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Glycoside modification of oleanolic acid derivatives as a novel class of anti-osteoclast formation agents

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ABSTRACT

Oleanolic acid, a natural product, possesses an anti-osteoclast formation activity. Targeting at discovery of novel and potent anti-bone resorption agents, 22 glycosides of oleanolic acid derivatives (including D-galactopyranosides, D-glucopyranosides, D-xylopyranoses, D-arabopyranoses and D-glycuronic acids) were synthesized at phase-transfer-catalyzed conditions (K_2CO_3, Bu_4NBr, CH_2Cl_2–H_2O) and their inhibitory activity on the formation of osteoclast-like multinucleated cells (OCLs) induced by 1α, 25-dihydroxy vitamin D_3 was evaluated in a co-culture assay system. The structure–activity relationships of these compounds were also discussed.

1. Introduction

Osteoporosis is widely recognized as a major public health problem, particularly among the postmenopausal women. Currently, it is estimated that over 200 million people worldwide have osteoporosis[1] and osteoporosis related fractures accounted for more disability-adjusted life years (DALYs) lost than common cancers except for lung cancer in Europe.[2] It is a common skeletal disease characterized by gradual loss of bone mass and disrupted bone architecture as a result of an imbalance between the bone resorption activity of osteoclasts and the bone formation activity of osteoblasts.[3] In a research project focused on finding new anti-osteoporosis agents, we discovered that the extracts from the root of *Achyranthes bidentata* Blume (Amaranthaceae) showed the anti-osteoporosis activity in vitro and in vivo and further research revealed that oleanolic acid (OA, **1**) and its glycosides were the active components of the extracts responsible for the anti-osteoporosis activity.[4,5] OA, a natural product, and its derivatives possess a variety of biological activities, such as anti-inflammatory,[6,7] hepatoprotective effects,[8,9] anti-HIV,[10,11] antitumour,[12,13] inhibitory effect of glycogen phosphorylase[14] and suppressive activity of nitric oxide production.[15,16] However, the effect of oleanolic acid and its derivatives on anti-osteoclast formation had not been studied sufficiently (Fig. 1).

Triterpenoid glycosides are a large family of natural products with a great variety of structures and bio-effects, especially some of them show prominent activities.[17] Several papers also reported that heterocyclic system such as indole, phenylhydrazone moieties produce effective anti-osteoporosis activity.[18,19] Those reports, together with our previous results of oleanolic acid as a potential anti-osteoporosis lead compound inspired us to incorporate indole, phenylhydrazone and saccharide segments in the syntheses of oleanolic acid derivatives to improve the inhibitory activity on osteoclast formation. In the present study, as a part of our continuing efforts targeting at discovery of more potent anti-osteoclast formation molecules, we report here the synthesis, activity evaluation of 22 glycosides of oleanolic acid derivatives.

2. Results and discussion

2.1. Chemistry

The preparation of compounds **2**–**4** is outlined in Scheme 1. 3-Keto oleanolic acid (**2**) was synthesized by Jones oxidation of oleanolic acid.[20] A mixture of 3-keto oleanolic acid (**2**) and phen-

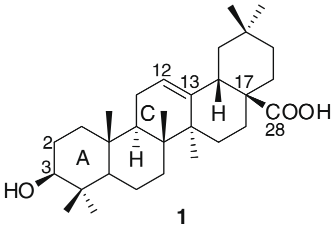

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Figure 1. Chemical structure of oleanolic acid.

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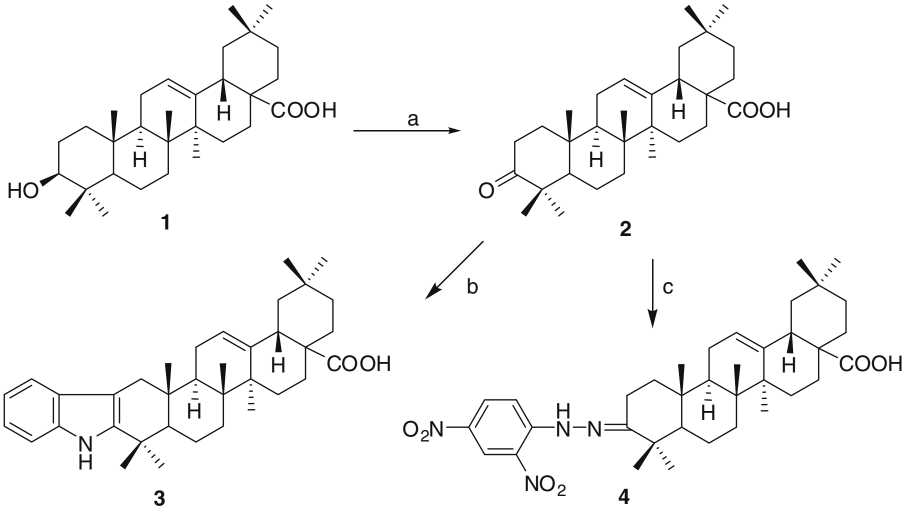


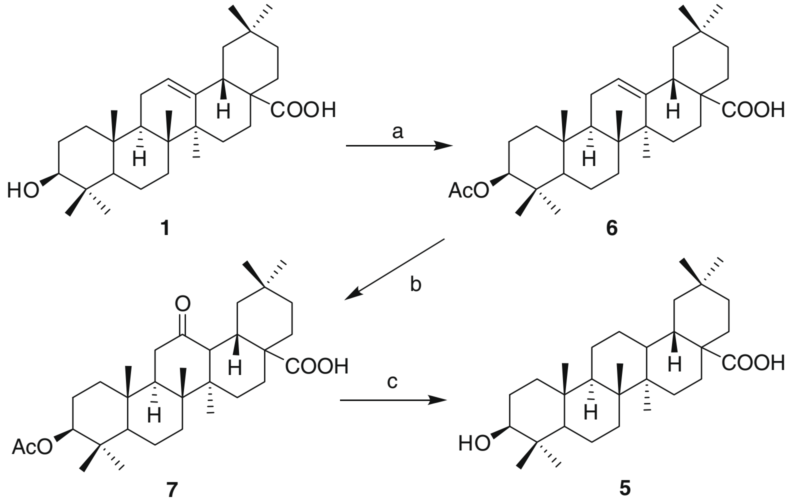
Scheme 1. Reagents and conditions: (a) Jones oxidation;[20] (b) phenylhydrazine, AcOH, reflux;[21] (c) 2,4-dinitrophenylhydrazine, AcOH, reflux.

ylhydrazine in the presence of acetic acid was refluxed under N_2 for 1–2 h to provide indole target compound **3**.[21] A mixture of 3-keto oleanolic acid (**2**) and *o*,*p*-dinitrophenylhydrazine in the presence of acetic acid was refluxed under N_2 for 1–2 h to afford phenylhydrazone **4**.

The optimized synthesis route of compound **5** is outlined in Scheme 2. Oleanolic acid was acetylated with 10 equiv of acetic anhydride in anhydrous pyridine to give 3-acetoxy-oleanolic acid (**6**). Oxidation of compound **6** with 30% H_2O_2 in formic acid at 50 °C overnight afforded 3-acetoxy-12-oxo-oleanolic acid (**7**). Dihydrooleanolic acid (**5**) was produced by Wolff–Kishner reduction of **7** in diethylene glycol solution. Although the synthesis of dihydrooleanolic acid (**5**) had been reported,[22,23] it is worth to indicate that in the present synthetic process: (i) Unprotection of the C-17 carboxyl group of **2** did not impact the yield of dihydrooleanolic acid **5**. (ii) Oxidation of **6** to 12-oxo compound **7** could be achieved by one step with 30% H_2O_2 in acidic medium (formic acid), which was superior to that with 3-chloroperbenzoic acid and then reflux with ethereal boron trifluoride (two steps, in acetic acid).

D-Sugar bromides **8**–**11** (Fig. 2) were prepared through benzoylation (BzCl in pyridine) and bromination (HBr in HOAc).[24,25] Protection of D-glucuronic acid was achieved by a one-pot method. Tosin reported when deprotection of an acetyl group at COOH of fully acetylated D-glucuronic acid in the presence of iodine followed by treatment with methanol, the reaction gave the methyl ester, not the desired carboxylic acid.[26] However, this result gave us a clue to protection of COOH. As expected, the acetylation of D-glucuronic acid in the presence of iodine in acetic anhydride followed by treatment with methanol in one pot gave target product, 1,2,3,4-tetra-*O*-acetyl-β-D-glycuronic acid-6-methyl ester. Thereafter, bromide **12** was synthesized with the same protocol as **8**–**11** (Fig. 2).

In recent years, a phase-transfer-catalyzed method has been applied for glycosidation due to its mild conditions and high yields.[27,28] The present glycosidations of the oleanolic acid derivatives were achieved by the reaction of acceptors (**1**–**5**) with donors (**8**–**12**) under the optimized phase-transfer-catalyzed conditions (K_2CO_3, Bu_4NBr, CH_2Cl_2–H_2O, reflux). Subsequent removal of the protecting groups (benzoyl) using sodium methoxide (cat.) in MeOH gave the corresponding glycosides **a**–**d**. Removal of the glycuronic acid protecting groups was slightly modified. The related compounds were treated with sodium methoxide in MeOH and then water to yield compounds **1e** and **5e**, respectively (Scheme 3).



Scheme 2. Reagents and conditions: (a) Ac_2O, Py, DMAP, rt; (b) formic acid, 30% H_2O_2, $CHCl_3$, 0–50 °C; (c) KOH, diethylene glycol, $NH_2NH_2{\cdot}H_2O$, reflux, 160–210 °C.

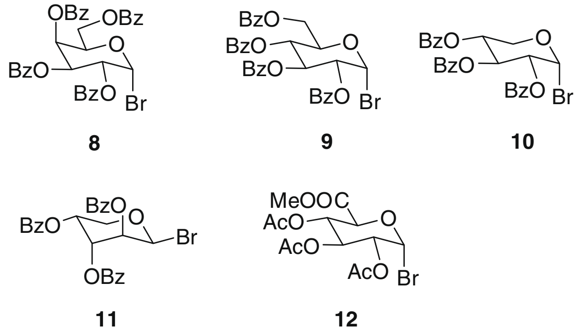
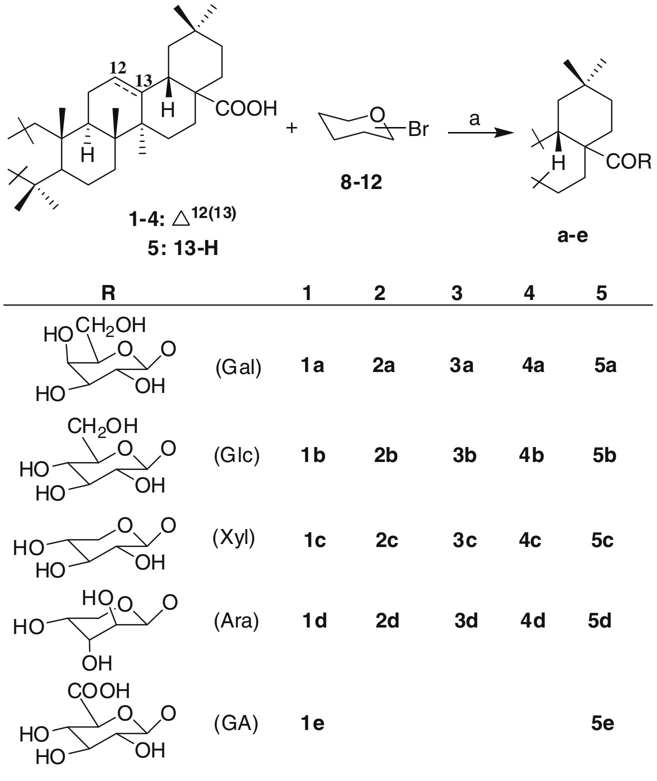


Figure 2. Five sugar donors (**8**–**12**).

For the stereochemistry of the glycosidic bonds, bromides **8**–**9** were α configuration which were identical with the reported data.[24,29] Bromides **10** and **12** were elucidated to be α configuration (H-1, 6.66–6.85 ppm, d, *J* = 3.7–3.9 Hz), while **11** was β configuration (H-1, 6.93 ppm, d, *J* = 3.2 Hz). To target glycosides, all of the glycosidic bonds were β configuration (**a**–**c** and **e** series: H-1′, 5.34–6.35 ppm, d, *J* = 6.4–8.5 Hz; **d** series, H-1′, 5.41–6.28 ppm, d, *J* = 6.1–7.9 Hz).

2.2. Biological activity

Since potentiated bone resorption by osteoclasts is one of the major causes of osteoporosis, inhibitors of osteoclasts may therefore present useful agents to prevent the excessive bone resorption associated with osteoporosis. Thus, an inhibitory activity of all compounds on TRAP-positive osteoclast-like multinucleated cells (OCLs) formation was assayed. The OCLs formation was induced by 1α, 25-dihydroxy vitamin D_3 [1α, $25(OH)_2D_3$] in a co-culture system with mouse bone marrow cells and osteoblast-like cells.[30]



Scheme 3. Reagents and conditions: (a) (i) CH_2Cl_2/H_2O (10:1), K_2CO_3, Bu_4NBr, reflux; (ii) MeONa (cat.), MeOH, rt or MeONa (cat.), MeOH, H_2O, rt.

As presented in Table 1, all of the tested compounds remained inhibitory effect on OCLs formation. Fortunately, except for heterocyclic derivative (compound **3**), the skeleton modifications of OA (compounds **2**, **4** and **5**) displayed a more potent activity than that OA did, especially compound **4** showed much stronger activity compared with OA.

A lot of papers have reported that glycosidation could improve the bioactivity of parent molecules.[31–33] As expected, introducing monosaccharide moieties including D-glucose, D-galactose, D-xylose and D-arabinose at C_{28}-position of parent compounds **1**–**4** greatly enhanced the inhibitory activity compared with their parent compounds, especially indole **3** and phenylhydrazone **4** series (**3a**–**d**, **4a**–**d**) exhibited quite strong activity, almost 100% inhibition on OCLs formation at 20 μM. Unfortunately, dihydrooleanolic glycosides (**5a, 5c**–**e**) showed a quite weaker activity compared with compound **5**. Together with our previous results,[4] it could be concluded that double bond at 12 and 13 position in C-ring is a key for the potent anti-OCLs formation activity of glycosides, any changes at the double bond would negatively impact the activity. Furthermore, the activity of the compounds at 2 μM was assayed. As can be seen in Table 1, most of the compounds displayed a weak activity. Meaningfully, the xylose and glycuronic acid glycosides improved the activity except for **1c**. However, for phenylhydrazone **4**, adding a glycone section at the C-28 position suppressed the inhibitory activity (about **4a**, 99.0%; **4b**, 91.3%; **4c**, 88.3%; **4d**, 59.4%). Encouragingly, compounds **1e**, **1f**, **2c**, **3a**, **5c**, **5e** displayed a quite strong activity compared with parent compounds even at 2 μM, among those compounds, **1e**, a glucuronic acid glycoside showed the strongest activity (about 16.0%). For the cytotoxicity of the tested compounds by a recovery test,[34] no significant toxicity for all of the tested compounds was observed.

From the above data, it is quite clear that any changes in double bond at C-ring of OA gave a negative impact on the anti-OCLs formation activity. The most glycosidations are of quite benefit to the improvement of the activity, especially, xylose and glycuronic acid glycosides. A phenylhydrazone moiety at A-ring of OA greatly improved the activity, while its glycosides did not bring any benefit on the activity enhancement.

In conclusion, we have synthesized a series of glycosides of oleanolic acid derivatives, and assayed their anti-OCLs formation activity. 12-Keto oleanolic acid (**7**) was afforded by one step with 30% H_2O_2 in acidic medium instead of the two steps method. Protection of D-glucuronic acid was achieved by a one pot reaction. Twenty two novel oleanolic acid type glycosides were synthesized at phase-transfer-catalyzed conditions (K_2CO_3, Bu_4NBr, CH_2Cl_2–H_2O). The preliminary biological evaluation results showed that compound **1e**, **2c** and **4** displayed quite strong inhibitory activity even at 2 μM. Our results revealed that the glycosides of OA derivatives represent an interesting class of compounds for further pharmacological studies and preclinical developments. Further syntheses of glycosides, detailed studies on compound **1e** and **2c** in vivo are in progress in our lab.

3. Experimental

3.1. General methods

All the solvents used were commercially available. Reactions were monitored by thin-layer chromatography (TLC) on silica gel plates, visualizing with ultraviolet light or iodine spray. NMR spectra (300 MHz ^1H and 75 MHz ^{13}C) were taken in $CDCl_3$, C_5D_5N-d_5 or MeOD solution on Bruker Avance II-300 spectrometer. Melting points were determined using X4 apparatus and uncorrected. ESI-MS spectra were obtained on a Finnigan TSQ-7000 mass spectrometer.

Table 1
Effects of tested compounds on 1α, $25(OH)_2D_3$ induced TRAP(+)-OCLs formation

Compound	OCLs (%)		Compound	OCLs (%)	
	20 μM	2 μM		20 μM	2 μM
Control	100.0 ± 9.7$^{\#}$	100.0 ± 6.2$^{\#}$	Normal	20.0 ± 5.0	20.0 ± 8.6
1	20.3 ± 5.9**	89.2 ± 10.2**	**3c**	0**	61.3 ± 13.7**
1a	0**	98.2 ± 5.2	**3d**	0**	96.7 ± 10.9*
1b	1.3 ± 0.2**	98.7 ± 5.2	**4**	39.8 ± 8.7**	29.7 ± 4.7**
1c	54.7 ± 2.9**	95.5 ± 5.0*	**4a**	0**	99.0 ± 6.7
1d	5.7 ± 0.5**	99.3 ± 5.2	**4b**	0**	91.3 ± 12.2**
1e	19.3 ± 1.1**	16.0 ± 0.8**	**4c**	0**	88.3 ± 8.2**
2	1.4 ± 3.4**	79.2 ± 12.0**	**4d**	0**	59.4 ± 18.3**
2a	0**	78.1 ± 9.3**	**5**	28.1 ± 7.0**	66.3 ± 3.5**
2b	0**	64.4 ± 12.8**	**5a**	77.1 ± 4.1**	87.2 ± 4 .6**
2c	0**	30.2 ± 10.7**	**5b**	0**	85.7 ± 4.5**
2d	9.5 ± 5.0**	98.4 ± 7.4	**5c**	60.3 ± 3.2**	56.5 ± 3.0**
3	51.1 ± 6.2**	94.6 ± 9.6**	**5d**	55.5 ± 2.9**	90.9 ± 4.8**
3a	0**	54.4 ± 15.6**	**5e**	41.9 ± 2.2**	54.6 ± 2.9**
3b	0**	98.5 ± 6.9			

Control: cultured with 1α, $25(OH)_2D_3$ (10^{-8} M). Normal: cultured without any additions. Samples: cultured with $25(OH)_2D_3$ (10^{-8} M) and each compound . Each value was expressed as mean ± SD, $n = 4$. The data of control group was pegged as 100%, while other data were calculated relative to it. Data were analyzed by student's *t* test. Significant differences in TRAP (+)-OCLs compared with control group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.01$ versus normal group.

3.2. 3-Keto oleanolic acid (2) and [3,2-*b*] indole oleanolic acid (3)

3-Keto oleanolic acid (**2**) and [3,2-*b*] indole acid (**3**) were prepared following the Gribble's method[20,21] and the ^{1}H NMR, ^{13}C NMR and MS data were identical with the reported ones.

3.3. 3-[2,4-Dinitrophenylhydrazone] oleanolic acid (4)

To 3-keto oleanolic acid (**2**) (8.67 g, 19.10 mmol) stirring in acetic acid (80 mL) was added 2,4-dinitrophenylhydrazine (3.97 g, 20 mmol). The mixture was refluxed for 1 h. The reaction mixture was pipetted into distilled water and then extracted with Et_2O (4 × 80 mL). After removing Et_2O, the residue was purified by column chromatography (silica gel; EtOAc/PE) to provide phenylhydrazone **4** (yield 65%). Orange solid; mp 272 °C; ^{1}H NMR ($CDCl_3$): δ 11.19 (s, 1H), 9.15 (s, 1H), 8.32 (d, 1H, *J* 9 Hz), 7.87 (d, 1H, *J* 9 Hz), 5.33 (s, 1H), 2.86 (d, 1H, *J* 12 Hz); ^{13}C NMR ($CDCl_3$): δ 184.7, 166.9, 145.5, 143.6, 137.4, 129.9, 128.9, 123.6, 122.3, 116.4; ESI-MS (negative) *m/z*: 633.23 $[M-H]^-$.

3.4. Preparation of 12,13-dihydrooleanolic acid (5)

3.4.1. 3-*O*-Acetyl oleanolic acid (6)

To OA (**1**) (456 mg, 1 mmol) stirring in pyridine (19 mL) in an ice bath was added acetic anhydride (9.45 mL). DMAP (122 mg, 1 mmol) was added and the mixture was stirred at room temperature for 5 h. The mixture was poured into ice water and was then extracted with CH_2Cl_2. The organic layer was washed with dilute aqueous HCl, water and brine, respectively. After removing the solution, the residue was purified by recrystallization to provide compound **6** (4.93 g, yield 99%). ^{1}H NMR ($CDCl_3$): δ 5.32 (s, 1H), 4.48 (t, 1H, *J* 7.9 Hz), 2.80 (dd, 1H, *J* 13.3, 3.5 Hz), 2.04 (s, 3H).

3.4.2. 3-*O*-Acetyl-12-oxo oleanolic acid (7)

To compound **6** (2.50 g, 5 mmol) in $CHCl_3$ (19 mL) was added H_2O_2 (10 mL, 30%) and formic acid (2.25 mL). The mixture was stirred at 50 °C for 24 h. After removal of the solvent, crude product was purified by recrystallization to give compound **7** (1.82 g, 71%).^{1}H NMR ($CDCl_3$): δ 4.47 (dd, 1H, *J* 10.5, 4.8 Hz), 2.71 (t, 1H, *J* 14.3 Hz), 2.54 (t, 1H, *J* 8.3 Hz), 2.37 (dd, 1H, *J* 14.6, 3.0 Hz).

3.4.3. 12,13-Dihydrooleanolic acid (5)

To compound **7** (10.28 g, 20 mmol) in diethylene glycol (200 mL) was added KOH (26 g, 0.46 mol) and hydrazine hydrate (100 mL). The mixture was refluxed at 160 °C for 3 h. After removal of water and hydrazine hydrate, the mixture was then stirred at 210 °C for 6 h. After cooling room temperature, the reaction mixture was neutralized with dilute aqueous HCl (pH <7). The mixture was poured into water and the resulting suspension was filtered. Crude product was purified by recrystallization to provide compound **5** (6.62 g, 72%). White solid; ^{1}H NMR ($CDCl_3$): δ 3.23 (dd, 1H, *J* 9.4, 6.3 Hz), 2.90 (dd, 1H, *J* 13.0, 3.4 Hz); ^{13}C NMR ($CDCl_3$): δ 174.5; MS (ESI) *m/z*: 457.41 $[M-H]^-$.

3.5. General procedure for preparation of glycosides of oleanolic acid derivatives a–d

To bromine glycoside (0.52 mmol) and oleanolic acid derivative (0.40 mmol) stirring in 22 mL CH_2Cl_2/H_2O (10:1) was added K_2CO_3 (138 mg) and Bu_4NBr (51.52 mg). The mixture was refluxed under nitrogen atmosphere. After completion (TLC) the reaction mixture was diluted with $CHCl_3$ and washed with water, then brine and dried. The crude was purified by recrystallization or column chromatography.

To the above compound (0.24 mmol) stirring in methanol was added sodium methoxide (cat.). The mixture was stirred at room temperature. After completion (TLC) the reaction mixture was neutralized with HCl (1 M). Water was added and the resulting suspension was filtered. Crude product was purified by recrystallization or column chromatography. Compounds **a**–**d** were deprotected following this protocol.

3.5.1. Oleanolic acid 28-*O*-[β-D-galactopyranosyl] ester (1a)

White solid; yield 91.0%; mp 243–245 °C; ^{1}H NMR ($CDCl_3$): δ 5.38 (d, 1H, *J* 8.2 Hz, Gal-1-H), 5.26 (s, 1H), 3.91 (s, 1H), 3.72–3.70 (m, 2H), 3.66 (d, 1H, *J* 8.2 Hz), 3.61 (dd, 1H, *J* 9.2, 5.9 Hz), 3.54 (dd, 1H, *J* 9.7, 2.7 Hz), 3.16 (dd, 1H, *J* 10.6, 4.0 Hz), 2.88 (dd, 1H, *J* 13.7, 3.0 Hz); ^{13}C NMR ($CDCl_3$): δ 177.3, 141.2, 122.3, 96.9; MS (ESI) *m/z*: 641.2 $[M+Na]^+$. Anal. Calcd for $C_{36}H_{58}O_8$: C, 69.87; H, 9.45. Found: C, 69.57; H, 9.37.

3.5.2. Oleanolic acid 28-*O*-[β-D-glucopyranosyl] ester (1b)

White solid; yield 87.6%; mp 239–241 °C; ^{1}H NMR (MeOD): δ 5.57 (d, 1H, *J* 7.8 Hz, Glc-1-H), 5.26 (s, 1H), 3.85 (d, 1H, *J* 11.9 Hz), 3.69 (d, 1H, *J* 12.1 Hz), 3.46–3.33 (m, 4H), 3.12 (dd, 1H, *J* 11.1, 5.7 Hz), 2.87 (d, 1H, *J* 13.7 Hz); ^{13}C NMR (MeOD): δ 185.2, 145.8, 122.7, 95.6; MS (ESI) *m/z*: 641.2 $[M+Na]^+$. Anal. Calcd for $C_{36}H_{58}O_8$: C, 69.87; H, 9.45. Found: C, 69.62; H, 9.40.

3.5.3. Oleanolic acid 28-*O*-[β-D-xylopyranosyl] ester (1c)

White solid; yield 95.0%; mp >280 °C; ^{1}H NMR (MeOD): δ 5.34 (d, 1H, *J* 6.4 Hz, Xyl-1-H), 5.25 (s, 1H), 3.90–3.84 (m, 2H), 3.54–3.46 (m, 2H), 3.39 (dd, 1H, *J* 8.1, 3.2 Hz), 3.14 (dd, 1H, *J* 11.8, 4.4 Hz), 2.87 (dd, 1H, *J* 13.9, 3.4 Hz); ^{13}C NMR (C_5D_5N-d_5): δ 178.1, 145.8, 122.4, 97.9; MS (ESI) *m/z*: 611.17 [M+Na]$^+$. Anal. Calcd for $C_{35}H_{56}O_7$: C, 71.39; H, 9.59. Found: C, 71.14; H, 9.63.

3.5.4. Oleanolic acid 28-*O*-[β-D-arabopyranosyl] ester (1d)

White solid; yield 78.2%; mp 260–262 °C; ^{1}H NMR (C_5D_5N-d_5): δ 6.21 (d, 1H, *J* 7.8 Hz, Aar-1-H), 5.25 (s, 1H), 4.63 (dd, 1H, *J* 7.6, 6.3 Hz), 4.35–4.27 (m, 2H), 4.24–3.83 (m, 2H), 3.14 (dd, 1H, *J* 10.3, 4.7 Hz), 2.81 (d, 1H, *J* 10.4 Hz); ^{13}C NMR (C_5D_5N-d_5): δ 178.1, 145.8, 121.9, 97.9; MS (ESI) *m/z*: 611.17 [M+Na]$^+$. Anal. Calcd for $C_{35}H_{56}O_7$: C, 71.39; H, 9.59. Found: C, 71.10; H, 9.53.

3.5.5. 3-Keto oleanolic acid 28-*O*-[β-D-galactopyranosyl] ester (2a)

White solid; yield 69%; mp 210 °C; ^{1}H NMR ($CDCl_3$): δ 5.41 (d, 1H, *J* 8.1 Hz, Gal-1-H), 5.30 (t, 1H, *J* 3.0 Hz), 3.90 (s, 1H), 3.75–3.70 (m, 2H), 3.64 (d, 1H, *J* 8.3 Hz), 3.60 (dd, 1H, *J* 9.0, 6.0), 3.52 (dd, 1H, *J* 9.3, 2.9); ^{13}C NMR ($CDCl_3$): δ 218.2, 176.5, 143.6, 122.3, 94.5, 74.1, 70.4, 68.3; ESI-MS *m/z*: 639.15 [M+Na]$^+$. Anal. Calcd for $C_{36}H_{56}O_8$: C, 70.10; H, 9.15. Found: C, 69.85; H, 9.11.

3.5.6. 3-Keto oleanolic acid 28-*O*-[β-D-glucopyranosyl] ester (2b)

White solid; yield 71%; mp 192 °C; ^{1}H NMR (C_5D_5N-d_5): δ 6.35 (d, 1H, *J* 8.1 Hz, Glc-1-H), 5.46 (s, 1H), 4.42 (d, 1H, *J* 11.9 Hz), 4.21 (d, 1H, *J* 12.1 Hz), 4.01–3.95 (m, 4H); ^{13}C NMR (C_5D_5N-d_5): δ 217.8, 178.0, 145.7, 124.2, 97.3, 80.9; ESI-MS *m/z*: 639.17 [M+Na]$^+$. Anal. Calcd for $C_{36}H_{56}O_8$: C, 70.10; H, 9.15. Found: C, 70.39; H, 9.18.

3.5.7. 3-Keto oleanolic acid 28-*O*-[β-D-xylopyranosyl] ester (2c)

White solid; yield 73%; mp 252 °C; ^{1}H NMR (C_5D_5N-d_5): δ 6.25 (d, 1H, *J* 7.9 Hz, Xyl-1-H), 5.47 (s, 1H), 4.42 (d, 1H, *J* 11.3 Hz), 4.30–4.18 (m, 3H), 3.90–3.80 (m, 1H), 2.47 (dd, 1H, *J* 3.4, 13.9 Hz); ^{13}C NMR (C_5D_5N-d_5): δ 217.7, 178.2, 145.7, 122.3, 97.8, 79.9, 75.26, 72.4, 69.3; ESI-MS *m/z*: 609.27 [M+Na]$^+$. Anal. Calcd for $C_{35}H_{54}O_7$: C, 71.64; H, 9.28. Found: C, 71.39; H, 9.22.

3.5.8. 3-Keto oleanolic acid 28-*O*-[β-D-arabopyranosyl] ester (2d)

White solid; yield 70%; mp 240 °C; ^{1}H NMR (C_5D_5N-d_5): δ 5.41 (d, 1H, *J* 7.5 Hz, Ara-1-H), 5.32 (s, 1H), 4.34–4.29 (m, 2H), 4.23 (dd, 1H, *J* 7.6, 3.4 Hz), 3.85 (dd, 1H, *J* 10.5, 1.6 Hz), 2.86 (dd, 1H, *J* 5.1, 13.9 Hz); ^{13}C NMR (C_5D_5N-d_5): δ 218.4, 176.3, 143.2, 122.3, 94.3, 73.1, 70.4, 67.8, 66.0; ESI-MS *m/z*: 587.19 [M+H]$^+$. Anal. Calcd for $C_{35}H_{54}O_7$: C, 71.64; H, 9.28. Found: C, 71.85; H, 9.32.

3.5.9. [3,2-*b*] Indole oleanolic acid 28-*O*-[β-D-galactopyranosyl] ester (3a)

Orange solid; yield 67%; mp 229 °C; ^{1}H NMR ($CDCl_3$): δ 7.74 (s, 1H), 7.43–7.03 (m, 4H), 5.49 (d, 1H, *J* 7.9 Hz, Gal-1-H), 5.42 (s, 1H), 3.98 (s, 1H), 3.78–3.72 (m, 2H), 3.69 (d, 1H, *J* 8.1 Hz), 3.63 (dd, 1H, *J* 9.3, 6.0 Hz), 3.56 (dd, 1H, *J* 9.2, 2.8 Hz); ^{13}C NMR ($CDCl_3$): δ 177.7, 143.9, 141.9, 136.3, 128.8, 124.5, 121.2, 119.0, 118.1, 110.4, 106.7, 95.6; ESI-MS *m/z*: 712.35 [M+Na]$^+$. Anal. Calcd for $C_{42}H_{59}NO_7$: C, 73.12; H, 8.62; N, 2.03. Found: C, 72.90; H, 8.59; N, 2.05.

3.5.10. [3,2-*b*] Indole oleanolic acid 28-*O*-[β-D-glucopyranosyl] ester (3b)

Orange solid; yield 68%; mp 212 °C; ^{1}H NMR ($CDCl_3$): δ 7.73 (s, 1H), 7.42–7.02 (m, 4H), 5.49 (d, 1H, *J* 8.1 Hz, Glc-1-H), 5.40 (s, 1H), 3.73 (d, 1H, *J* 11.7 Hz), 3.62 (d, 1H, *J* 12.3 Hz), 3.43–3.32 (4H, m); ^{13}C NMR ($CDCl_3$): δ 176.7, 143.8, 140.9, 136.1, 128.5, 124.5, 121.2, 119.0, 118.1, 110.4, 106.7, 94.5, 76.7, 72.1, 69.5, 65.6; ESI-MS *m/z*: 712.27 [M+Na]$^+$. Anal. Calcd for $C_{42}H_{59}NO_7$: C, 73.12; H, 8.62; N, 2.03. Found: C, 72.85; H, 8.60; N, 2.04.

3.5.11. [3,2-*b*] Indole oleanolic acid 28-*O*-[β-D-xylopyranosyl] ester (3c)

Orange solid; yield 69%; mp 196 °C; ^{1}H NMR ($CDCl_3$): δ 7.76 (s, 1H), 7.43–7.05 (m, 4H), 5.55 (d, 1H, *J* 6.0 Hz, Xyl-1-H), 5.43 (s, 1H), 3.95–3.85 (m, 2H), 3.58–3.46 (m, 2H), 3.41 (dd, 1H, *J* 8.6, 2.3 Hz); ^{13}C NMR ($CDCl_3$): δ 176.4, 143.0, 140.9, 136.2, 128.7, 123.2, 121.0, 119.0, 118.1, 110.4, 106.9, 94.4, 76.7, 72.1, 69.5, 65.6; ESI-MS *m/z*: 682.11 [M+Na]$^+$. Anal. Calcd for $C_{41}H_{57}NO_6$: C, 74.62; H, 8.71; N, 2.12. Found: C, 74.90; H, 8.75; N, 2.13.

3.5.12. [3,2-*b*] Indole oleanolic acid 28-*O*-[β-D-arabopyranosyl] ester (3d)

Orange solid; yield 68%; mp 193 °C; ^{1}H NMR ($CDCl_3$): δ 7.76 (s, 1H), 7.42–7.03 (m, 4H), 5.49 (d, 1H, *J* 7.9 Hz, Ara-1-H), 5.41 (s, 1H), 4.01 (dd, 1H, *J* 7.1, 6.0 Hz), 3.95–3.83 (m, 2H), 3.69–3.60 (m, 2H); ^{13}C NMR ($CDCl_3$): δ 176.4, 143.0, 140.9, 136.2, 128.3, 123.2, 121.1, 119.0, 118.1, 110.4, 107.0, 94.4, 73.3; ESI-MS *m/z*: 682.19 [M+Na]$^+$. Anal. Calcd for $C_{41}H_{57}NO_6$: C, 74.62; H, 8.71; N, 2.12. Found: C, 74.37; H, 8.68; N, 2.14.

3.5.13. 3-[2,4-Dinitrophenylhydrazone] oleanolic acid 28-*O*-[β-D-galactopyrano-syl] ester (4a)

Orange solid; yield 71%; mp 215 °C; ^{1}H NMR (C_5D_5N-d_5): δ 11.20 (s, 1H), 9.14 (s, 1H), 8.42 (d, 1H, *J* 9.0 Hz), 8.05–8.02 (m, 1H), 6.29 (d, 1H, *J* 8.2 Hz, Gal-1-H), 5.47 (t, 1H, *J* 3 Hz), 4.70–4.61 (m, 2H), 4.49 (dd, 1H, *J* 12.9, 5.9 Hz), 4.26 (dd, 1H, *J* 13.0, 6.1 Hz), 4.22–4.16 (m, 2H); ^{13}C NMR (C_5D_5N-d_5): δ 178.1, 169.3, 147.3, 145.7, 139.2, 131.7, 131.0, 124.2, 118.1, 97.8, 79.3, 77.3, 73.0, 71.6; ESI-MS *m/z*: 798.21 [M+H]$^+$. Anal. Calcd for $C_{42}H_{60}N_4O_{11}$: C, 63.30; H, 7.59; N, 7.03. Found: C, 63.03; H, 7.52; N, 7.06.

3.5.14. 3-[2,4-Dinitrophenylhydrazone] oleanolic acid 28-*O*-[β-D-glucopyranosyl] ester (4b)

Orange solid; yield 70%; mp 210 °C; ^{1}H NMR (C_5D_5N-d_5): δ 11.16 (s, 1H), 9.12 (s, 1H), 8.28 (d, 1H, *J* 9.0 Hz), 7.96–7.91 (m, 1H), 5.50 (d, 1H, *J* 8.2 Hz, Glc-1-H), 5.32 (s, 1H), 4.45 (d, 1H, *J* 11.9 Hz), 4.26 (d, 1H, *J* 12.1 Hz), 4.01–3.85 (m, 4H); ^{13}C NMR (C_5D_5N-d_5): δ 176.7, 167.1, 145.6, 143.8, 137.6, 130.2, 129.0, 123.7, 122.4, 116.6, 93.9; ESI-MS *m/z*: 798.18 [M+H]$^+$. Anal. Calcd for $C_{42}H_{60}N_4O_{11}$: C, 63.30; H, 7.59; N, 7.03. Found: C, 63.01; H, 7.52; N, 7.05.

3.5.15. 3-[2,4-Dinitrophenylhydrazone] oleanolic acid 28-*O*-[β-D-xylopyranosyl] ester (4c)

Orange solid; yield 71%; mp 171 °C; ^{1}H NMR (C_5D_5N-d_5): δ 11.17 (s, 1H), 9.13 (s, 1H), 8.42 (d, 1H, *J* 9.0 Hz), 8.03–7.99 (m, 1H), 6.22 (d, 1H, *J* 7.9 Hz, Xyl-1-H), 5.45 (s, 1H), 4.32 (d, 1H, *J* 11.5 Hz), 4.15–4.01 (m, 3H), 3.81–3.71 (m, 1H); ^{13}C NMR (C_5D_5N-d_5): δ 178.2, 169.2, 147.3, 145.7, 139.2, 131.7, 124.3, 118.1, 97.3; ESI-MS *m/z*: 767.11 [M+H]$^+$. Anal. Calcd for $C_{41}H_{58}N_4O_{10}$: C, 64.21; H, 7.62; N, 7.31. Found: C, 63.96; H, 7.57; N, 7.35.

3.5.16. 3-[2,4-Dinitrophenylhydrazone] oleanolic acid 28-*O*-[β-D-arabopyranosyl] ester (4d)

Orange solid; yield 72%; mp 178 °C; ^{1}H NMR (C_5D_5N-d_5): δ 11.16 (s, 1H), 9.12 (s, 1H), 8.29 (d, 1H, *J* 9.0 Hz), 7.87–7.84 (m, 1H), 6.25 (d, 1H, *J* 7.8 Hz, Ara-1-H), 5.32 (s, 1H), 4.59 (dd, 1H, *J* 7.4, 6.2 Hz), 4.34–4.29 (m, 2H), 4.23 (dd, 1H, *J* 7.6, 3.4 Hz), 3.85 (dd, 1H, *J* 10.5, 1.6 Hz); ^{13}C NMR (C_5D_5N-d_5): δ 178.2, 169.2, 147.3, 145.7, 139.2, 131.7, 131.0, 122.5, 118.1, 97.8; ESI-MS (negative) m/z: 767.12 $[M+H]^+$. Anal. Calcd for $C_{41}H_{58}N_4O_{10}$: C, 64.21; H, 7.62; N, 7.31. Found: C, 62.99; H, 7.58; N, 7.34.

3.5.17. 12,13-Dihydrooleanolic acid 28-*O*-[β-D-galactopyranosyl] ester (5a)

White solid; yield 92.9%; mp 259–261 °C; ^{1}H NMR (C_5D_5N-d_5): δ 6.30 (d, 1H, J 8.5 Hz, Gal-1-H), 4.61–4.55 (m, 2H), 4.42 (dd, 1H, J 13.0, 6.4 Hz), 4.28 (dd, 1H, J 13.0, 6.6 Hz), 4.16–4.10 (m, 2H), 3.33–3.27 (m, 1H); ^{13}C NMR (C_5D_5N-d_5): δ 181.3, 98.2; MS (ESI) m/z: 643.16 $[M+Na]^+$. Anal. Calcd for $C_{36}H_{60}O_8$: C, 69.64; H, 9.74. Found: C, 69.93; H, 9.78.

3.5.18. 12,13-Dihydrooleanolic acid 28-*O*-[β-D-glucopyranosyl] ester (5b)

White solid; yield 90.2%; mp 249–251 °C; ^{1}H NMR (MeOD): δ 5.51 (d, 1H, J 8.1 Hz, Glc-1-H), 3.83 (d, 1H, J 12.1 Hz), 3.71 (dd, 1H, J 12.3, 2.3 Hz), 3.43–3.36 (m, 4H), 3.14 (dd, 1H, J 11.3, 5.5 Hz); ^{13}C NMR (MeOD): δ 185.2, 95.6; MS (ESI) m/z: 643.16 $[M+Na]^+$. Anal. Calcd for $C_{36}H_{60}O_8$: C, 69.64; H, 9.74. Found: C, 69.39; H, 9.69.

3.5.19. 12,13-Dihydrooleanolic acid 28-*O*-[β-D-xylopyranosyl] ester (5c)

White solid; yield 91.8%; mp 149–152 °C; ^{1}H NMR (C_5D_5N-d_5): δ 6.21 (d, 1H, J 7.5 Hz, Xyl-1-H), 4.24 (d, 1H, J 11.7 Hz), 4.10–3.90 (m, 3H), 3.72–3.69 (m, 1H), 3.30 (m, 1H); ^{13}C NMR (C_5D_5N-d_5): δ 179.2, 97.8; MS (ESI) m/z: 613.22 $[M+Na]^+$. Anal. Calcd for $C_{35}H_{58}O_7$: C, 71.15; H, 9.89. Found: C, 71.46; H, 9.92.

3.5.20. 12,13-Dihydrooleanolic acid 28-*O*-[β-D-arabopyranosyl] ester (5d)

White solid; yield 82.5%; mp 238–240 °C; ^{1}H NMR (C_5D_5N-d_5): δ 6.28 (d, 1H, J 6.1 Hz, Ara-1-H), 4.52 (dd, 1H, J 7.3, 6.5 Hz), 4.33–4.28 (m, 2H), 4.24 (dd, 1H, J 7.7, 3.1 Hz), 3.83 (dd, 1H, J 10.9, 1.3 Hz), 3.30 (t, 1H, J 7.8 Hz); ^{13}C NMR (C_5D_5N-d_5): δ 179.2, 97.8; MS (ESI) m/z: 613.22 $[M+Na]^+$. Anal. Calcd for $C_{35}H_{58}O_7$: C, 71.15; H, 9.89. Found: C, 70.87; H, 9.84.

3.6. General procedure for preparation of oleanolic acid glycoside derivatives 1e and 5e

To bromine glycoside (0.52 mmol) and oleanolic acid derivative (0.40 mmol) stirring in 22 mL CH_2Cl_2/H_2O (10:1) was added K_2CO_3 (138 mg) and Bu_4NBr (51.52 mg). The mixture was refluxed under nitrogen atmosphere. After completion (TLC) the reaction mixture was diluted with $CHCl_3$ and washed with water, then brine and dried. The crude was purified by recrystallization or column chromatography.

Deprotection of the compounds was conducted by following method. To the above compound (0.20 mmol) stirring in methanol was added sodium methoxide (cat.). The mixture was stirred at room temperature for 10 h. And then 20 mL water was added and the mixture was stirred at room temperature for 5 h. After completion (TLC) the reaction mixture was neutralized with HCl (1 M). Water was added and the resulting suspension was filtered. Crude products were purified by recrystallization to provided **1e** and **5e**, respectively.

3.6.1. Oleanolic acid 28-*O*-[β-D-glycuronic acid] ester (1e)

White solid; yield 66.8%; mp 194–196 °C; ^{1}H NMR ($CDCl_3$): δ 5.73 (d, 1H, J 6.8 Hz, GA-1-H), 5.27 (s, 1H), 3.59 (d, 1H, J 6.2 Hz), 3.43–3.23 (m, 3H), 3.13 (m, 1H), 2.80 (d, 1H, J 9.9 Hz); ^{13}C NMR (C_5D_5N-d_5): δ 178.1, 175.5, 145.8, 123.1, 97.9; MS (ESI) m/z: 631.64 $[M-H]^-$. Anal. Calcd for $C_{36}H_{56}O_9$: C, 68.33; H, 8.92. Found: C, 68.63; H, 8.99.

3.6.2. 12,13-Dihydrooleanolic acid 28-*O*-[β-D-glycuronic acid] ester (5e)

White solid; yield 71.3%; mp 200–202 °C; ^{1}H NMR ($CDCl_3$): δ 5.80 (d, 1H, J 7.4 Hz, GA-1-H), 3.49 (d, 1H, J 5.8 Hz), 3.22–3.16 (m, 3H), 3.10 (m, 1H); ^{13}C NMR (C_5D_5N-d_5): δ 178.2, 175.3, 98.4; MS (ESI) m/z: 633.23 $[M-H]^-$. Anal. Calcd for $C_{36}H_{58}O_9$: C, 68.11; H, 9.21. Found: C, 68.31; H, 9.28.

3.7. Formation of OCLs

A co-culture assay using mouse bone marrow cells and osteoblast-like cells was used for the evaluation of the effects of the compounds on OCLs formation.[27] Briefly, osteoblast-like cells were prepared from calvaria of 2-day-old ICR mice. Mouse bone marrow cells were obtained from tibiae of 6-week-old male ICR mouse. Osteoblast-like cells (1×10^4/well) and bone marrow cells (1×10^5/well) were co-cultured for 6 days with 1α, $25(OH)_2D_3$ (10^{-8} M) and each compound. The medium was replaced every 2 days. After 6 days, adherent cells on the well surface were fixed with 10% formalin-phosphate buffered saline (PBS, pH 7.2) for 10 min and dehydrated with ethanol–acetone (1:1, v/v) for 1 min. The cells were stained by tartrate-resistant acid phosphatase (TRAP) for 12 min at room temperature and TRAP-positive cells containing three or more nuclei were counted. The percentages of TRAP(+)-OCLs cultured with compounds were compared to that only with 1α, $25(OH)_2D_3$ group.

All procedures involving animals were approved by the Jiangsu Animal Care and Use Committee, China and followed the national and institutional rules considering animal experiments. Mice were housed in a climate-controlled room, 12 h light/dark photoperiod, water and food ad libitium.

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