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Synthesis of oleanolic acid saponins mimicking components of Chinese folk medicine Di Wu

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ABSTRACT

3,28-Di-*O*-rhamnosylated oleanolic acid saponins, mimicking components of Chinese folk medicine Di Wu, have been designed and synthesized. One-pot glycosylation and 'inverse procedure' technologies have been applied thus significantly simplifying the preparation of desired saponins. The cytotoxic activity of compounds 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl]oleanolic acid 28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (**3**), 3-*O*-[α-L-rhamnopyranosyl]oleanolic acid 28-*O*-[α-L-rhamnopyranosyl- (1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (**4**), 3-*O*-[α-L-rhamnopyranosyl]oleanolic acid 28-*O*-[α-L-rhamnopyranosyl] ester (**5**), and 3-*O*-[α-L-rhamnopyranosyl]oleanolic acid 28-*O*-[6-*O*-(α-L-rhamnopyranosyl)hexyl] ester (**6**) was preliminarily evaluated against HL-60 human promyelocytic leukemia cells. The natural saponin **3** and designed saponin **4** exhibited comparable moderate cytotoxic activity under our testing conditions.

1. Introduction

Saponins, notably steroid or triterpene plant glycosides, have been used worldwide in the treatments of diseases and health care practices by taking advantages of their generally non-ionic surfactant and membrane-disrupting properties.[1–5] The diversity of structures, the challenges of isolation and purification, their wide abundance in the plant kingdom and their broad spectrum of biological and pharmacological activities have driven chemists to the research field of saponins.[6] It is surprising that more than half of the triterpene saponins are glycosides of oleanolic acid or its derivatives, with one sugar chain attached through an ether linkage at C-3 and another through an ester linkage at C-28.[7] In our ongoing project of Di Wu,[8] a Chinese folk medicine from dry rhizome of *Anemone flaccida* Fr. Schmidt, we proved that the major components (Scheme 1) of Di Wu are 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]oleanolic acid 28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (**1**), 3-*O*-[α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl] oleanolic acid 28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (**2**), and 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl]oleanolic acid 28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (**3**).[9–11]

Literature searching suggested that α-L-rhamnopyranosyl moiety of some natural soponins may play a crucial role in triggering cell death by apoptosis.[12] Several hypotheses have been proposed for the mechanism of their activity.[13–15] However, the related SAR study is sporadic and unsystematic due to the difficulty in obtaining adequate analogical glycosides from nature. We have recently synthesized compound **3** which inhibited ConA-induced lymphocyte proliferation.[8] However, the structural complexity, especially having 2′-OH branched sugar chain on C-3 of oleanolic acid, has hindered the large-scale preparation of this type of bidesmosidic triterpene saponins.[6,16–23] To simplify the structure and investigate the role of L-rhamnopyranosyl residues in oleanane-type saponins, especially in Di Wu saponins, we here report the design, synthesis, and bioactivity of some structural analogues.

2. Results and discussion

The target molecules are shown in Scheme 2. To prepare compound **4**, trisaccharide **10** was first synthesized via one-pot glycosylation technology (Scheme 3). Thus, 2,3,4-tri-*O*-benzoyl-α-L-rhamnopyranosyl trichloroacetimidate (**8**, 1.4 equiv)[24] was slowly added into ethyl 2,3-di-*O*-acetyl-6-*O*-benzoyl-1-thio-β-D-glucopyranoside[25] (**7**, 1.2 equiv) in dry CH_2Cl_2 in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf, 0.17 equiv) at −70 °C. The reaction mixture was warmed up to rt and stirred at this condition for another 30 min, then acceptor 4-methoxyphenyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranoside (**9**, 1.0 equiv)[8,26] and

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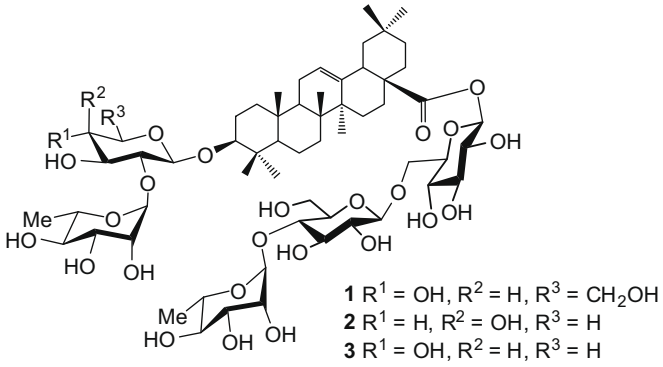
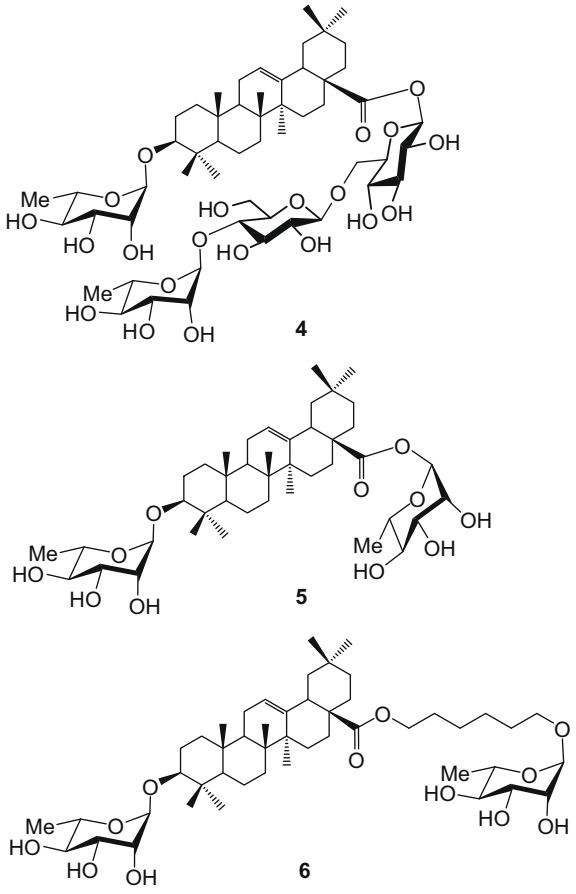


Scheme 1. Natural saponins isolated from Di Wu.



Scheme 2. Designed saponins mimicking natural Di Wu saponins.

N-iodosuccinimide (NIS) in dry CH_2Cl_2, respectively, were added at −15 °C, obtaining 4-methoxyphenyl 2,3,4-tri-*O*-benzoyl-α-L-rhamnopyranosyl-(1→4)-2,3-di-*O*-acetyl-6-*O*-benzoyl-β-D-glucopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-β-D-glucopyranoside (**10**) in a total yield of 53%. Treatment of **10** with ceric ammonium nitrate (CAN) in CH_3CN–H_2O (4:1, v/v), followed by trichloroacetimidation (Cl_3CCN, DBU, CH_2Cl_2),[27] furnished the trisaccharide donor **11** in 80% yield. Coupling of oleanolic trityl ester **12** with donor **8** was smoothly completed within 30 min in the presence of a catalytic amount of TMSOTf at −35 °C, obtaining key intermediate **13** in 92% yield (Scheme 4). Sugar-ester formation between trityl-ester **13** and trisaccharide donor **11** was achieved using a so-called 'Inverse Procedure'[28,29] in dry dichloromethane under the promotion of TMSOTf leading to the fully protected saponin derivative **14** in excellent yield. Selective removal of the acetate and benzoate protecting groups in **14** with catalytic amount of NaOMe (CH_2Cl_2–MeOH, 2:1, v/v), taking advantage of a kinetic steric effect of the neighboring quaternary center on C-28,[16] furnished the target 3-*O*-α-L-rhamnopyranosyl oleanolic acid 28-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**4**) in 70% yield from **12**.

Similarly (Scheme 5), condensation of **13** with **8** as described in the preparation of compound **14** gave derivative **15**, which was subjected to deacylation with NaOMe in CH_2Cl_2–MeOH to give desired saponin 3-*O*-α-L-rhamnopyranosyl oleanolic acid 28-*O*-α-L-rhamnopyranoside (**5**) in excellent overall yield.

In the preparation of compound **6** (Scheme 6), 3-*O*-acetyl oleanolic acid **16**[30] was treated with oxalyl chloride in dry CH_2Cl_2, the resulting acid chloride was then immediately reacted with 1,6-hexanediol in the presence of triethylamine affording 3-*O*-acetyl oleanolic acid 28-*O*-(6-hydroxyhexyl) ester (**17**). Removal of acetyl group with NaOMe in CH_2Cl_2/MeOH (→**18**), followed by glycosylation with **8** using the same procedure applied in the synthesis of **13**, gave diglycosylated compound **19** in 90% yield. Global debenzoylation of **19** with catalytic amount of NaOMe (CH_2Cl_2–MeOH, 2:1, v/v) in the presence of the C-28 ester glycosidic linkage gave the target compound **6** in 60% yield from **16**.

The cytotoxic activities of compounds **3**–**6**, and oleanolic acid were evaluated[31,32] against HL-60 human promyelocytic leukemia cells (Table 1). Saponins **3** and **4** exhibited comparable moderate cytotoxic activity with IC_{50} values at 2.7 and 3.0 μg/mL, respectively. Compared to **4**, structural analogues **5** and **6** showed only weak activities under the same testing conditions, suggesting that sugar moieties (or may be polyhydroxyl linkers) are important between α-L-rhamnopyranosyl residue and C-28 carboxyl group. Oleanolic acid itself was not cytotoxic under our assay conditions. The current results should be valuable for the related molecule design, synthesis, and bioactivity screening.[33,34]

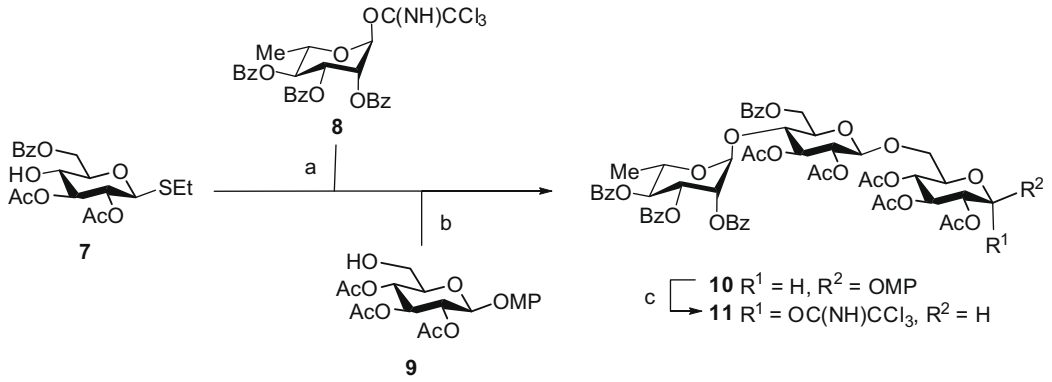
3. Experimental

3.1. General methods

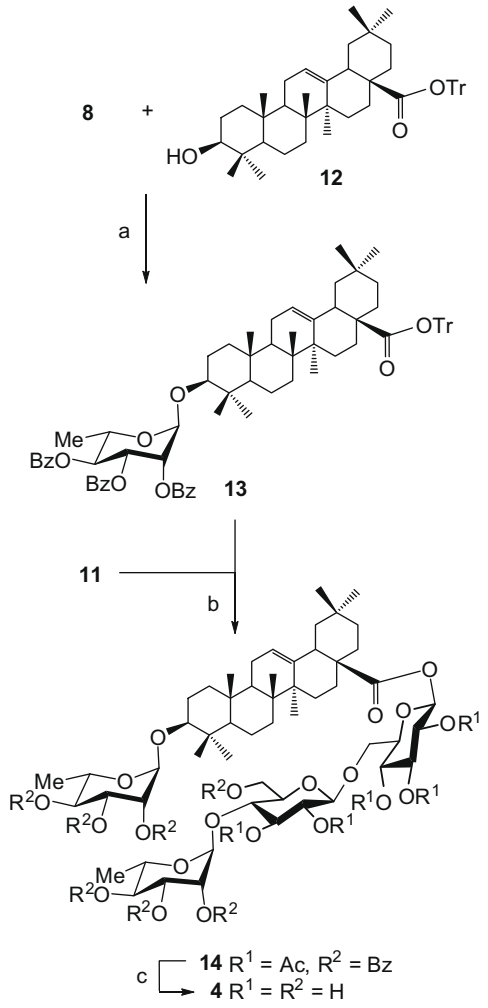
Optical rotations were determined at 25 °C with a Perkin–Elmer Model 241-Mc automatic polarimeter. ^{1}H NMR, ^{13}C NMR, and ^{1}H–^{1}H, ^{1}H–^{13}C COSY spectra were recorded with a Bruker ARX 400 spectrometer for solutions in $CDCl_3$, MeOD, or DMSO-d_6. Chemical shifts are given in ppm downfield from internal Me_4Si. Mass spectra were measured using a MALDI-TOF-MS with α-cyano-4-hydroxycinnamic acid as matrix. Thin-layer chromatography (TLC) was performed on silica gel HF_{254} with detection by charring with 30% (v/v) H_2SO_4 in MeOH, or by UV detector. Column chromatography was conducted by elution of a column of silica gel (100 mesh) with EtOAc–petroleum ether (60–90 °C) as the eluent, or a column of Biogel P2 with water as the eluent. Solutions were concentrated at <50 °C under reduced pressure.

3.2. 4-Methoxyphenyl 2,3,4-tri-*O* -benzoyl-α-L-rhamnopyranosyl-(1→4)-2,3-di-*O*-acetyl-6-*O* -benzoyl-β-D-glucopyranosyl-(1→6)-2,3,4-tri-*O* -acetyl-β-D-glucopyranoside (10)

A suspension of **7** (495 mg, 1.2 mmol), **8** (870 mg, 1.4 mmol), and 4 Å MS in dry CH_2Cl_2 (15 mL) was stirred at rt for 0.5 h, then cooled to −70 °C, and a solution of TMSOTf (30 μL, 0.17 mmol) in CH_2Cl_2 (0.2 mL) was added under N_2 protection. The reaction mixture was allowed to stir at these conditions for about 3 h, at the end of which time, TLC (petroleum ether–EtOAc 3:1) indicated a complete consumption of **8**. To the above mixture was added



Scheme 3. One-pot synthesis of trisaccharide **10**. Reagents and conditions: (a) TMSOTf, CH_2Cl_2, −70 °C, 3 h; (b) NIS, CH_2Cl_2, −15 °C, 2 h, 53% overall yield; (c) CAN, CH_3CN–H_2O (4:1, v/v); then Cl_3CCN, DBU, CH_2Cl_2, rt, 80%.



Scheme 4. Synthesis of saponin **4**. Reagents and conditions: (a) TMSOTf, CH_2Cl_2, −35 °C, 92%; (b) TMSOTf, CH_2Cl_2, 0 °C, 1 h, then **11**, −35 °C, 30 min, 90%; (c) NaOMe, CH_2Cl_2–MeOH, 2:1, v/v, 85%.

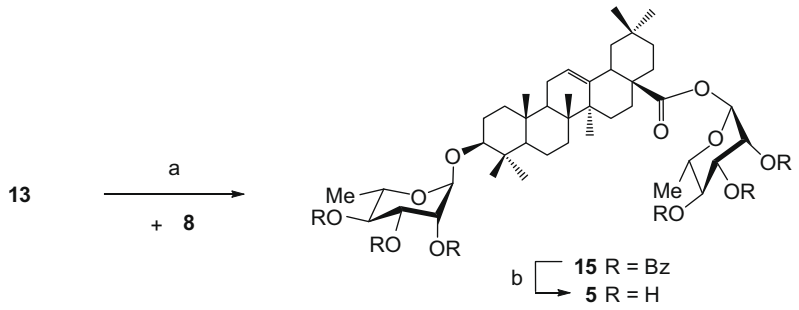
compound **9** (412 mg, 1.0 mmol) in dry CH_2Cl_2 (2.5 mL) at −15 °C, followed by adding NIS (450 mg, 2.0 mmol), and a further portion of TMSOTf (10 μL, 0.055 mmol) in CH_2Cl_2. The reaction mixture was stirred at these conditions for 2 h, then quenched by Et_3N, diluted with CH_2Cl_2, washed with satd aq $NaHCO_3$ and water. The organic layer was combined, dried, and concentrated. Purification by column chromatography (1:1 petroleum ether–EtOAc) gave trisaccharide **10** as a foamy solid (640 mg, 53% overall yield), which presented the same physical data as that reported before {$[\alpha]_D^{25}$ +75 (*c* 2, $CHCl_3$), lit.[8] $[\alpha]_D^{25}$ +74 (*c* 2.1, $CHCl_3$)}.

3.3. 3-*O*-[2,3,4-Tri-*O*-benzoyl-α-L-rhamnopyranosyl]oleanolic acid 28-*O*-trityl ester (13)

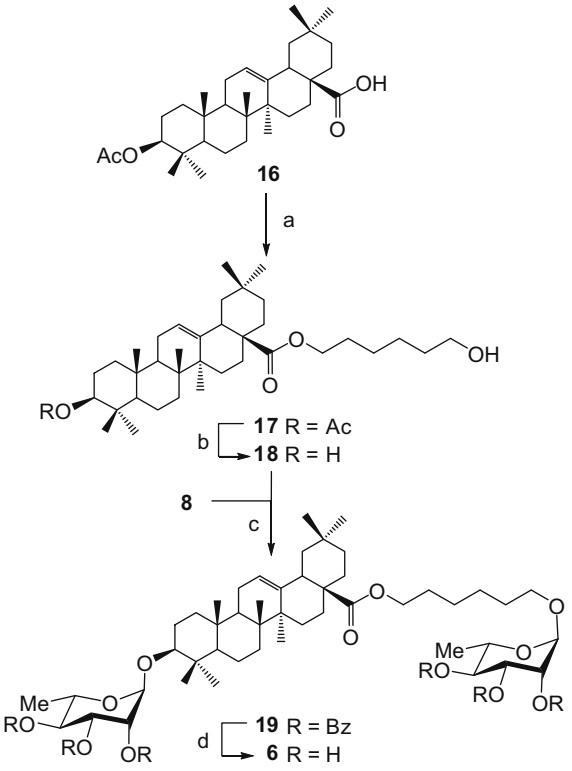
To a mixture of compounds **8** (220 mg, 0.35 mmol) and **12** (200 mg, 0.29 mmol) in anhyd CH_2Cl_2 (5 mL) was added TMSOTf (7 μL, 0.04 mmol) under an N_2 atmosphere at −35 °C. The mixture was stirred under these conditions for 30 min, at the end of which time TLC (3:1 petroleum ether–EtOAc) indicated that all starting materials were consumed. The reaction mixture was neutralized with Et_3N and concentrated. Column chromatography (4:1 petroleum ether–EtOAc) of the residue gave **13** as a foamy solid (305 mg, 92%): $[\alpha]_D^{25}$ +45 (*c* 1, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): δ 0.35, 0.82, 0.88, 0.90, 0.91, 1.11, 1.29, 1.31 (8s, 8 × 3H, $8CH_3$), 0.78–1.79 (m, 22H), 2.83 (dd, 1H, *J* 4.0, 13.3 Hz, H-18 of oleanolic acid), 3.25 (dd, 1H, *J* 4.6, 11.5 Hz, H-3 of oleanolic acid), 4.28–4.31 (m, 1H, H-5), 5.07 (d, 1H, *J* 2.0 Hz, H-1), 5.24 (br s, 1H, H-12 of oleanolic acid), 5.62–5.69 (m, 2H, H-2, H-4), 5.82 (dd, 1H, *J* 3.2, 10.1 Hz, H-3), 7.20–8.09 (m, 30H, 6Ph). Anal. Calcd for $C_{76}H_{84}O_{10}$: C, 78.86; H, 7.31; Found: C, 79.14; H, 7.22.

3.4. 3-*O*-[2,3,4-Tri-*O*-benzoyl-α-L-rhamnopyranosyl]oleanolic acid 28-*O*-[2,3,4-tri-*O*-benzoyl-α-L-rhamnopyranosyl-(1→4)-2,3-di-*O*-acetyl-6-*O*-benzoyl-β-D-glucopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl] ester (14)

To a solution of compound **13** (243 mg, 0.21 mmol) in dry dichloromethane (5 mL) was added TMSOTf (20 μL, 0.11 mmol) under an N_2 atmosphere at 0 °C. The mixture was stirred at this condition for 1 h, then cooled to −35 °C, and trisaccharide **11** (306 mg, 0.24 mmol) in anhyd CH_2Cl_2 (3 mL) was added dropwise. The reaction mixture was stirred under these conditions for further 30 min, quenched by Et_3N, and concentrated. The residue was purified by silica gel column chromatography (7:2, petroleum ether–EtOAc) to give compound **14** as a foamy solid (381 mg, 90%): $[\alpha]_D^{25}$ +98 (*c* 1.1, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): 0.76, 0.85, 0.89, 0.95, 0.99, 1.12, 1.17, 1.29, 1.56 (9s, 9 × 3H, $9CH_3$), 0.76–1.79 (m, 22H), 1.99, 2.00, 2.02, 2.08, 2.11 (5s, 5 × 3H, 5Ac), 2.80 (dd, 1H, *J* 4.2, 10.1 Hz, H-18 of oleanolic acid), 3.22 (dd, 1H, *J* 7.3, 11.6 Hz, H-3 of oleanolic acid), 3.60 (dd, 1H, *J* 5.6, 11.5 Hz, H-6a^{Glc}), 3.79–3.89 (m, 3H), 4.05 (t, 1H, *J* 9.3 Hz, H-5^{Glc}), 4.14–4.16 (m, 1H, H-5^{Glc}), 4.30–4.33 (m, 1H, H-5^{Rha}), 4.60–4.64 (m, 2H), 4.86–4.99 (m, 3H), 5.07 (d, 1H, *J* 2.1 Hz, H-1^{Rha}), 5.13–5.33 (m, 5H), 5.51 (br s, 1H, H-12 of oleanolic acid), 5.55–5.80 (m, 6H), 7.23–8.09 (m, 35H, 7Ph). ^{13}C NMR (100 MHz, $CDCl_3$): 175.9, 170.8, 170.7, 170.5,



Scheme 5. Synthesis of saponin **5**. Reagents and conditions: (a) TMSOTf, CH_2Cl_2, 0 °C, 1 h, then **8**, −35 °C, 30 min, 93%; (b) NaOMe, CH_2Cl_2–MeOH, 2:1, v/v, 92%.



Scheme 6. Synthesis of saponin **6**. Reagents and conditions: (a) oxalyl chloride, CH_2Cl_2; 1,6-hexanediol, triethylamine, 82%; (b) NaOMe, MeOH, 91%; (c) TMSOTf, CH_2Cl_2, −35 °C, 90%; (d) NaOMe, CH_2Cl_2–MeOH, 2:1, v/v, 89%.

Table 1
Cytotoxicity of compounds **3**–**6** and oleanolic acid against HL-60 Cells[a]

Entry	Compound	IC_{50} (μg/mL)
1	**3**	2.7
2	**4**	3.0
3	**5**	20.1
4	**6**	14.5
5	Oleanolic acid	>30

[a] The cells were continuously treated with each sample for 72 h, and the cell growth was evaluated using MTT reduction assay. Data are mean values of three experiments performed in triplicate.

170.0, 169.5, 166.5, 166.3, 166.3, 166.2, 166.0, 166.0, 142.0, 134.0, 134.1, 133.8, 133.7, 133.6, 130.5, 130.3, 130.3, 130.1, 130.0, 129.9, 129.8, 129.8, 129.7, 129.1, 129.0, 129.0, 128.8, 124.0, 100.1 (C-1), 99.8 (C-1), 99.7 (C-1), 92.1 (C-1), 89.5, 74.8, 74.5, 73.6, 73.5, 72.6, 72.5, 72.0, 71.8, 71.7, 70.8, 70.6, 70.0, 69.4, 68.7, 68.2, 67.4, 56.0, 54.4, 48.2, 47.3, 46.4, 42.4, 41.7, 39.9, 39.6, 39.0, 37.4, 34.3, 33.5, 32.3, 31.1, 29.9, 28.9, 28.4, 26.2, 26.0, 24.1, 23.5, 21.7, 21.3, 21.2, 18.9, 18.1, 18.0, 17.6, 17.2, 16.0. Anal. Calcd for $C_{113}H_{126}O_{33}$: C, 67.45; H, 6.31; Found: C, 67.21; H, 6.27.

3.5. 3-*O*-[α-L-Rhamnopyranosyl]oleanolic acid 28-*O*-[α-L-rhamnopyranosyl- (1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (4)

To a solution of **14** (202 mg, 0.1 mmol) in dry CH_2Cl_2–MeOH (1:2, v/v, 15 mL) was added 1.0 M NaOMe in MeOH at 0 °C. The mixture was stirred at rt until all starting materials were consumed, and then neutralized with Dowex H^+ resin. The mixture was filtered and filtrate was concentrated under diminished pressure. The resulting residue was purified by Biogel P2 column chromatography using H_2O as eluent to afford, after freeze drying, compound **4** as an amorphous solid (91 mg, 85%): $[\alpha]_D^{25}$ +7.8 (*c* 1, MeOH); ^{1}H NMR (400 MHz, MeOD): 0.81 (s, 6H, $2CH_3$), 0.92-0.97 (m, 12H, $4CH_3$), 1.17 (s, 3H, CH_3), 1.24 (d, 3H, *J* 6.2 Hz), 1.27 (d, 3H, *J* 6.4 Hz), 0.81−1.71 m, 22H), 2.86 (dd, 1H, H-18 of oleanolic acid), 3.09 (dd, 1H, *J* 6.9, 11.1 Hz, H-3 of oleanolic acid), 3.23–3.84 (m, 19H), 3.96–3.98 (m, 1H), 4.10 (d, 1H, *J* 10.8 Hz), 4.74 (d, 1H, *J* 7.8 Hz), 4.41 (d, 1H, *J* 7.8 Hz), 5.27 (br s, 1H, H-12 of oleanolic acid), 5.35 (d, 1H, *J* 8.0 Hz). ^{13}C NMR (100 MHz, MeOD): 171.2, 144.1, 122.4, 103.0 (C-1), 102.9 (C-1), 101.5 (C-1), 94.3 (C-1), 89.0, 78.7, 77.4, 77.2, 76.0, 75.9, 74.5, 73.3, 73.0, 72.9, 71.8, 71.7, 71.6, 71.4, 79.1, 69.8, 69.1, 68.6, 61.2, 55.4, 46.4, 42.1, 41.7, 39.9, 39.2, 38.9, 37.1, 34.1, 33.1, 32.8, 32.4, 30.8, 28.0, 25.6, 23.4, 18.7, 17.1, 17.1, 17.0, 16.3, 15.3. MALDITOF-MS: calcd for $C_{54}H_{88}O_{21}$: 1072.58 $[M]^+$; found, 1095.6 $[M+Na]^+$. Anal. Calcd for $C_{54}H_{88}O_{21}$: C, 60.43; H, 8.26; Found: C, 60.67; H, 8.32.

3.6. 3-*O*-[2,3,4-Tri-*O* -benzoyl-α-L-Rhamnopyranosyl]oleanolic acid 28-*O*-[2,3,4-tri-*O*-benzoyl-α-L-rhamnopyranosyl] ester (15)

Coupling of compounds **8** (248 mg, 0.4 mmol) and **13** (417 mg, 0.36 mmol) using the same procedure as described in the preparation of **14** from **11** and **13** obtained compound **15** as a foamy solid (460 mg, 93%): $[\alpha_D^{25}$ +43 (*c* 1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): 0.79, 0.87, 0.88, 0.90, 1.00, 1.01, 1.14 (7s, 7 × 3H), 1.26 (d, 3H, *J* 6.2 Hz, H-6)), 1.30 (d, 3H, *J* 6.2 Hz, H-6)), 0.73–1.86 (m, 22H), 2.97 (dd, 1H, *J* 3.6, 13.4 Hz, H-18 of oleanolic acid), 3.17 (dd, 1H, *J* 6.9, 10.8 Hz, H-3 of oleanolic acid), 4.19–4.23 (m, 2H, 2H-5), 5.02 (d, 1H, *J* 1.9 Hz, H-1), 5.36 (br s, 1H, H-12 of oleanolic acid), 5.57–5.79 (m, 6H), 6.22 (d, 1H, *J* 1.8 Hz, H-1), 7.19–8.04 (m, 30H, 6Ph). ^{13}C NMR (100 MHz, $CDCl_3$): 175.4, 166.5, 166.3, 166.2 (2C), 166.1, 165.8, 144.5, 134.2, 134.1, 134.0, 133.8 (2C), 133.6, 131.5, 130.6, 130.5, 130.3, 130.2, 130.0, 129.9, 129.8 (2C), 129.7, 129.4, 129.2 (2C), 129.1, 129.0, 128.9, 128.8, 124.0, 100.3 (C-1), 90.9 (C-1), 73.4, 72.6, 71.8, 71.1, 70.8, 70.5, 70.3, 69.8, 69.7, 67.4, 66.1, 48.1, 48.0, 46.1, 42.6, 42.5, 40.1, 39.6, 39.1, 37.4, 34.3, 33.7, 33.4, 33.2, 31.4, 31.2, 30.3, 29.0, 28.2, 26.5, 26.0, 24.1, 23.7, 21.6, 19.8,

18.9, 18.4, 18.3, 18.2, 17.8, 17.2, 16.0, 14.3. Anal. Calcd for $C_{84}H_{92}O_{17}$: C, 73.45; H, 6.75; Found: C, 73.72; H, 6.68.

3.7. 3-*O*[α-L-Rhamnopyranosyl]oleanolic acid 28-*O*[α-L-rhamnopyranosyl] ester (5)

Compound **15** (215 mg, 0.16 mmol) was treated with NaOMe, as described in the preparation of **4**, affording compound **5** (108 mg, 92%) as a foamy solid: ^{1}H NMR (400 MHz, MeOD): [α_D^{25} +11 (*c* 1.5, H_2O); δ 0.79–1.91 (m, 49H), 2.90 (dd, 1H, H-18 of oleanolic acid), 3.09 (dd, 1H, H-3 of oleanolic acid), 3.35 (t, 1H, *J* 9.5 Hz), 3.43 (t, 1H, *J* 9.5 Hz), 3.61–3.72 (m, 4H), 3.75 (br s, 1H), 3.82 (br s, 1H), 4.72 (d, 1H, *J* 2.1 Hz, H-1), 5.31 (br s, 1H, H-12 of oleanolic acid), 5.92 (d, 1H, *J* 2.3 Hz, H-1). ^{13}C NMR (100 MHz, DMSO-d_6): 175.3, 143.6, 122.7, 103.1 (C-1), 93.6 (C-1), 87.8, 72.4, 71.8, 71.4, 71.1, 71.0, 70.9, 69.9, 68.8, 55.2, 47.3, 46.9, 45.7, 41.6, 41.5, 38.4, 36.7, 33.5, 33.1, 32.8, 32.5, 30.8, 28.3, 27.35, 25.98, 26.0, 25.3, 23.7, 23.4, 22.8, 18.2, 18.1, 17.3, 16.8, 15.5. MALDITOF-MS: calcd for $C_{42}H_{68}O_{11}$: 748.5 [M]$^+$; found, 771.88 [M+Na]+. Anal. Calcd for $C_{42}H_{68}O_{11}$: C, 67.35; H, 9.15; Found: C, 67.60; H, 9.08.

3.8. 3-*O*-Acetyl oleanolic acid 28-*O*-(6-hydroxyhexyl) ester (17)

To a stirred solution of **16** (500 mg, 1.0 mmol) and *N*,*N*-dimethylformamide (125 μL, 1.50 mmol) in dry CH_2Cl_2 (5 mL) was added oxalyl chloride (430 μL, 5.01 mmol) at room temperature under nitrogen atmosphere. The reaction mixture was stirred at this condition for 1 h, then the organic layer was transferred into a solution of 1,6-hexanediol (354 mg, 3.00 mmol), triethylamine (0.85 mL, 6.00 mmol), and 4-methylaminopyridine (15 mg) in dry CH_2Cl_2 (20 mL) at rt under N_2 protection. The mixture was stirred for 6 h, then poured into cold water, and the organic phase was washed with $NaHCO_3$ aqueous solution, dried over Na_2SO_4, and concentrated. The crude product was purified by flash column chromatography (petroleum ether–EtOAc 4:1) to give **17** (491 mg, 82%) as an amorphous solid: [α_D^{25} +65 (*c* 0.5, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): 0.74, 0.86, 0.87, 0.90, 0.93, 0.93, 1.13 (7s, 7 × 3H, 7CH_3), 0.74–1.87 (m, 30H), 2.05 (s, 3H, Ac), 2.87 (dd, 1H, *J* 4.0, 13.8 Hz, H-18 of oleanolic acid), 4.49 (t, 1H, *J* 8.1 Hz, H-3 of oleanolic acid), 3.63-3.67 (m, 2H, CH_2OH), 3.99–4.03 (m, 2H, CH_2OC=O), 5.28 (br s, 1H, H-12 of oleanolic acid). Anal. Calcd for $C_{38}H_{62}O_5$: C, 76.21; H, 10.43; Found: C, 75.96; H, 10.40.

3.9. Oleanolic acid 28-*O*-(6-hydroxyhexyl) ester (18)

1 M NaOMe was added into a solution of compound **17** (443 mg, 0.74 mmol) in MeOH/CH_2Cl_2 (3 mL/7 mL). The reaction mixture was stirred under reflux for 72 h at pH 9.5, then neutralized with Dowex-50 (H^+) ion exchange resin. The mixture was filtered, the filtrate concentrated, and the resulting residue was purified by silica gel column chromatography (petroleum ether–EtOAc 2:1) to afford compound **18** as a white foam (375 mg, 91%): [α_D^{25} +72 (*c* 1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): 0.73, 0.77, 0.90, 0.90, 0.92, 0.98, 1.13 (7s, 7 × 3H, 7CH_3), 0.73–2.01 (m, 30H), 2.86 (dd, 1H, *J* 4.4, 13.9 Hz, H-18 of oleanolic acid), 3.21 (dd, 1H, *J* 4.3, 10.8 Hz, H-3 of oleanolic acid), 3.63–3.67 (m, 2H, CH_2OH), 3.98–4.03 (m, 2H, CH_2OC=O), 5.27 (br s, 1H, H-12 of oleanolic acid). Anal. Calcd for $C_{36}H_{60}O_4$: C, 77.65; H, 10.86; Found: C, 77.91; H, 10.78.

3.10. 3-*O*-[2,3,4-Tri-*O*-benzoyl-α-L-Rhamnopyranosyl]oleanolic acid 28-*O*-[6-*O* -(2,3,4-tri-*O* -benzoyl-α-L-rhamnopyranosyl)-hexyl] ester (19)

To a solution of compounds **8** (480 mg, 0.77 mmol) and **18** (200 mg, 0.36 mmol) in anhyd CH_2Cl_2 (5 mL) was added TMSOTf (16 μL, 0.09 mmol) under an N_2 atmosphere at −35 °C. The reaction mixture was stirred under these conditions for 30 min, at the end of which time TLC (petroleum ether–EtOAc 5:2) indicated that all starting materials were consumed. The reaction mixture was neutralized with Et_3N and concentrated. Column chromatography of the residue gave **19** as a white foam (477 mg, 90%): [α_D^{25} +89 (*c* 0.5, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): 0.79, 0.88, 0.92, 0.96, 1.00, 1.08, 1.16 (7s, 7 × 3H, 7CH_3), 1.34, 1.39 (2d, 2 × 3H, *J* 6.3 Hz, 2H-6^{Rha}), 0.79–1.82 (m, 30H), 2.90 (dd, 1H, H-18 of oleanolic acid), 3.25 (dd, 1H, H-3 of oleanolic acid), 3.55–3.58 (m, 1H, CH_2OH), 3.81-3.84 (m, 1H, CH_2OH), 4.08 (m, 2H, CH_2OC=O), 4.20 (m, 1H, H-5^{Rha}), 4.31-4.33 (m, 1H, H-5^{Rha}), 5.03 (d, 1H, *J* 2.4 Hz, H-1^{Rha}), 5.09 (d, 1H, *J* 2.1 Hz, H-1^{Rha}), 5.32 (br s, 1H, H-12 of oleanolic acid), 5.66-5.88 (m, 6H), 7.28–8.14 (m, 30H, 6Ph). ^{13}C NMR (100 MHz, $CDCl_3$): 178.4, 166.5, 166.3, 166.2 (2C), 166.1, 166.0, 144.5, 134.0 (2C), 133.9, 133.7 (2C), 130.5, 130.3 (2C), 130.2, 130.1, 130.0, 129.9, 129.8 (2C), 129.4, 129.2, 129.0, 128.9, 124.5, 100.3 (C-1), 98.2 (C-1), 90.0, 73.4, 72.5 (2C), 71.8, 71.6, 70.8, 70.7, 68.9, 67.4, 67.2, 64.8, 56.0, 48.2, 47.3, 46.5, 42.3, 41.9, 40.0, 39.6, 39.0, 37.4, 34.5, 33.7, 33.3, 33.1, 31.3, 30.0, 29.2, 29.0, 28.2, 26.6, 26.4, 26.0, 24.2, 24.1, 23.6, 19.8, 18.9, 18.3, 18.1, 17.7, 17.2, 16.0. Anal. Calcd for $C_{90}H_{104}O_{18}$: C, 73.35; H, 7.11; Found: C, 73.06; H, 7.18.

3.11. 3-*O*-[α-L-Rhamnopyranosyl]oleanolic acid 28-*O*-[6-*O*-(α-L-rhamnopyrano syl)hexyl] ester (6)

To a solution of **19** (148 mg, 0.1 mmol) in dry CH_2Cl_2/MeOH (1:2, v/v, 9 mL) was added 1 M NaOMe (12 μL, 0.012 mmol) in MeOH. The mixture was stirred at rt until all starting materials were consumed, and then neutralized with Dowex-50 (H^+) ion exchange resin. Filtration, concentration of the filtrate, and purification of the resulting residue by LH-20 column chromatography using H_2O as eluent afforded compound **6** (76 mg, 89%) as a white powder: [α_D^{25} +6 (*c* 1.2, water); ^{1}H NMR (400 MHz, MeOD): 0.77, 0.80, 0.92, 0.95, 0.96, 0.97, 1.18 (7s, 7 × 3H, 7CH_3), 1.24, 1.27 (2d, 2 × 3H, *J* 6.3 Hz, H-6^{Rha}), 0.77–2.02 (m, 30H), 2.91 (dd, 1H, H-18 of oleanolic acid), 3.10 (dd, 1H, *J* 4.6, 11.5 Hz, H-3 of oleanolic acid), 3.31–3.41 (m, 3H), 3.56–3.72 (m, 5H), 3.79–3.82 (m, 1H), 3.83–3.85 (m, 1H), 4.03 (t, 2H, *J* 6.2 Hz), 4.66 (d, 1H, *J* 1.6 Hz, H-1^{Rha}), 4.74 (d, 1H, *J* 1.4 Hz, H-1^{Rha}), 5.27 (br s, 1H, H-12 of oleanolic acid). ^{13}C NMR (100 MHz, MeOD): 178.7, 144.3, 123.0, 103.6 (C-1), 100.8 (C-1), 89.5, 73.3, 73.2, 71.7, 71.5, 69.1, 68.9, 67.6, 64.7, 56.8, 47.6, 42.1, 42.0, 39.9, 39.2, 38.8, 37.1, 34.0, 33.1, 32.9, 32.7, 30.8, 29.9, 28.9, 28.0, 26.3, 26.1, 25.8, 25.6, 23.8, 23.2, 18.6, 17.3, 17.1, 17.0, 16.2, 15.2. MALDITOF-MS: calcd for $C_{48}H_{80}O_{12}$: 848.56 [M]$^+$; found, 871.72 [M+Na]$^+$. Anal. Calcd for $C_{48}H_{80}O_{12}$: C, 67.89; H, 9.50; Found: C, 67.63; H, 9.44.

3.12. Cell culture assay

HL-60 cells were maintained in the RPMI 1640 medium containing 10% fetal bovin serum supplemented with L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. The leukemia cells were washed and suspended in the above medium to 1 × 10^5 cells/mL, and 100 μL of this cell suspension was placed in each well of a 96-well plate. The cells were incubated in 5% CO_2/air for 24 h at 37 °C. After incubation, 3 μL/mL of DMSO–H_2O (1:20, v/v) solution containing the sample was added to give the final concentration of 0.1–30 μL/mL, while 3 μL/mL of DMSO–H_2O (1:20, v/v) was added into control wells. The cells were further incubated for 72 h in the presence of each agent, and then cell growth was evaluated using modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, and the IC_{50} values were calculated accordingly.[31,32]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2009.04.005.

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