Hz), 0.89 (s, 3H, CH_3), 1.90–0.70 (m, 28H, lupane protons); δ_C (150 MHz, $CDCl_3$) 165.8 (C), 165.7 (C), 165.6 (C), 151.2 (C), 133.4, 133.2, 133 0, 129.9–128.2 (aromatic carbons), 109.2 (CH_2), 99.7 (C-1′), 90.1, 72.0, 71.2, 70.2, 66.7, 55.5, 50.4, 49.8, 47.4, 45.7 (C), 42.5 (C), 40.9 (C), 39.2 (C), 38.6 (CH_2), 37.0, 36.9 (C), 35.8 (CH_2), 34.2 (CH_2), 31.2 (CH_2), 30.1 (CH_2), 30.0 (CH_2), 28.2, 27.2 (CH_2), 25.7 (CH_2), 25.1 (CH_2), 21.0 (CH_2), 19.3, 18.3 (CH_2), 17.6, 17.0 (CH_2), 16.4, 16.1, 16.0, 15.4, 14.9.

*4.2.9.7. 28-C-Propyllupeol 3β-O-(2,3,4,6-tetra-O-benzoyl-α-D-mannopyranoside) (**35a**).* Yield 61%; $[\alpha]_D^{20}$ −8.8 (*c* 0.3, $CHCl_3$); ν_{max} (film) 2950, 2869, 1731, 1451, 1266, 1109, 1068, 757, 710 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 8.11–7.25 (m, 20H, Ar), 6.09 (t, 1H, *J* 10.1 Hz, H-4′), 5.91 (dd, 1H, *J* 3.2, 10.1 Hz, H-3′), 5.62 (dd, 1H, *J* 1.8, 3.2 Hz, H-2′), 5.29 (br s, 1H, H-1′), 4.71–4.66 (m, 2H, H-29, H-6′), 4.58–4.57 (m, 1H, H-29), 4.57–4.53 (m, 1H, H-5′), 4.48 (dd, 1H, *J* 5.1, 11.9 Hz, H-6′), 3.35 (dd, 1H, *J* 4.3, 11.8 Hz, H-3), 2.45–2.40 (m, 1H), 1.70 (s, 3H, CH_3), 1.11 (s, 3H, CH_3), 1.05 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.93 (t, 3H, *J* 7.4 Hz, CH_3), 0.88 (s, 3H, CH_3), 1.90–0.65 (m, 30H, lupane protons); δ_C (150 MHz, $CDCl_3$) 166.1 (C), 165.6 (C), 165.5 (C), 151.1 (C), 133.4, 133.1, 133.0, 129.9–128.3 (aromatic carbons), 109.3 (CH_2), 94.3 (C-1′), 84.2, 71.6, 70.2, 69.4, 67.0, 63.1 (CH_2), 55.7, 50.5, 49.9, 47.4, 45.6 (C), 42.5 (C), 40.9 (C), 38.6 (C), 38.2 (CH_2), 37.1 (C), 37.0, 35.8 (CH_2), 34.2 (CH_2), 31.2 (CH_2), 30.1 (CH_2), 28.8, 27.3 (CH_2), 27.0 (CH_2), 26.0 (CH_2), 25.1 (CH_2), 23.9 (CH_2), 22.2 (CH_2), 21.0 (CH_2), 19.3, 18.3 (CH_2), 16.5, 16.1, 16.0, 14.9, 14.2; HRMS (ESI): MNa^+, found 1069.5801. $C_{67}H_{82}NaO_{10}$ requires 1069.5806.

*4.2.9.8. 28-C-Propyllupeol 3β-O-(2,3,4-tri-O-benzoyl-α-L-arabinopyranoside) (**35c**).* Yield 79%; [found: C, 77.89; H, 8.09. $C_{59}H_{76}O_8$ requires C, 77.60; H, 8.39%]; $[\alpha]_D^{20}$ 68.9 (*c* 0.3, $CHCl_3$); ν_{max} (film) 2944, 2869, 1730, 1451, 1282, 1262, 1104, 1093, 758, 710 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 8.07–7.25 (m, 15H, Ar), 5.76 (dd, 1H, *J* 6.4, 8.8 Hz, H-2′), 5.67 (m, 1H, H-4′), 5.59 (dd, 1H, *J* 3.6, 8.8 Hz, H-3′), 4.78 (d, 1H, *J* 6.4 Hz, H-1′), 4.68 (br d, 1H, *J* 2.2 Hz, H-29), 4.56 (s, 1H, H-29), 4.32 (dd, 1H, *J* 3.9, 13.0 Hz, H-5′), 3.87 (dd, 1H, *J* 2.0, 13.0 Hz, H-5′), 3.13 (dd, 1H, *J* 4.7, 11.6 Hz, H-3), 2.44–2.38 (m, 1H), 1.68 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.91 (t, 3H, *J* 7.3 Hz, CH_3), 0.81 (s, 3H, CH_3), 0.77 (s, 3H, CH_3), 0.65 (s, 3H, CH_3), 1.90–0.60 (m, 30H, lupane protons); δ_C (150 MHz, $CDCl_3$) 165.8 (C), 165.6 (C), 165.2 (C), 151.1 (C), 133.3, 133.2, 133.1, 129.9–128.3 (aromatic carbons), 109.2 (CH_2), 103.0 (C-1′), 90.1, 70.7, 70.3, 68.7, 62.6 (CH_2), 55.6, 50.4, 49.8, 47.4, 45.6 (C), 42.5 (C), 40.9 (C), 39.0 (C), 38.7 (CH_2), 37.0, 36.9 (C), 35.7 (CH_2), 34.2 (CH_2), 31.1 (CH_2), 30.1 (CH_2), 27.7, 27.2 (CH_2), 26.9 (CH_2), 26.1 (CH_2), 26.0 (CH_2), 25.1 (CH_2), 23.8 (CH_2), 21.0 (CH_2), 19.3, 18.1 (CH_2), 16.0, 15.9, 14.8, 14.2.

*4.2.9.9. 28-C-Propyllupeol 3β-O-(2,3,4-tri-O-benzoyl-α-L-rhamnopyranoside) (**35e**).* Yield 78%; [found: C, 77.59; H, 8.55. $C_{60}H_{78}O_8$ requires C, 77.72; H, 8.48%]; $[\alpha]_D^{20}$ 75.6 (*c* 0.3, $CHCl_3$); ν_{max} (film) 2954, 2865, 1731, 1450, 1265, 1107, 757, 710 cm^{-1}; δ_H (600 MHz, $CDCl_3$) 8.10–7.23 (m, 15H, Ar), 5.82 (dd, 1H, *J* 3.3, 10.1 Hz, H-3′), 5.67 (dd, 1H, *J* 9.9, 10.1 Hz, H-4′), 5.63 (dd, 1H, *J* 1.8, 3.3 Hz, H-2′), 5.07 (br s, 1H, H-1′), 4.67 (br d, 1H, *J* 2.2 Hz, H-29), 4.55 (s, 1H, H-29), 4.30 (dq, 1H, *J* 9.9, 6.2 Hz, H-5′), 3.19 (dd, 1H, *J* 7.6, 8.7 Hz, H-3), 2.44–2.38 (m, 1H), 1.67 (s, 3H, CH_3), 1.32 (d, 3H, *J* 6.2 Hz, H-6′), 1.04 (s, 6H, 2× CH_3), 0.95 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.92 (t, 3H, *J* 7.4 Hz), 0.89 (s, 3H, CH_3), 1.90–0.70 (m, 30H, lupane protons); δ_C (150 MHz, $CDCl_3$) 165.8 (C), 165.7 (C), 165.5 (C), 151.2 (C), 133.4, 133.2, 133.0, 129.9–128.2 (aromatic carbons), 109.2 (CH_2), 99.7 (C-1′), 90.1, 72.0, 71.2, 70.2, 66.7, 55.5, 50.4, 49.8, 47.4, 45.6 (C), 42.5 (C), 40.9 (C), 39.1 (C), 38.6 (CH_2), 37.0, 36.9 (C), 35.7 (CH_2), 34.2 (CH_2), 31.1 (CH_2), 30.1 (CH_2), 28.2, 27.2 (CH_2), 27.0 (CH_2), 26.0 (CH_2), 25.7 (CH_2), 25.1 (CH_2), 23.9 (CH_2), 19.3, 18.3 (CH_2), 17.6, 16.4, 16.1, 16.0, 14.9, 14.2.

4.2.10. General procedure for the debenzoylation reaction. A suspension of protected saponin (0.10 mM) and K_2CO_3 (20 mg) in MeOH (3 mL) was stirred overnight, then neutralized with Amberlyst 15 resin (H^+ form) and filtered through a PTFE syringe filter (MeOH as eluent), and the filtrate was evaporated to dryness. The residual methyl benzoate was removed by adding water (3 mL) and freeze-drying to afford the free saponin as a white powder.

*4.2.10.1. 28-C-Methyllupeol 3β-O-α-D-mannopyranoside (**33b**).* Yield 96%; [found: C, 72.25; H, 10.51. $C_{37}H_{62}O_6 \times ½H_2O$ requires C, 72.63; H, 10.38%]; $[\alpha]_D^{20}$ 60.0 (*c*, 0.3, $CHCl_3$); ν_{max} (film) 3380 (br), 2942, 2871, 1455, 1376, 1104, 1059, 1027, 758 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRMS (ESI): MNa^+, found 625.4445. $C_{37}H_{62}NaO_6$ requires 625.4444.

*4.2.10.2. 28-C-Methyllupeol 3β-O-α-L-arabinopyranoside (**33d**).* Yield 60%; [found: C, 74.02; H, 10.85. $C_{36}H_{60}O_5\times½H_2O$ requires C, 74.31; H, 10.57%]; $[\alpha]_D^{20}$ 3.5 (*c*, 0.25, $CHCl_3$); ν_{max} (film) 3411 (br), 2944, 2871, 1454, 1376, 1087, 1070, 1000, 759 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRMS (ESI): MNa^+, found 595.4340. $C_{36}H_{60}NaO_5$ requires 595.4338.

*4.2.10.3. 28-C-Methyllupeol 3β-O-α-L-rhamnopyranoside (**33f**).* Yield 94%; [found: C, 74.37; H, 10.86. $C_{37}H_{62}O_5\times½H_2O$ requires C, 74.58; H, 10.66%]; $[\alpha]_D^{20}$ −19.0 (*c* 0.3, $CHCl_3$+10% methanol); ν_{max} (film) 3398 (br), 2937, 2867, 1452, 1131, 1105, 1069, 1052, 982, 757 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRMS (ESI): MNa^+, found 609.4493. $C_{37}H_{62}NaO_5$ requires 609.4495.

*4.2.10.4. 28-C-Ethyllupeol 3β-O-α-D-mannopyranoside (**34b**).* Yield 97%; [found: C, 73.14; H, 10.87. $C_{38}H_{64}O_6\times½H_2O$ requires C, 72.92; H, 10.47%]; $[\alpha]_D^{20}$ 52.5 (*c*, 0.3, $CHCl_3$); ν_{max} (film) 3386 (br), 2943, 2870, 1454, 1102, 1060, 1026, 977, 882, 759 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRMS (ESI): MNa^+, found 639.4598. $C_{38}H_{64}NaO_6$ requires 639.4601.

*4.2.10.5. 28-C-Ethyllupeol 3β-O-α-L-arabinopyranoside (**34d**).* Yield 96%; [found: C, 73.81; H, 10.84. $C_{37}H_{62}O_5\times H_2O$ requires C, 73.47; H, 10.66%]; $[\alpha]_D^{20}$ −3.8 (*c* 0.3, $CHCl_3$); ν_{max} (film) 3416 (br), 2951, 2870, 1454, 1375, 1141, 1087, 1070, 999, 882, 759 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRMS (ESI): MNa^+, found 609.4498. $C_{37}H_{62}NaO_5$ requires 609.4495.

*4.2.10.6. 28-C-Ethyllupeol 3β-O-α-L-rhamnopyranoside (**34f**).* Yield 87%; [found: C, 74.86; H, 10.89. $C_{38}H_{64}O_5\times½H_2O$ requires C, 74.83; H, 10.74%]; $[\alpha]_D^{20}$ −27.3 (*c* 0.3, $CHCl_3$+10% methanol); ν_{max} (film) 3401 (br), 2942, 2870, 1453, 1376, 1127, 1053, 982, 881, 758 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2.

*4.2.10.7. 28-C-Propyllupeol 3β-O-α-D-mannopyranoside (**35b**).* Yield 93%; [found: C, 72.61; H, 10.63. $C_{39}H_{66}O_6\times H_2O$ requires C, 72.18; H, 10.56%]; $[\alpha]_D^{20}$ 53.8 (*c*, 0.3, $CHCl_3$); ν_{max} (film) 3387 (br), 2941, 2870, 1455, 1376, 1103, 1060, 1026, 979, 882, 759 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRMS (ESI): MNa^+, found 653.4753. $C_{39}H_{66}NaO_6$ requires 653.4757.

*4.2.10.8. 28-C-Propyllupeol 3β-O-α-L-arabinopyranoside (**35d**).* Yield 98%; [found: C, 73.55; H, 10.89. $C_{38}H_{64}O_5\times H_2O$ requires C, 73.74; H, 10.75%]; $[\alpha]_D^{20}$ −1.0 (*c* 0.25, $CHCl_3$); ν_{max} (film) 3408 (br), 2943, 2870, 1454, 1376, 1141, 1087, 1070, 1000, 882, 759 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRMS (ESI): MNa^+, found 623.4664. $C_{38}H_{64}NaO_5$ requires 623.4651.

*4.2.10.9. 28-C-Propyllupeol 3β-O-α-L-rhamnopyranoside (**35f**).* Yield 92%; [found: C, 74.00; H, 10.98. $C_{39}H_{66}O_5\times H_2O$ requires C, 74.00; H, 10.83%]; $[\alpha]_D^{20}$ −26.5 (*c* 0.2, $CHCl_3$+10% methanol); ν_{max} (film) 3388 (br), 2937, 2868, 1452, 1377, 1277, 1131, 1069, 1051, 984 cm^{-1}; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HRMS (ESI): MNa^+, found 637.4813. $C_{39}H_{66}NaO_5$ requires 637.4808.

4.3. Biological evaluation

4.3.1. Cell culture. Stock solutions (10 mmol/L) of saponins were prepared by dissolving an appropriate quantity of each substance in DMSO. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin were purchased from Sigma (MO, USA). Calcein AM was obtained from Molecular Probes (Invitrogen Corporation, CA, USA).

The screening cell lines (T-lymphoblastic leukemia cell line CEM, breast carcinoma cell line MCF7, cervical carcinoma cell line HeLa, and human fibroblasts BJ) were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in DMEM medium (Sigma, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 10,000 U penicillin and 10 mg/mL streptomycin. The cell lines were maintained under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment. Cells were subcultured twice (fibroblasts) or three times a week using the standard trypsinization procedure, and used from up to thirtieth passage.

4.3.2. Calcein AM assay. Suspensions of tested cell lines (ca. 1.0×10^5 cells/mL) were placed in 96-well microtiter plates and after 24 h of stabilization (time zero) the tested compounds were added (in four 20 μL aliquots) in serially diluted concentrations in dimethylsulfoxide (DMSO). Control cultures were treated with DMSO alone, and the final concentration of DMSO in the incubation mixtures never exceeded 0.6%. The tested compounds were typically evaluated at six three-fold dilutions and the highest final concentration was generally 50 μM. After 72 h incubation, 100 μl Calcein AM solution (Molecular Probes, Invitrogen, CA, USA) was added, and incubation was continued for a further hour. The fluorescence of viable cells was then quantified using a Fluoroskan Ascent instrument (Labsystems, Finland). The percentage of surviving cells in each well was calculated by dividing the intensity of the fluorescence signals from the exposed wells by the intensity of signals from control wells and multiplying by 100. These ratios were then used to construct dose-response curves from which IC_{50} values, the concentrations of the respective compounds that were lethal to 50% of the tumor cells, were calculated. The results obtained for selected compounds are shown in Table 1.

4.3.3. Flow cytometric analysis of the cell cycle and apoptosis. HeLa cervical carcinoma cells were trypsinized, seeded in 100-mm culture dishes, and immediately incubated with the test compounds. After 24 h, the cells were again detached with trypsin, washed, fixed and stained in 0.1% [v/v] Triton X-100, 0.2 mg/mL RNase A and 10 μg/mL propidium iodide in PBS. Their DNA contents were then assessed with a flow cytometer (Cell Lab Quanta SC—MPL, Beckman Coulter, CA USA), and the distribution of cells in subG$_1$ ('apoptotic cells'), G$_0$/G$_1$, S and G$_2$/M peaks were quantified, by histogram analysis, using MultiCycle AV software (Phoenix Flow Systems, CA, USA).

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