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Carbohydrate Research 343 (2008) 995–1003
Carbohydrate RESEARCH

Synthesis of lupane-type saponins bearing mannosyl and 3,6-branched trimannosyl residues and their evaluation as anticancer agents

Piotr Cmoch,[a] Zbigniew Pakulski,[a,*] Jana Swaczynová[b] and Miroslav Strnad[b]

[a]*Institute of Organic Chemistry, Polish Academy of Sciences, Kasprzaka 44/52, PL-01-224 Warszawa, Poland*
[b]*Laboratory of Growth Regulators, Palacky University & Institute of Experimental Botany ASCR, Slechtitelu 11, CZ-783 71 Olomouc, Czech Republic*

Received 10 October 2007; received in revised form 11 February 2008; accepted 12 February 2008
Available online 20 February 2008

Abstract—The saponins modified with mono- or trimannosyl residues can provide a convenient means of delivering drugs to certain human cells via interactions with mannose receptors. In the study reported therein, we developed a convenient approach for the synthesis of 3-*O*-mannoside and branched trimannoside derivatives of the saponin lupeol and of C-28 acyl esters of 3-*O*-acetyl-betulinic acid bearing the same mannosyl entities. Lupeol and 3-*O*-acetyl-betulinic acid were mannosylated with tetra-*O*-benzoyl- or tetra-*O*-acetyl-α-D-mannopyranosyl trichloroacetimidates. De-esterification followed by regioselective dimannosylation of the unprotected monosaccharide derivatives with 2 equiv of tetra-*O*-benzoyl-α-D-mannopyranosyl trichloroacetimidate selectively yielded O-3,O-6-linked trimannosides. The cytotoxic activity of selected lupane-type saponins (derivatives of lupeol, betulinic acid, and betulin) toward normal human fibroblasts and various cancer cell lines was also compared.

Keywords: Saponin; Betulin; Lupeol; Betulinic acid; Glycosylation

1. Introduction

Terminal α-D-mannopyranosyl units that are recognized by the receptors of macrophages[1] and dendritic cells[2] commonly occur in glycoproteins of pathogenic bacteria, yeasts, viruses, and various parasites. The branched trisaccharide 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranosyl ligand binds with especially high affinity to these mannose receptors,[3] providing a possible way to transport antigens or drugs into human dendritic cells or macrophages. The interaction of carbohydrates with proteins responsible for such effects is intensively studied, and information regarding trimannoside binding has been published in several recent studies.[4]

Saponins are a large family of steroid or triterpenoid glycosides, widely distributed in plants and in some marine organisms,[5] in which hydrophilic mono- or oligosaccharides are attached to a hydrophobic sapogenin backbone. They have received considerable attention because of their diverse, promising biological and pharmaceutical properties, including antitumor,[6] antiviral,[7] antifungal,[8] antiinflamatory,[9] and other[10] activities. They have also been shown recently to have significant effects on plant growth.[11]

Lupeol [**1**, 3-β-hydroxy-lup-20(29)-ene, Chart 1] is found in many plant species,[10,12] betulin (3-β-28-dihydroxylup-20(29)-ene) is a highly abundant component of birch bark, and betulinic acid [**2**, 3β-hydroxyl-up-20(29)-ene-28-oic acid], which has very interesting and promising biological properties,[13] can also be isolated from various plants,[12c,e,13d,f] or easily prepared from betulin.[14] Natural saponins based on lupeol[15] and betulinic acid[16] are rarely found in nature, and reports

* Corresponding author. E-mail: pakul@icho.edu.pl

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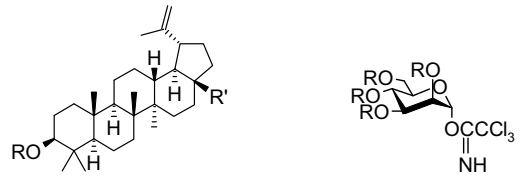
doi:10.1016/j.carres.2008.02.011



1: R = H, R' = CH_3 (lupeol)
2: R = H, R' = CO_2H (betulinic acid)
3: R = Ac, R' = CH_3
4: R = Ac, R' = CO_2H

5: R = Bz
6: R = Ac

Chart 1.

on their synthesis are also sporadic.[17] We have discussed various aspects of the chemistry and biological activity of betulin and its derivatives in a previous paper.[18]

Promisingly, for their potential clinical use, these triterpenoids have shown no hemolytic activity at high concentrations (100 mmol/L), and very weak hemolytic activity even at extremely high concentrations (500 mmol/L).[10a] For instance, for some derivatives of betulin and betulinic acid, which showed high anti-HIV activity, EC_{50} (concentration which produces 50% of the maximum response) values $<6.6 \times 10^{-4}$ μM and remarkably high therapeutic index (TI), exceeding 20,000, have been obtained, compared to 1.5 μM and 12,000, respectively, for azidothymidine (AZT).[19] Some derivatives, for which IC_{50} (concentrations leading to 50% inhibition of viability) values were approx. 20–80 μM, have also shown significant cytotoxicity and anti-tumor properties.[20] Although betulin and lupeol themselves are usually inactive, betulinic acid was found to be selectively cytotoxic against several cancer cell lines.[13a]

Recently, we were interested in the preparation of saponins from several lupane-type triterpenes (lupeol, betulinic acid, and betulin). Attaching trimannoside moieties to sapogenins as a hydrophilic transport-facilitating functional group could both improve their ability to enter target cells via interactions with mannose receptors and increase their solubility, thus providing a convenient drug delivery strategy. Additionally, due to the presence of the sugar fragment and potential affinity with the dendritic cells, these derivatives may be considered as components of carbohydrate-based cancer vaccines.[21]

Several formal routes leading to 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranose have been reported, including glycosylation of partially protected mannopyranoside derivatives.[22] A direct approach for synthesizing this mannotriose derivative has also been reported via the selective O-3,O-6 mannosylation of unprotected mannosides.[18,23] In this paper, as a continuation of our studies on regioselective glycosylation,[18,23d,h] we report a convenient method for synthesizing lupeol trimannoside and trimannosyl betulinate, which are potential antitumor and antiviral agents.

2. Results and discussion

2.1. Synthesis of lupeol mannosides

For the synthesis of saponins having mannosyl or trimannosyl residues connected with lupeol and betulinic acid, the synthetic strategy previously applied by us for the preparation of betulin derivatives was used.[18] Reacting lupeol (**1**) with 2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl trichloroacetimidate (**5**) in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) gave the expected glycoside **7** in good yield (95%). Debenzoylation with potassium carbonate in methanol afforded the free mannoside **8** (79%), which was further mannosylated with 2 equiv of **5** to yield the (1→3, 1→6)-linked trisaccharide **9** purified by chromatography as its diacetate **10** (27% after two steps). According to NMR spectra, this product was contaminated by small amounts (approx. 15%) of inseparable 1-*O*-acetyl-2,3,4,6-tetra-*O*-benzoyl-D-mannopyranose.

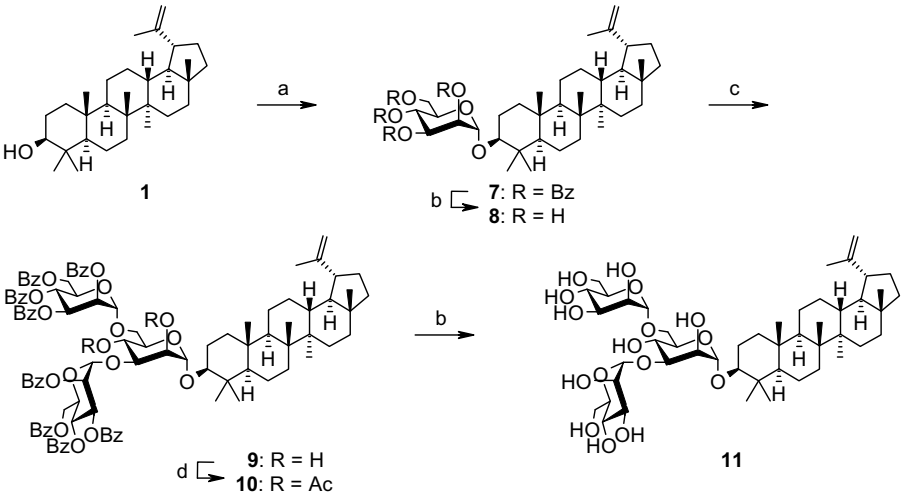
No other regioisomer was detected in the above reaction, in accordance with our previous observations that *O*-benzoyl-protected donors afforded (1→3, 1→6)-linked mannosides regioselectively.[18,23h] Typical deprotection with potassium carbonate in methanol afforded the final saponin **11** (88%), slightly contaminated by small amounts of free mannose (Scheme 1).

2.2. Synthesis of betulinic acid mannosyl esters

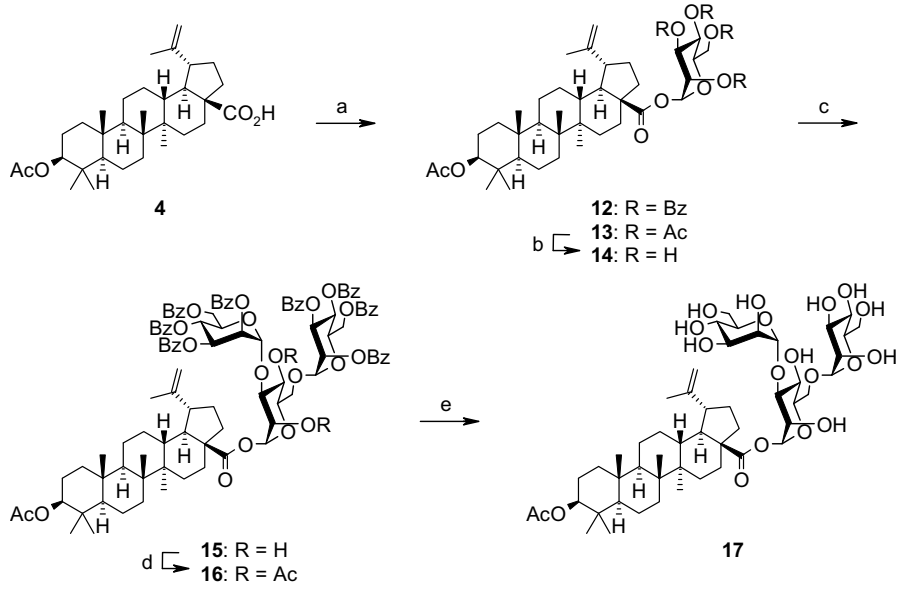
Very similar transformations were used to prepare saponins **14** and **17**. Glycosylation of 3-*O*-acetyl betulinic acid (**4**) with **5** in the presence of TMSOTf gave betulinic acid derivative **12** (91%). However, its debenzoylation with potassium carbonate gave a mixture of the expected product **14** together with the compound deacetylated at the triterpene O-3 position. Repetition of mannosylation of **4** with 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl trichloroacetimidate (**6**) in the presence of TMSOTf afforded derivative **13** (93%). Its deprotection with sodium methoxide in methanol gave **14** (90%), without affecting the acetyl group at the triterpene O-3 position.

Treatment of **14** with 2 equiv of **5** as described above, followed by acetylation of crude **15**, purification of diacetate **16** (41% after two steps), and removal of benzoyl protecting groups gave the saponin glycosyl-ester **17** (quant., Scheme 2).

Observed reactivity of the acetyl group at the betulinic acid's O-3 position is rather unexpected. It was not affected under Zemplen conditions (NaOMe/MeOH, in the case of **13**) but partially deprotected when treated with potassium carbonate in methanol (in the case of **12**). Previously, we showed that acetyl groups at the O-3 and O-28 positions of betulin derivatives were resis-



Scheme 1. Reagents and conditions: (a) **5** (1 equiv), CH_2Cl_2, TMSOTf, −40 °C; (b) K_2CO_3, MeOH; (c) **5** (2 equiv), MeCN–CH_2Cl_2, TMSOTf, −40 °C; (d) Ac_2O, pyridine.



Scheme 2. Reagents and conditions: (a) **5** or **6** (1 equiv), CH_2Cl_2, TMSOTf, −40 °C; (b) NaOMe, MeOH; (c) **5** (2 equiv), acetonitrile–CH_2Cl_2, TMSOTf, −40 °C; (d) Ac_2O, pyridine; (e) K_2CO_3, MeOH.

tant to alkaline hydrolysis (potassium carbonate in methanol).[18]

We decided to leave the O-3 acetyl group at the sapogenin portion to improve the solubility of the final compound **17**. This acetate group should be removed by the action of esterases, thereby releasing the free betulinic acid derivative inside the target cells.

2.3. Configurational assignments

The ^{1}H NMR spectra of diacetates **10** and **16** showed the H-2 and H-4 resonances of the central mannose residues being deshielded by ca. 0.9–1.2 ppm with respect to those of H-3. The ^{13}C NMR spectra of **10** and **16** indicated characteristic deshielding effects of the C-3 and C-6 resonances.

Moreover, results of HMBC analyses of **10** showed that ^{1}H signals of H-3 at 4.39 ppm and both protons H-6 at 3.70 and 4.02 ppm were correlated with the ^{13}C signals at 99.0 (C-1′) and 97.3 ppm (C-1″), respectively. These observations clearly proved that the glycosidic linkages were at O-3 and O-6 of the central mannose ring. In addition, $^1J(^1H–^{13}C)$ coupling constants of 171.3, 173.5, and 173.5 Hz observed for the anomeric carbon atoms (C-1, C-1′, and C-1″, respectively) indicate the presence of α-linkages.[24]

Similarly, HMBC analysis of **16** showed correlations between ^{1}H signals of H-3 (4.38 ppm) and H-6 protons

(4.02 and 3.76 ppm) with ^{13}C signals of C-1′ (99.4 ppm) and C-1″ (98.0 ppm), respectively. Observed $^{1}J(^{1}H–^{13}C)$ coupling constants for C-1 (179.3 Hz), C-1′ (174.2 Hz), and C-1″ (175.5 Hz) proved the anomeric configuration to be α.[24]

2.4. Biological activity

Anticancer activity was tested for parent triterpenoids [lupeol (**1**) and its acetate (**3**), betulinic acid (**2**) and its acetate (**4**), betulin (**18**) and its acetylated derivatives (**19**–**21**)], mono- and trisaccharide derivatives of lupeol (**8**, **11**), and betulinic acid (**14**, **17**) prepared above as well as mono- and trimannoside of betulin (**22**–**25**), which were obtained during our previous studies (Chart 2).[18]

Several model normal and cancer cell lines were cultured and used in experiments to examine the structure–activity relationships of lupane-type saponins 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, lung carcinoma A-549, multiple myeloma RPMI 8226, cervical carcinoma HeLa, and malignant melanoma G361 lines. Cells of all of these lines were exposed to six serial 4-fold dilutions of each drug for 72 h, the proportions of surviving cells were then estimated and IC_{50} values were calculated. The results obtained from Calcein AM assays are presented in Table 1.

The most potent compounds were monosaccharide derivatives (**8**, **14**, **22**, **24**) that showed cytotoxic activity against all of the tumor cell lines. However, these compounds also had significant toxicity toward normal cells (BJ fibroblasts). Compound **4** was also tested using the panel of cancer cells. The cytotoxicity values of **4** varied between 6.7 and 43 μM and were comparable with those of the monosaccharide analogues. A striking observation from these 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.[25] Trisaccharides (**11**, **17**, **23**, **25**) showed no cytotoxicity

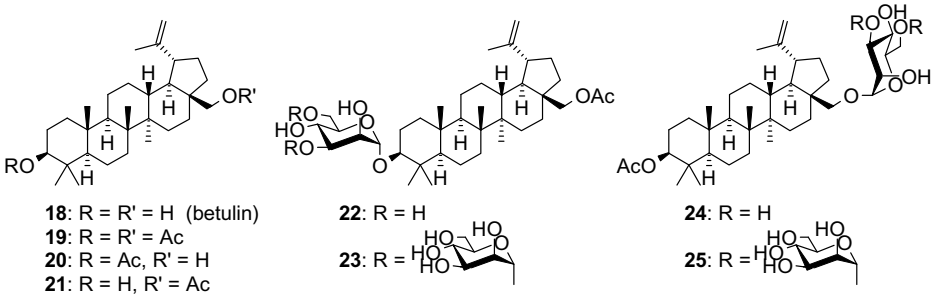


Chart 2.

Table 1. 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

Compound	Cell lines (IC_{50}, μM)						
	CEM	MCF 7	A-549	HeLa	BJ-H-tert	RPMI 8226	G 361
1	27.6 ± 1.4	>50	>50	>50	>50	37.5 ± 5.6	>50
2	40 ± 2.8	>50	>50	47.6 ± 1.9	>50	34.6 ± 1.5	>50
3	>50	>50	>50	>50	>50	44.1 ± 2.2	>50
4	10 ± 0.2	21.8 ± 5.5	43 ± 0.6	14.5 ± 4.3	>50	6.7 ± 0.4	32.3 ± 0.6
8	33.3 ± 4.7	34.1 ± 0.1	45.1 ± 1.4	32.1 ± 0.4	33.5 ± 5.3	26.2 ± 1.5	31.8 ± 1.7
11	>50	>50	>50	>50	>50	>50	>50
14	10.4 ± 0.7	22.7 ± 2.2	43.3 ± 0.3	34.7 ± 2.5	38.7 ± 0.5	19.4 ± 0.5	22.7 ± 1.4
17	>50	>50	>50	>50	>50	>50	>50
18	21.2 ± 3.4	>50	>50	>50	48.6 ± 0.1	12.9 ± 1.6	13.4 ± 0.8
19	34.8 ± 0.2	>50	>50	38.4 ± 4.6	>50	28.1 ± 0.5	>50
20	30.4 ± 2.2	>50	>50	>50	>50	19.9 ± 2.3	42.1 ± 1.9
21	30.2 ± 0.7	46.7 ± 0.9	>50	37.9 ± 0.1	>50	22.4 ± 1.7	35.9 ± 0.1
22	12.9 ± 1.1	35.5 ± 1.9	44.6 ± 0.2	42.8 ± 1.3	43.1 ± 0.5	18.5 ± 0.5	27.9 ± 0.1
23	>50	>50	>50	>50	>50	>50	>50
24	27.7 ± 5.3	39.2 ± 5.7	44.6 ± 0.2	45.7 ± 3.5	35.6 ± 7.9	29.2 ± 3.8	22.3 ± 3.9
25	>50	>50	>50	>50	>50	>50	>50

Betulinic acid (**2**) was used as a positive control.

toward the cancer and normal cell lines used, even when tested at concentrations up to 50 μM. Parent compounds (**1**, **18**–**21**) were more active against leukemia cell lines CEM and RPMI 8226 (IC_{50} 12.9–34.8 μM) than the other types of cancer cells. The results also show that nonmalignant cells (BJ-H-tert fibroblasts) tolerated higher doses of most of the tested compounds than tumor cells, demonstrating that they have preferential cytotoxicity for malignant cells.[26]

3. Conclusions

In summary, a rapid, efficient method for synthesizing novel lupeol α-D-mannopyranosides and 3-*O*-acetylbetulinic acid α-D-mannopyranosyl esters has been developed. Structure–activity analysis showed that lupane-type saponins can inhibit the growth of various cancer cell lines at micromolar concentrations, despite having limited effects on normal human fibroblasts. Monosaccharide derivatives of lupeol (**8**), betulinic acid (**14**), and betulin (**22**, **24**) were strongly cytotoxic, whereas trisaccharide analogues were inactive.

4. Experimental

4.1. General methods

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 303 K (500 and 125 MHz, respectively) with a Bruker Avance DRX-500 spectrometer. An internal TMS was used as the ^1H and ^{13}C NMR chemical shift standard. Signals of the aromatic groups observed for the typical values were omitted for simplicity. High resolution mass spectra (HRMS) were acquired with a MARINER mass spectrometer. Optical rotations were measured with a JASCO P-1020 automatic polarimeter. Configurational assignments were based on NMR measurements including DEPT and two-dimensional techniques, including *gradient-selected* COSY, ^1H–^{13}C gradient selected HSQC (g-Heteronuclear Single Quantum Correlation; C, H correlation via double INEPT transfer in the phase sensitive mode), ^1H–^{13}C gradient selected HMBC (g-Heteronuclear Multiple Bond Correlation; long-range correlation), as well as TOCSY experiments.

4.2. Lupeol [3β-hydroxy-lup-20(29)-ene] (1)

A sample (300 g) of the outer bark of a white birch tree collected in Poraj (South Poland) was cut into small pieces, air dried for 7 days and extracted with MeOH in a Soxhlet apparatus for 8 h. The extract was concentrated to afford 58.0 g of crude betulin, which was purified by acetylation in a refluxed mixture of Ac_2O (120 mL), acetic acid (50 mL), and pyridine (1 mL) for 2 h. Solvents were evaporated to dryness and the residue was purified by column chromatography (40:1→5:1 hexane–diethyl ether) as described earlier.[18] The fastest moving fraction during chromatographic separation of individual components contained lupeol acetate (**3**). It was further purified by column chromatography (50:1 hexane–EtOAc) to afford pure **3** (4.0 g). ^1H NMR spectral data matched those reported.[27] ^{13}C NMR ($CDCl_3$) δ: 170.9 (C=O), 150.9 (C-20), 109.3 (C-29), 81.0 (C-3), 55.4, 50.4, 48.3, 48.0, 43.0 (C), 42.8 (C), 40.9 (C), 40.0 (CH_2), 38.4 (CH_2), 38.1, 37.8 (C), 37.1 (C), 35.6 (CH_2), 34.2 (CH_2), 29.8 (CH_2), 27.9, 27.4 (CH_2), 25.1 (CH_2), 23.7 (CH_2), 21.3, 20.9 (CH_2), 19.3, 18.2 (CH_2), 18.0, 16.5, 16.2, 16.0, 14.5.

A soln of lupeol acetate (**3**, 3.93 g, 8.4 mmol) and potassium hydroxide (1.25 g, 22 mmol) in EtOH (30 mL) was refluxed for 5 h, cooled to room temperature, and concentrated. The oily residue was suspended in CH_2Cl_2 and filtered through a short silica pad (5:1 hexane–EtOAc). The filtrate was concentrated and the residue was purified by column chromatography (9:1 hexane–EtOAc) to yield lupeol (**1**, 3.06 g, 85%): mp 209–211 °C; lit.[28] mp 215–216 °C; $[\alpha]_D^{20}$ +25.0 (*c* 1.15, $CHCl_3$); lit.[28] $[\alpha]_D^{20}$ +26.4 ($CHCl_3$). ^1H and ^{13}C NMR spectral data matched those reported.[12a,27]

4.3. 3β-*O*-(2,3,4,6-Tetra-*O*-benzoyl-α-D-mannopyranosyl)-lup-20(29)-ene (7)

A soln of **5** (572 mg, 0.77 mmol) and lupeol (**1**, 320 mg, 0.75 mmol) in CH_2Cl_2 (15 mL) was stirred for 30 min at room temperature over molecular sieves (4 Å, 700 mg, finely ground), then cooled to −40 °C and TMSOTf (50 μL) 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 (9:1→5:1 hexane–EtOAc) of the residue gave **7** (716 mg, 95%) as a foam: $[\alpha]_D^{20}$ −2.4 (*c* 0.5, $CHCl_3$). ^1H NMR ($CDCl_3$) δ: 6.08 (t, 1H, $J_{4,3} = J_{4,5} = 10.1$ Hz, H-4), 5.92 (dd, 1H, $J_{3,2}$ 3.2 Hz, H-3), 5.62 (dd, 1H, $J_{2,1}$ 1.8 Hz, H-2), 5.28 (d, 1H, H-1), 4.70 (d, 1H, *J* 2.0 Hz, lupene-H-29), 4.67 (dd, 1H, $J_{6,5}$ 2.3, $J_{6,6'}$ 11.9 Hz, H-6), 4.56 (m, 2H, H-5, lupene-H-29), 4.48 (dd, 1H, $J_{6,5}$ 5.1 Hz, H-6), 3.35 (dd, 1H, *J* 4.3, 11.7 Hz, lupene-H-3), 2.38 (td, 1H, *J* 5.7, 11.0 Hz, lupene-H-19), 1.78–1.98 (m, 2H), 0.66–1.75 (m), 1.70 (s, 3H, CH_3), 1.11 (s, 3H, CH_3), 1.05 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.89 (s, 3H, CH_3), 0.80 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ: 166.1 (C=O), 165.6 (C=O), 165.6 (C=O), 165.5 (C=O), 150.9 (lupene-C-20), 109.4 (lupene-C-29), 94.4 (C-1), 84.3 (lupene-C-3), 71.6, 70.3, 69.5, 67.1, 63.1 (CH_2), 55.7, 50.5, 48.3, 48.0, 43.0 (C), 42.9 (C), 40.9 (C), 40.0 (CH_2), 38.6 (C), 38.3 (CH_2), 38.1, 37.1 (C), 35.6 (CH_2), 34.3 (CH_2), 29.9 (CH_2),

28.8, 27.5 (CH_2), 25.2 (CH_2), 22.2 (CH_2), 21.0 (CH_2), 19.3, 18.3 (CH_2), 18.0, 16.5, 16.2, 16.0, 14.6. HRESIMS: calcd for $C_{64}H_{76}NaO_{10}$ $[M+Na]^+$, 1027.5331. Found: *m*/*z* 1027.5388.

4.4. 3β-*O*-(α-D-Mannopyranosyl)-lup-20(29)-ene (8)

To a soln of **7** (910 mg, 0.91 mmol) in MeOH (20 mL), K_2CO_3 (200 mg) was added, the mixture was stirred overnight, and concentrated to dryness. Column chromatography (5:1 hexane–EtOAc then 5:3:1→1:1:1 hexane–EtOAc–MeOH) of the residue gave **8** (420 mg, 79%) as white crystals: mp 244–245 °C; $[\alpha]_D^{20}$ +77.5 (*c* 0.5, $CHCl_3$–MeOH). ^1H NMR (1:1 $CDCl_3$–CD_3OD) δ: 4.94 (br s, 1H), 4.70 (d, 1H, *J* 2.0 Hz, lupene-H-29), 4.57 (m, 1H), 3.72–3.84 (m, 5H), 3.68 (m. 1H), 3.35 (m, 1H), 3.23 (dd, 1 H, *J* 4.3, 11.7 Hz, lupene-H-3), 2.40 (td, 1H, *J* 5.8, 11.1 Hz, lupene-H-19), 1.92 (m, 2H), 0.66–1.80 (m), 1.69 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.81 (s, 3H, CH_3), 0.78 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$–CD_3OD 1:1) δ: 151.3 (lupene-C-20), 109.7 (lupene-C-29), 97.0 (C-1), 83.1 (lupene-C-3), 73.6, 72.3, 72.1, 67.6, 62.0 (CH_2), 56.2, 51.0, 48.9, 48.6, 43.5 (C), 43.3 (C), 41.4 (C), 40.5 (CH_2), 38.9 (C), 38.8 (CH_2), 38.6, 37.6 (C), 36.1 (CH_2), 34.8 (CH_2), 30.3 (CH_2), 28.9, 27.9 (CH_2), 25.7 (CH_2), 22.4 (CH_2), 21.5 (CH_2), 19.5, 18.8 (CH_2), 18.3, 16.6, 16.5, 16.4, 14.9. HRESIMS: calcd for $C_{36}H_{60}NaO_6$ $[M+Na]^+$, 611.4282. Found: *m*/*z* 611.4311. Anal. Calcd for $C_{36}H_{60}O_6{\cdot}H_2O$: C, 71.25; H, 10.30. Found: C, 71.02; H, 10.38.

4.5. 3β-*O*-(2,3,4,6-Tetra-*O*-benzoyl-α-D-mannopyranosyl)-(1→3)-[(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-(1→6)]-α-D-mannopyranosyl-lup-20(29)-ene (9)

A soln of **8** (295 mg, 0.5 mmol) in 1:1 CH_2Cl_2–MeCN (20 mL) was stirred at room temperature over molecular sieves (4 Å, 500 mg, finely ground) for 30 min, then cooled to −40 °C and TMSOTf (55 μL) was added followed by **5** (815 mg, 1.1 mmol) in CH_2Cl_2 (10 mL) dropwise over 15 min. The soln was stirred for a further 30 min, neutralized with Et_3N (0.2 mL), and concentrated to dryness. Column chromatography (5:1→7:3 hexane–EtOAc, then 5:3:1 hexane–EtOAc–MeOH) afforded 430 mg of crude **9**, which was used in the next step without further purification.

4.6. 3β-*O*-(2,3,4,6-Tetra-*O*-benzoyl-α-D-mannopyranosyl)-(1→3)-[(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-(1→6)]-2,4-di-*O*-acetyl-α-D-mannopyranosyl-lup-20(29)-ene (10)

The above crude product (**9**) was acetylated under standard conditions (Ac_2O, Py) and purified by column chromatography (5:1→7:3 hexane–EtOAc) to yield **10** (251 mg, 27% after two steps) as an amorphous glass. ^1H NMR ($CDCl_3$) δ: 6.23 (t, 1H, $J_{4,3} = J_{4,5} = 10.0$ Hz, H-4′), 6.14 (t, 1H, $J_{4,3} = J_{4,5} = 10.1$ Hz, H-4″), 5.91 (dd, 1H, $J_{3,2}$ 3.4 Hz, H-3″), 5.84 (dd, 1H, $J_{3,2}$ 3.2 Hz, H-3′), 5.78 (dd, 1H, $J_{2,1}$ 1.8 Hz, H-2″), 5.56 (dd, 1H, $J_{2,1}$ 2.0 Hz, H-2′), 5.49 (t, 1H, $J_{4,3} = J_{4,5} = 10.1$ Hz, H-4), 5.40 (d, 1H, H-1′), 5.34 (dd, 1H, $J_{2,3}$ 3.3, $J_{2,1}$ 1.7 Hz, H-2), 5.14 (d, 1H, H-1″), 5.08 (d, 1H, H-1), 4.48–4.75 (m, 8H, H-5′, 5″, 6′, 6″, lupene-H-29), 4.39 (dd, 1H, H-3), 4.23 (m, 1H, H-5), 4.02 (dd, 1H, $J_{6,5}$ 6.4, $J_{6,6'}$ 10.7 Hz, H-6), 3.70 (dd, 1H, $J_{6',5}$ 2.5 Hz, H-6), 3.33 (dd, 1H, *J* 4.3, 11.7 Hz, lupene-H-3), 2.31 (s, 3H, CH_3), 2.30 (s, 3H, CH_3), 0.75–1.63 (m, other protons). ^{13}C NMR ($CDCl_3$) δ: 170.8 (C=O), 170.3 (C=O), 166.1 (C=O), 166.0 (C=O), 165.4 (C=O), 165.3 (C=O), 165.2 (C=O), 165.2 (C=O), 165.1 (C=O), 150.8 (lupene-C-20), 109.3 (lupene-C-29), 99.0 ($^1J_{C-H}$ 173.5 Hz, C-1′), 97.3 ($^1J_{C-H}$ 173.5 Hz, C-1″), 94.3 ($^1J_{C-H}$ 171.3 Hz, C-1), 84.4 (lupene-C-3), 75.2 (C-3), 72.0 (C-2), 70.7 (C-2′), 70.3, 70.2 and 70.2 (C-2″,3″,5), 69.6 (C-5′), 69.4 (C-3′), 68.7 (C-5″), 68.4 (C-4), 66.9 (C-6), 66.8 (C-4″), 66.3 (C-4′), 62.9 (C-6″), 62.4 (C-6′), 55.3, 50.1, 48.2, 47.9, 42.9 (C), 42.5 (C), 40.7 (C), 39.9 (CH_2), 38.6 (C), 38.3 (CH_2), 37.9, 37.0 (C), 35.4 (CH_2), 34.1 (CH_2), 29.8 (CH_2), 28.9, 27.2 (CH_2), 24.9 (CH_2), 22.5 (CH_2), 21.1 (CH_3), 20.9 (CH_3), 18.2 (CH_2), 17.9, 16.5, 16.0, 15.9, 14.0. HRESIMS: calcd for $C_{108}H_{116}NaO_{26}$ $[M+Na]^+$, 1851.7647. Found: *m*/*z* 1851.7730.

4.7. 3β-*O*-(α-D-Mannopyranosyl)-(1→3)-[(α-D-mannopyranosyl)-(1→6)]-α-D-manno-pyranosyl-lup-20(29)-ene (11)

A suspension of **10** (190 mg, 0.1 mM) and K_2CO_3 (40 mg) in MeOH (5 mL) was stirred for 2 h, then neutralized with Amberlyst 15 resin (H^+ form), filtered through a short silica pad (MeOH as eluent), and the filtrate was evaporated to dryness. The residual methyl benzoate was removed by adding water (3 mL) and was freeze dried to afford **11** (84 mg, 88%) as a white powder. ^{13}C NMR (pyridine-d_5) δ: 151.1 (lupene-C-20), 109.9 (lupene-C-29), 103.8 (C-1), 101.8 (C-1), 98.6 (C-1), 82.9, 81.1, 75.5, 75.1, 74.5, 73.2, 73.1, 72.4, 72.1, 72.1, 69.5, 69.3, 67.2 (CH_2), 67.1, 63.2 (CH_2), 63.0 (CH_2), 55.8, 52.0, 50.5, 48.6, 48.3, 43.2 (C), 43.1 (C), 41.1 (C), 40.3 (CH_2), 38.7 (C), 38.5 (CH_2), 38.3, 37.3 (C), 35.8 (CH_2), 34.7 (CH_2), 30.2 (CH_2), 27.8 (CH_2), 25.6 (CH_2), 22.7 (CH_2), 21.1 (CH_2), 19.5, 18.6 (CH_2), 18.2, 16.7, 16.3, 16.2, 14.7. HRESIMS: calcd for $C_{48}H_{80}NaO_{16}$ $[M+Na]^+$, 935.5339. Found: *m*/*z* 935.5343.

4.8. 1-*O*-[3-β-Acetoxy-lup-20(29)-ene-28-oyl)-2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl (12)

Betulinic acid acetate[18] (**4**, 500 mg, 1.0 mM) was converted into the glycosyl ester **12** using **5** and the

procedure described for **7** to yield **12** (985 mg, 91%) as a foam. $[\alpha]_D^{25}$ −18.6 (*c* 0.6, $CHCl_3$). ^1H NMR ($CDCl_3$) δ: 6.44 (d, 1H, $J_{1,2}$ 2.0 Hz, H-1), 6.19 (t, 1H, $J_{4,3} = J_{4,5} = 10.1$ Hz, H-4), 5.87 (dd, 1H, $J_{3,2}$ 3.3 Hz, H-3), 5.72 (dd, 1H, H-2), 4.80 (br s, 1H, lupene-H-29), 4.66 (m, 2H), 4.42–4.52 (m, 3H), 3.05 (ddd, 1H, *J* 5.0, 11.1 Hz), 2.42 (m, 1H), 2.31 (m, 1H), 2.12 (m, 1H), 2.04 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ: 173.1 (C=O), 171.0 (C=O), 166.1 (C=O), 165.5 (C=O), 165.3 (C=O), 165.1 (C=O), 150.0 (lupene C-20), 110.0 (lupene C-29), 90.3 (C-1), 80.9 (lupene C-3), 71.5, 70.0, 69.3, 66.3, 62.6 (C-6), 57.1 (C), 55.5, 50.5, 49.4, 46.9, 42.5 (C), 40.7 (C), 38.4 (CH_2), 38.2, 37.8 (C), 37.1 (C), 37.0 (CH_2), 34.2 (CH_2), 32.4 (CH_2), 30.5 (CH_2), 29.6 (CH_2), 27.9, 25.5 (CH_2), 23.7 (CH_2), 21.3 (CH_3), 20.8 (CH_2), 19.4, 18.2 (CH_2), 16.4 (CH_3), 16.1 (CH_3), 16.0 (CH_3), 14.7 (CH_3). HRESIMS: calcd for $C_{66}H_{76}NaO_{13}$ $[M+Na]^+$, 1099.5178. Found: *m*/*z* 1099.5142.

4.9. 1-*O*-[3-β-Acetoxy-lup-20(29)-ene-28-oyl]-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl (13)

Betulinic acid acetate (**4**, 500 mg, 1.0 mM) and **6** were converted into the glycosyl ester **13** using the procedure described for **7** to yield **13** (772 mg, 93%) as a foam: $[\alpha]_D^{20}$ +32.4 (*c* 0.6, $CHCl_3$). ^1H NMR ($CDCl_3$) δ: 6.13 (d, 1H, $J_{1,2}$ 2.0 Hz, H-1), 5.35 (t, 1H, $J_{4,3} = J_{4,5} = 10.0$ Hz, H-4), 5.29 (dd, 1H, $J_{3,2}$ 3.3 Hz, H-3), 5.24 (dd, 1H, H-2), 4.74 (d, 1H, *J* 1.8 Hz, lupene H-29), 4.61 (br s, lupene H-29), 4.46 (dd, 1H, *J* 8.0 and 10.3 Hz, lupene H-17), 4.29 (dd, 1H, $J_{6,5}$ 4.9, $J_{6,6'}$ 12.4 Hz, H-6), 4.06 (dd, 1H, $J_{6',5}$ 2.5 Hz, H-6′), 3.98 (m, 1H, H-5), 2.94 (m, 1H, lupene H-19), 2.18 (s, 3H, CH_3), 2.08 (s, 3H, CH_3), 2.05 (s, 3H, CH_3), 2.03 (s, 3H, CH_3), 1.99 (s, 3H, CH_3), 1.68 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.83 (s, 6H, 2 × CH_3), 0.81 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ: 172.9 (C=O), 171.0 (C=O), 170.6 (C=O), 169.9 (C=O), 169.7 (C=O), 169.5 (C=O), 149.8 (lupene C-20), 110.0 (lupene C-29), 90.1 (C-1), 80.9 (lupene C-3), 71.1, 69.0, 68.3, 65.4, 62.2 (C-6), 56.9 (C), 55.4, 50.5, 49.3, 46.8, 42.4 (C), 40.7 (C), 38.4 (CH_2), 38.1, 37.8 (C), 37.1 (C), 36.8 (CH_2), 34.2 (CH_2), 32.2 (CH_2), 30.3 (CH_2), 29.5 (CH_2), 27.9, 25.4 (CH_2), 23.7 (CH_2), 21.3, 20.8 (CH_2), 20.7, 20.7, 20.6, 20.5, 19.3, 18.1 (CH_2), 16.4, 16.1, 16.0, 14.6. HRESIMS: calcd for $C_{46}H_{68}NaO_{13}$ $[M+Na]^+$, 851.4552. Found: *m*/*z* 851.4578.

4.10. 1-*O*-[3-β-Acetoxy-lup-20(29)-ene-28-oyl]-α-D-mannopyranosyl (14)

To a soln of **13** (1.27 g, 1.53 mM) in MeOH (15 mL), a soln of freshly prepared sodium methoxide in MeOH (0.24 M, 0.2 mL) was added and stirred for 90 min. The soln was neutralized with Amberlyst 15 resin (H^+ form), filtered through a short silica pad (MeOH as eluent), and the filtrate was evaporated to dryness. Column chromatography (7:3 hexane–EtOAc, then 5:3:1→1:1:1 hexane–EtOAc–MeOH) of the residue gave **14** (910 mg, 90%) as a foam; $[\alpha]_D^{20}$ +48.4 (*c* 0.6, 1:1 $CHCl_3$–MeOH). ^1H NMR (1:1 $CDCl_3$–CD_3OD) δ: 6.04 (d, 1H, $J_{1,2}$ 1.8 Hz, H-1), 2.01 (s, 3H, CH_3), 1.66 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 0.81 (s, 6H, 2 × CH_3). ^{13}C NMR (1: 1 $CDCl_3$–CD_3OD,) δ: 173.7 (C=O), 171.3 (C=O), 149.5 (lupene C-20), 109.1 (lupene C-29), 92.7 (C-1), 80.9 (lupene C-3), 75.0, 70.8, 69.3, 65.7, 60.6 (C-6), 56.3 (C), 54.9, 50.0, 48.7, 46.4, 41.9 (C), 40.1 (C), 37.8 (CH_2), 37.7, 37.1 (C), 36.5 (C), 36.1, 33.7 (CH_2), 31.4 (CH_2), 29.7 (CH_2), 29.0 (CH_2), 27.1 (CH_2), 24.9, 23.0 (CH_2), 20.3 (CH_2), 20.2, 18.3, 17.5 (CH_2), 15.6, 15.3, 15.2, 13.9. HRESIMS: calcd for $C_{38}H_{60}NaO_9$ $[M+Na]^+$, 683.4130. Found: 683.4161. Anal. Calcd for $C_{38}H_{60}O_9 \cdot 1.5H_2O$: C, 66.35; H, 9.23. Found: C, 66.35; H, 9.14.

4.11. 1-*O*-[3-β-Acetoxy-lup-20(29)-ene-28-oyl]-(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-(1→3)-[(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-(1→6)]-α-D-mannopyranosyl (15)

Mannoside **14** (330 mg, 0.5 mM) and **5** (815 mg, 1.1 mM) were converted into trimannoside **15** using the procedure described for **9**. The product was used in the next reaction without further purification.

4.12. 1-*O*-[3-β-Acetoxy-lup-20(29)-ene-28-oyl]-(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-(1→3)-[(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-(1→6)]-2,4-di-*O*-acetyl-α-D-mannopyranosyl (16)

The crude product **15** was acetylated under standard conditions (Ac_2O, Py) and purified by column chromatography (7:3 hexane–EtOAc then 5:3:0.2→5:3:0.5 hexane–EtOAc–MeOH) to yield **16** (391 mg, 41% after two steps) as an amorphous glass; $[\alpha]_D^{20}$ −13.9 (*c* 0.5, $CHCl_3$). ^1H NMR ($CDCl_3$) δ: 6.27 (t, 1H, $J_{4,3} = J_{4,5} = 10.1$ Hz, H-4″), 6.25 (d, 1H, $J_{1,2}$ 1.9 Hz, H-1), 6.17 (m, 1H, *J* 9.6, 10.9 Hz, H-4′), 5.83 (m, 3H, H-2′, 3′, 3″), 5.63 (t, 1H, $J_{4,3} = J_{4,5} = 9.9$ Hz, H-4), 5.56 (dd, 1H, $J_{2,1}$ 2.0, $J_{2,3}$ 3.1 Hz, H-2″), 5.45 (dd, 1H, $J_{2,3}$ 3.3 Hz, H-2), 5.39 (d, 1H, H-1″), 5.14 (br s, 1H, H-1′), 4.75 (m, 2H, H-6′, lupene H-29), 4.44–4.65 (m, 6H, H-5′, 5″, 6′, 6″, 6″, lupene H-29), 4.38 (m, 2H, H-3, lupene H-3), 4.02 (m, 2H, H-5, 6), 3.76 (dd, 1H, $J_{6,5}$ 2.3, $J_{6,6'}$ 10.6 Hz, H-6), 2.98 (m, 1H, *J* 4.7, 11.1 Hz), 2.38 (s, 3H, CH_3), 2.33 (s, 3H, CH_3), 2.01 (s, 3H, CH_3), 1.69 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.87 (s, 3H, CH_3), 0.66 (s, 3H, CH_3), 0.61 (s, 3H, CH_3), 0.57 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ: 173.3 (C=O), 171.0 (C=O), 170.4 (C=O), 170.1 (C=O), 166.2 (C=O), 166.0 (C=O), 165.5 (C=O), 165.4 (C=O),

165.4 (C=O), 165.3 (2 × C=O), 165.2 (C=O), 149.8 (lupene C-20), 110.2 (lupene C-29), 99.4 ($^1J_{C-H}$ 174.2 Hz, C-1′), 98.0 ($^1J_{C-H}$ 175.5 Hz, C-1″), 90.2 ($^1J_{C-H}$ 179.3 Hz, C-1), 80.9 (lupene C-3), 75.5 (C-3), 72.3 (C-5), 70.7 (C-2″), 70.4 (C-3′ or C-3″), 70.1 (C-2′), 69.8 (C-5″), 69.8 (C-2), 69.4 (C-3′ or C-3″), 68.9 (C-5′), 67.6 (C-4), 67.2 (C-6), 66.6 (C-4′), 66.4 (C-4″), 62.8 (C-6′), 62.4 (C-6″), 57.1 (C), 55.4, 50.4, 49.3, 47.1, 42.5 (C), 40.7 (C), 38.4, 38.2 (CH_2), 37.6 (C), 37.1 (CH_2), 37.1 (C), 36.9 (C), 34.3 (CH_2), 32.4 (CH_2), 30.5 (CH_2), 29.5 (CH_2), 27.8, 25.5 (CH_2), 23.6 (CH_2), 21.3, 21.0, 20.8, 20.8 (CH_2), 19.3, 18.0, 16.3, 16.1, 16.0, 14.6. HRE-SIMS: calcd for $C_{110}H_{116}NaO_{29}$ $[M+Na]^+$, 1923.7495. Found: *m/z* 1924.7516. Anal. Calcd for $C_{110}H_{116}O_{29}\cdot 2H_2O$: C, 68.17; H, 6.24. Found: C, 68.05; H, 6.29.

4.13. 1-*O*-[3-β-Acetoxy-lup-20(29)-en-28-oyl]-(α-D-mannopyranosyl)-(1→3)-[(α-D-mannopyranosyl)-(1→6)]-α-D-mannopyranosyl (17)

A suspension of **16** (160 mg, 0.084 mM) and K_2CO_3 (40 mg) in MeOH (5 mL) was stirred for 2 h, neutralized with Amberlyst 15 resin (H^+ form), filtered through a short silica pad (MeOH as eluent), and the filtrate was evaporated to dryness. The residual methyl benzoate was removed by adding water (3 mL) and was freeze dried to afford **17** (82 mg, quant.) as a white powder. ^{13}C NMR (pyridine-d_5) δ: 174.3 (C=O), 170.7 (C=O), 150.7 (lupene C-20), 110.2 (lupene C-29), 104.1 (C-1), 102.2 (C-1), 95.1 (C-1), 80.8, 80.5, 76.8, 75.5, 75.2, 73.1, 73.1, 72.3, 72.0, 70.3, 69.5, 69.2, 66.9 (CH_2), 66.5, 63.1 (CH_2), 63.0 (CH_2), 57.2 (C), 55.7, 52.0, 50.8, 49.7, 47.6, 42.8 (C), 41.1 (C), 38.6 (CH_2), 38.0 (C), 37.3 (C), 37.2 (CH_2), 34.6 (CH_2), 32.5 (CH_2), 30.9 (CH_2), 29.9 (CH_2), 28.1, 26.0 (CH_2), 24.1 (CH_2), 21.2 (CH, CH_2), 19.4, 18.5 (CH_2), 16.8, 16.3, 14.9. HR-MS (ESI) calcd for $C_{50}H_{80}NaO_{19}$ $[M+Na]^+$, 1007.5186. Found: 1007.5177. Anal. Calcd for $C_{50}H_{80}O_{19}\cdot H_2O$: C, 59.86; H, 8.24. Found: C, 59.77; H, 8.21.

4.14. Calcein AM cytotoxicity assays

The cell lines (T-lymphoblastic leukemia cell line CEM; breast carcinoma cell lines MCF-7, lung carcinoma cell lines A-549, chronic myelogenous leukemia cell lines K562, epitheloid carcinoma cell line HeLa, malignant melanoma cell lines G361, and human fibroblast BJ-H-tert) were cultured in DMEM medium (Gibco BRL) supplemented with 10% fetal calf serum, 4 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37 °C in a fully humidified atmosphere containing 5% CO_2. Suspensions of these lines (ca. 1.25×10^5 cells/mL) were placed in 96-well microtiter plates and after 3 h of stabilization the tested saponins were added in serially diluted concentrations. Saponins were dissolved in Me_2SO before addition to cultures. Control cultures were treated with Me_2SO alone. The final concentration of Me_2SO in the reaction mixtures never exceeded 0.6%. Fourfold dilutions of the intended test concentration were added at time zero in 20 μL aliquots to the microtiter plate wells. Usually, each test compound was evaluated at six 4-fold dilutions and in routine testing, the highest well concentration was 50 μM, although this varied in a few cases, depending on the test compound. After 72 h of culture, the cells were incubated with Calcein AM solution (Molecular Probes) for 1 h. The fluorescence of viable cells was quantified using a Fluoroscan Ascent instrument (Microsystems). The percentage of surviving cells in each well was calculated from the equation $IC_{50} = (OD_{\text{drug exposed well}}/\text{mean } OD_{\text{control wells}}) \times 100\%$. The IC_{50} value was calculated from the obtained dose-response curves.

Acknowledgments

This work was supported by grant 3 TO9A 110 29 from the Ministry of Science and Higher Education, Poland, and Grant MSM 6198959216 from the Ministry of Education of the Czech Republic.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.02.011.

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