Arch. Pharm. Res.
DOI 10.1007/s12272-016-0868-8

Archives of Pharmacal Research
www.springer.com/12272

RESEARCH ARTICLE

Synthesis of novel oleanolic acid and ursolic acid in C-28 position derivatives as potential anticancer agents

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Received: 3 July 2016 / Accepted: 28 November 2016

Abstract A series of nitrogen-containing derivatives of oleanolic acid and ursolic acid were prepared by a modification at C-28 position via esterification with 2-hydroxyacetic acid followed by amidation with amines, such as piperazine, *N*-methylpiperazine, and alkane-1, 2-diamines, alkane-1, 4-diamines, alkane-1, 6-diamines. In vitro antiproliferative activities of the compounds prepared towards MCF-7, Hela and A549 cell lines were evaluated by a MTT method to show that OA-5a, OA-5b, OA-5c and UA-5a showed somewhat improved antiproliferative activities against MCF-7, Hela and A549 cells comparing to that of the positive control, gefitinib.

Keywords Oleanolic acid · Ursolic acid · Antiproliferative activity · Structure–activity relationships

Electronic supplementary material The online version of this article (doi:10.1007/s12272-016-0868-8) contains supplementary material, which is available to authorized users.

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Introduction

Cancer is a widespread, complex, and lethal disease. Worldwide there were 14.1 million new cancer cases, 8.2 million deaths and 32.6 million people living with cancer (within 5 years of diagnosis) in 2012 (WHO Media Centre 2015). Chemotherapy is the most widely used cancer treatment, but is associated with serious side effects and other problems (Alam and Lee 2016). Despite efforts to develop small molecule anticancer drugs (Samadi et al. 2012; You et al. 2011; Dayyani et al. 2011; Hoelder et al. 2012) and drugs that target epidermal growth factor receptor mutations (Sharma et al. 2007; Ciardiello and Tortora 2008), their efficacy is limited. Thus, it is highly desirable to develop new anticancer agents with improved tumor selectivity, efficacy and safety.

A major advancement during the last decade has been the synthesis of derivatives of natural triterpenoids such as oleanolic acid (OA). A literature survey revealed that the activity of these pentacyclic triterpenoids is related to their basic triterpenoid skeletal structure, and the attached functional groups offer opportunities for chemical modification and for improvement of activity (Shanmugam et al. 2012; Zhao et al. 2007, 2014; Meng et al. 2012). For example, the OA derivative 2-cyano-3, 12-dioxo-oleana-1, 9(11)-dien-28-oic acid (CDDO, Fig. 1a), its C-28 methyl ester (CDDO-Me, Fig. 1b) and C-28 imidazolide (CDDO-Im, Fig. 1c) in clinical trials (Muthu et al. 2014) were more potent than the parent compound in terms of anti-inflammatory and antitumor activities (Suh et al. 1999; Honda et al. 2000). OA, a pentacyclic triterpenoid derived from tropical medicinal plants, has various bioactivities, such as antitumor (Liu 2005), anti-oxidant (Somova et al. 2003), anti-inflammatory (Zhang and Shen 2011), hepatoprotective (Sheng and Sun 2011) and hypoglycemic (Ali et al.

Published online: 18 January 2017

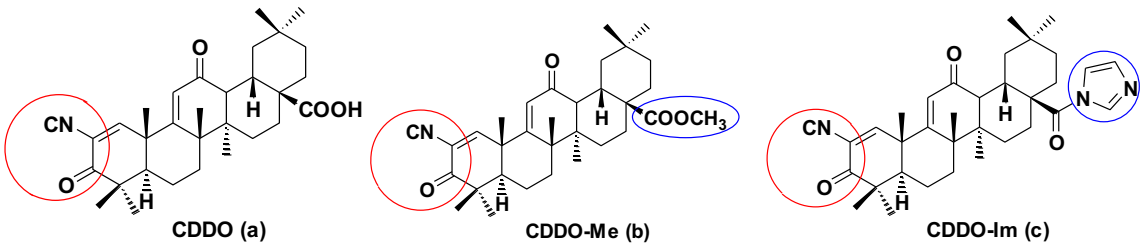


Fig. 1 Structures of several reported anticancer agents whose functional groups offer opportunities for chemical modification

2002) activities. Ursolic acid (UA), its isomer, has similar pharmacological properties (Shanmugam et al. 2013; Yadav et al. 2010), and both contain hydroxyl and carboxylic acid groups, as shown in Fig. 2. OA and UA have been shown to act at various stages of tumor development to inhibit tumor initiation and promotion, as well as to induce tumor cell differentiation and apoptosis (Tian et al. 2002; Zhang et al. 2008).

The low water solubility of OA and UA, however, results in low bioavailability. Efforts have been made to improve their water solubility by chemical modification. For instance, Ma and coworkers (Ma et al. 1999) found that introduction of a moiety at the C-3 position of OA, such as a carboxyl group, significantly improved the IC_{50} value. Piperazine derivatives also possess anticancer properties. Many currently notable anticancer agents, including imatinib (Shah et al. 2004) and dasatinib (Johnson et al. 2005) contain a piperazine ring as part of their molecular structure (Fig. 3). From previous studies in our group on pentacyclic triterpenoids, related analogs of OA and UA were shown to inhibit the growth of human non-small cell lung cancer (NSCLC) A549 and PC9/G (acquired resistance to gefitinib) cell lines in vitro (Chen et al. 2016; Zhao et al. 2015; Wang et al. 2013). Based on these results, we modified OA and UA by introduction of an acetyl group at the C-3 position, and attaching piperazine and *N*-methyl piperazine moieties at the C-28 position via a glycolic acid linker. A series of six OA and UA derivatives was designed as shown in Fig. 4.

Since antitumor activity was significantly increased after introducing piperazinyl moiety to C-28 position (Shah et al. 2004; Johnson et al. 2005), alternatively we selected straight-chain diamine instead of piperazine ring to expect improving anti-tumor activities. Ethylenediamine, butanediamine and hexanediamine were introduced at the C-28 position of OA and UA as shown in Fig. 4.

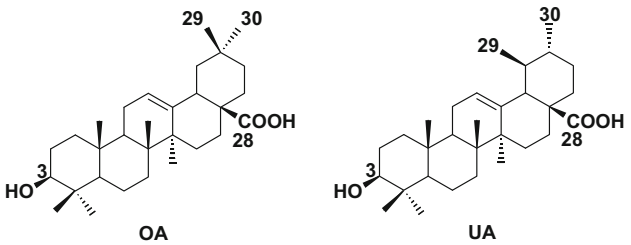


Fig. 2 Structure of OA and UA

Materials and methods

General

Synthesized compounds were purified on a silica gel column (200–300 mesh, Qingdao Marine Chemical Factory, China) using petroleum ether/ethyl acetate or methylene chloride/anhydrous methanol as eluent. Their structures were confirmed using nuclear magnetic resonance (NMR) on an AVANCE 500 MHz spectrometer (BRUKER, Switzerland) in $CDCl_3$. Chemical shifts are expressed in ppm and the coupling constants (J) in Hz. Infrared (IR) spectra were recorded in KBr pellets on a WGH-30 (Shanghai Yonggui Analysis Instrument co., Ltd.). Melting points were determined on an X-5 fiber melting point detector (temperature-controlled, Beijing Tektronix Instrument Co., Ltd.). Mass spectra were recorded on a GC-TOF high-resolution mass spectrometer (HR-MS) and an Agilent 6540 RRLC/Q-TOF (Agilent, Santa Clara, Ca, USA) MS. Most chemicals and solvents were purchased from commercial sources. Further purification and drying by standard methods were employed when necessary. All the reagents and chemicals were of analytical grade or chemically pure. The synthetic routes are presented in Schemes 1 and 2.

Chemistry

Synthesis and characterization of oleanolic acid derivatives

3β-Acetoxyolean-12-ene-28-oic acid (OA-1)

A solution of OA (3.0 g, 6.57 mmol) and DMAP (963.2 mg, 7.88 mmol) dissolved in anhydrous THF, stirring 3 h at room temperature, then adding acetic anhydride

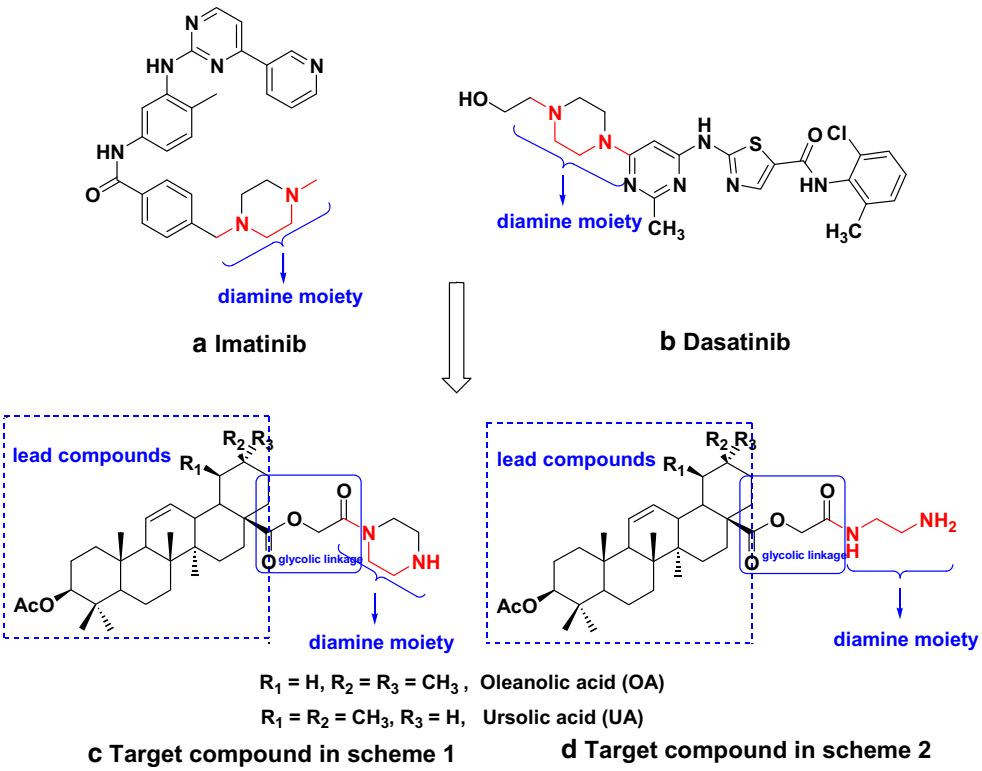


Fig. 3 The representative diamine moiety compounds

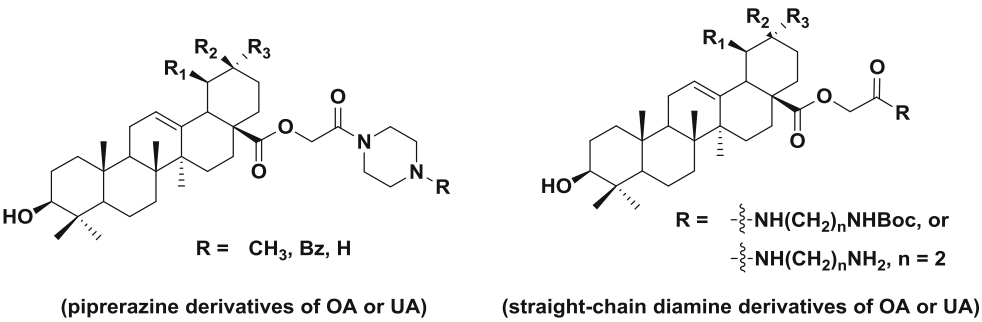
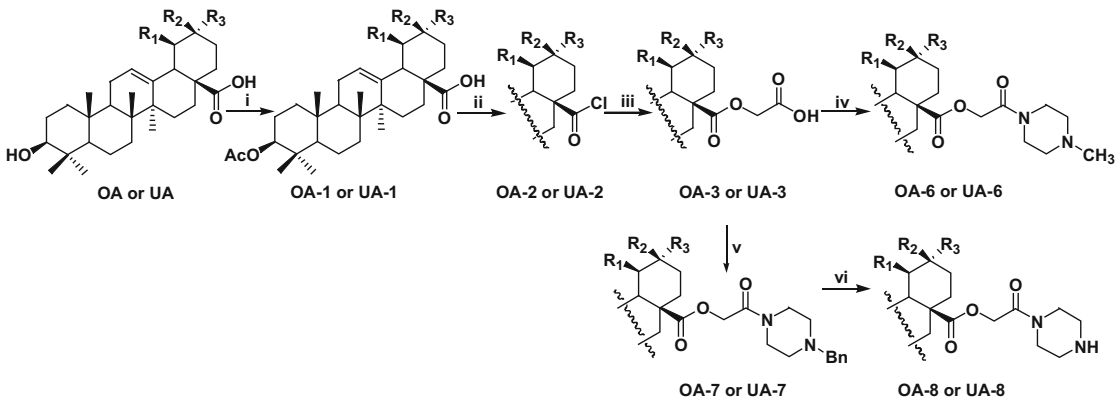


Fig. 4 Strategy for design of OA and UA derivatives

(1.6 mL, 9.71 mmol) to mixture, stirring 2 h at room temperature. The solvent was evaporated under reduced pressure to remove THF. The residue was extracted with ethyl acetate and water, washing to neutral with saturated NaCl solution. The organic layer was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which was purified by silica gel chromatography with a gradient elution of ethyl acetate-petroleum ether (1:10, v/v) to yield a white solid (2.42 g, 73%), m.p.259.5–261.5 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.27 (s, 1H, H-12), 4.49 (t, J = 6.8 Hz, 1H, H-3), 2.81 (dd, J_1 = 13.7 Hz, J_2 = 4.2 Hz, 1H, H-18), 2.04 (s, 3H, CH_3CO–), 1.13, 0.94, 0.93, 0.90, 0.87, 0.85, 0.75 (each s,3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 184.22, 171.01, 143.59, 122.54, 80.93, 55.30, 47.55, 46.54, 45.83, 41.54, 40.91, 39.29, 38.07, 37.68, 36.99, 33.79, 33.04, 32.53, 32.44, 30.64, 28.03, 27.66, 25.89, 23.57, 23.51, 23.38, 22.86, 21.26, 18.17, 17.14, 16.64, 15.36; IR (KBr) ν_{max} 2932, 2861 (CH), 1724(C=O), 1256 (C–O–C) cm^{-1}.

Acetic acid 3β-(olean-12-ene-28-chlorocarbonyl) ester (OA-2) and 2-(3β-Acetoxyolean-12-ene- 28-acetoxy) acetic acid (OA-3)

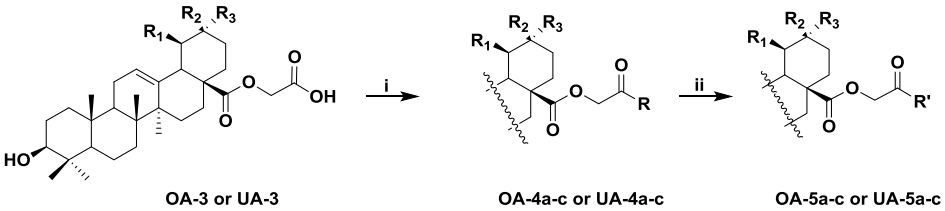
To a stirred solution of **OA-1** (4.0 g, 8.02 mmol) and oxalyl chloride (2.7 mL, 32.08 mmol) in anhydrous methylene chloride, the reaction mixture was stirred overnight at room temperature. The solvent was evaporated under reduced pressure to remove methylene chloride, gave light yellow solid (**OA-2**), which was used directly in subsequent reaction without further purification due to its hydroscopic property. A solution of glycolic acid (1.2 g, 16.04 mmol) and TEA (11.2 mL, 80.21 mmol) in anhydrous THF was stirred 7 h at room temperature. Reduced pressure



Reagents and conditions: i) Ac_2O, DMAP, THF, rt; ii) $(COCl)_2$, CH_2Cl_2, rt; iii) $HOCH_2COOH$, TEA, rt; iv) *N*-methyl piperazine, EDCI, DMAP, CH_2Cl_2, 0 °C to rt; v) Benzylpiperazine, EDCI, DMAP, CH_2Cl_2, 0 °C to rt; vi) 10 % Pd/C, H_2, anhydrous ethanol, rt.

Scheme 1 Synthesis of OA and UA Piperazine Derivatives

Scheme 2 Synthesis of OA and UA Diamine Derivatives



R = -NH(CH$_2$)$_n$NHBoc, n = 2, OA-4a or UA-4a
R' = -NH(CH$_2$)$_n$NH$_2$, n = 2, OA-5a or UA-5a
R = -NH(CH$_2$)$_n$NHBoc, n = 4, OA-4b or UA-4b
R' = -NH(CH$_2$)$_n$NH$_2$, n = 4, OA-5b or UA-5b
R = -NH(CH$_2$)$_n$NHBoc, n = 6, OA-4c or UA-4c
R' = -NH(CH$_2$)$_n$NH$_2$, n = 6, OA-5c or UA-5c

Reagents and conditions: i) *N*-Boc-Diamine, EDCI, DMAP, CH_2Cl_2, 0 °C to rt; ii) TFA, CH_2Cl_2, 0 °C to rt.

distillation to remove the solvent, the reaction mixture was extracted with ethyl acetate and water, washing to neutral with saturated NaCl solution. The organic layer was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which was purified by silica gel chromatography with a gradient elution of ethyl acetate-petroleum ether (1:5, v/v) to yield a white solid (1.37 g, 31%), m.p.244.5–258.7 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.29 (t, J = 3.5 Hz, 1H, H-12), 4.64 (d, J = 16.3 Hz, 1H, –COOCH$_2$COOH), 4.49 (dd, J_1 = 10.0 Hz, J_2 = 8.2 Hz, 1H, H-3), 2.87 (dd, J_1 = 13.7 Hz, J_2 = 4.3 Hz, 1H, H-18), 2.04 (s, 3H, CH_3-CO–), 0.92 (s, 6H, CH_3–), 1.13, 0.90, 0.86, 0.72 (each s,3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 176.96, 171.93, 171.10, 143.49, 122.52, 80.99, 60.72, 59.93, 55.31, 47.55, 46.81, 45.90, 41.75, 41.28, 39.35, 38.15, 37.69, 36.93, 33.85, 33.02, 32.69, 32.19, 30.65, 28.04, 27.59, 25.75, 23.60, 23.41, 23.11, 21.27, 18.22, 16.89, 16.68, 15.38; IR (KBr) ν_{max} 2944, 2866 (CH), 1734(C=O), 1248 (C–O–C) cm^{-1}.

3β-Acetoxyolean-12-ene-28-oic acid [1-(2-N-Boc-amino-ethylamino)-1-oxo]ethyl ester (OA-4a)

To a stirred solution of **OA-3** (250.12 mg, 0.45 mmol), *N*-Boc-ethylenediamine (86.29 mg, 0.54 mmol) and DMAP (65.85 mg, 0.54 mmol) in anhydrous methylene chloride was added EDCI (344.23 mg, 1.80 mmol) which dissolved in anhydrous methylene chloride at 0 °C. The reaction mixture was stirred for 5 min, and then at room temperature for 3 h. The reaction mixture was extracted with methylene chloride and water, washing to neutral with saturated NaCl solution. The organic layer was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which was purified by silica gel chromatography with a gradient elution of

ethyl acetate-petroleum ether (1:2, v/v) to yield a white solid (244.02 mg, 78%), m.p.200.4–200.7 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.68 (s, 1H, –CONH–), 5.34 (s, 1H, H-12), 4.86 (s, 1H, –NH-Boc), 4.66 (d, J = 15.6 Hz, 1H, –OCH_2CO–), 4.50–4.47 (m, 1H, H-3), 4.35 (d, J = 15.6 Hz, 1H, –OCH_2CO–), 3.53–3.49 & 3.35–3.25 (m, 4H, –$NHCH_2CH_2NH$–), 2.88 (dd, J_1 = 13.7 Hz, J_2 = 3.5 Hz, 1H, H-18), 2.04 (s, 3H, CH_3CO–), 1.44 (s, 9H, –$C(CH_3)_3$), 1.15, 0.95, 0.89, 0.88, 0.86, 0.85, 0.69 (each s,3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 176.19, 170.92, 168.00, 156.47, 144.38, 122.29, 80.81, 79.61, 62.81, 55.26, 47.43, 46.94, 45.72, 41.78, 41.48, 40.36, 40.01, 39.27, 38.06, 37.64, 36.88, 33.72, 32.97, 32.54, 32.25, 30.62, 29.63, 28.30, 27.99, 27.54, 26.87, 25.83, 23.60, 23.44, 23.37, 23.13, 21.22, 18.12, 16.89, 16.62, 15.31; IR (KBr) ν_{max} 3428 (NH), 2932, 2861 (CH), 1734, 1681 (C=O), 1253 (C–O–C) cm^{-1}. HR-MS calcd for $C_{41}H_{66}N_2O_7$ $[M+Na]^+$ 721.4870, found 721.4879.

3β-Acetoxyolean-12-ene-28-oic acid [1-(2-amino-ethylamino)-1-oxo]ethyl ester (OA-5a)

To a stirred solution of **OA-4a** (200.12 mg, 0.29 mmol) in anhydrous methylene chloride was added TFA (0.85 mL, 11.45 mmol) at 0 °C. The reaction mixture was stirred for 2 h at room temperature. After the reaction, with 0.1 mol L^{-1} $NH_3{\cdot}H_2O$ adjust pH to 9 under the ice water bath. The reaction mixture was extracted with methylene chloride and water. The organic layer was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which was purified by silica gel chromatography with a gradient elution of methylene chloride-anhydrous methanol (5:1 v/v) to yield a white solid (131.26 mg, 77%), m.p.134.9–135.4 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.07 (t, J = 5.6 Hz, 1H, –CONH–), 5.30–5.29 (m, 1H, H-12), 4.62 (d, J = 15.5 Hz, 1H, –OCH_2-CO–), 4.50–4.47 (m, 1H, H-3), 4.42 (d, J = 15.5 Hz, 1H, –OCH_2CO–), 3.58–3.46 (m, 4H, –$NHCH_2CH_2NH_2$), 3.08(s, 2H, –NH_2), 2.84 (dd, J_1 = 13.5 Hz, J_2 = 3.7 Hz, 1H, H-18), 2.04 (s, 3H, CH_3CO–), 1.14, 0.92, 0.91, 0.91, 0.86, 0.85, 0.69 (each s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 176.67, 170.97, 169.65, 144.29, 122.24, 80.82, 62.62, 55.28, 47.44, 46.97, 45.76, 41.82, 41.45, 40.35, 39.29, 38.42, 38.11, 37.67, 36.89, 33.73, 32.99, 32.59, 32.27, 30.61, 28.03, 27.55, 25.82, 23.53, 23.42, 23.17, 21.26, 18.16, 16.85, 16.67, 15.32; IR (KBr) ν_{max} 3429 (NH), 2949, 2869 (CH), 1736, 1680 (C=O), 1254 (C–O–C) cm^{-1}. HR-MS calcd for $C_{36}H_{58}N_2O_5$ $[M+H]^+$ 599.4346, found 599.4351.

3β-Acetoxyolean-12-ene-28-oic acid [1-(4-N-Boc-amino-butylamino)-1-oxo]ethyl ester (OA- 4b)

The **OA-3** (250.03 mg, 0.45 mmol) was connected with *N*-Boc-1, 4-diaminobutane hydrochloride (121.13 mg, 0.54 mmol) according to the same procedure used in the preparation of **OA-4a**, to give **OA-4b** (276.6 mg, 85%) as a white solid: m.p.112.8–113.4 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.23 (s, 1H, –CONH–), 5.29 (t, J = 3.2 Hz, 1H, H-12), 4.63 (d, J = 15.5 Hz, 1H, –OCH_2CO–), 4.47 (s, 1H, –NH-Boc), 4.50–4.47 (m, 1H, H-3), 4.37 (d, J = 15.5 Hz, 1H, –OCH_2CO–), 3.39–3.34 & 3.28–3.21 (m, 2H, –$NHCH_2$-$CH_2CH_2CH_2NH$–), 3.14 (d, J = 5.7 Hz, 2H, –$NHCH_2$-$CH_2CH_2CH_2$– NH–), 2.85 (dd, J_1 = 13.6 Hz, J_2 = 4.1 Hz, 1H, H-18), 2.06 (s, 3H, CH_3CO–), 1.44 (s, 9H, –$C(CH_3)_3$), 1.15, 0.95, 0.94, 0.92, 0.86, 0.85, 0.68 (each s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 176.12, 170.97, 167.41, 155.96, 144.65, 122.20, 80.81, 62.96, 55.28, 47.44, 47.00, 45.72, 41.85, 41.61, 39.31, 38.85, 38.10, 37.67, 36.90, 33.69, 32.95, 32.53, 32.46, 30.65, 28.41, 28.02, 27.51, 27.00, 26.90, 25.88, 23.57, 23.47, 23.40, 23.28, 21.26, 18.14, 16.93, 16.65, 15.32; IR (KBr) ν_{max} 3421 (NH), 2940, 2869 (CH), 1734 (C=O), 1254 (C–O–C) cm^{-1}. HR-MS calcd for $C_{43}H_{70}N_2O_7$ $[M+H]^+$ 727.5183, found 727.5192.

3β-Acetoxyolean-12-ene-28-oic acid [1-(4-amino-butylamino)-1-oxo]ethyl ester (OA-5b)

The **OA-4b** (200.00 mg, 0.27 mmol) was reacted with TFA (0.82 mL, 11.00 mmol) according to the same procedure used in the preparation of **OA-5a**, to give **OA-5b** (121.89 mg, 71%) as a white solid: m.p.106.9–107.7 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.40 (s, 1H, –CONH–), 5.29 (t, J = 3.3 Hz, 1H, H-12), 4.64 (d, J = 15.5 Hz, 1H, –OCH_2CO–), 4.50–4.47 (m, 1H, H-3), 4.37 (d, J = 15.5 Hz, 1H, –OCH_2CO–), 3.39–3.23 (m, 4H, –$NHCH_2CH_2CH_2$-CH_2NH_2), 2.86 (d, J = 4.0 Hz,1H, H-18), 2.83–2.81 (m, 2H, –NH_2), 2.04 (s, 3H, CH_3CO–), 1.15, 0.94, 0.92, 0.91, 0.86, 0.85, 0.69 (each s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ:176.23, 170.79, 167.66, 144.63, 122.18, 80.80, 62.87, 55.28, 47.44, 46.99, 45.75, 41.85, 41.57, 40.83, 39.31, 38.79, 38.10, 37.67, 36.90, 33.71, 32.96, 32.54, 32.44, 30.65, 29.67, 28.02, 27.51, 26.90, 25.87, 23.58, 23.47, 23.41, 23.26, 21.26, 18.14, 16.93, 16.66, 15.32; IR (KBr) ν_{max} 3429 (NH), 2941, 2870 (CH), 1735, 1672 (C=O), 1247 (C–O–C) cm^{-1}. HR-MS calcd for $C_{38}H_{62}N_2O_5$ $[M+H]^+$627.4659, found 627.4662.

3β-Acetoxyolean-12-ene-28-oic acid [1-(6-N-Boc-amino-hexylamino)-1-oxo]ethyl ester (OA-4c)

The **OA-3** (267.29 mg, 0.48 mmol) was connected with *N*-Boc-1, 6-hexanediamine hydrochloride (145.62 mg, 0.57 mmol) according to the same procedure used in the preparation of **OA-4a**, to give **OA-4c** (215.33 mg, 60%) as a white solid: m.p.90.3–90.7 °C; ^{1}H-NMR (500 MHz, $CDCl_3$)δ: 6.20(s, 1H, –CONH–), 5.29–5.28 (m, 1H, H-12), 4.63 (d, J = 15.5 Hz, 1H, –OCH_2 CO–), 4.50–4.47 (m, 2H,

H-3 × 1 & –NH-Boc × 1), 4.37 (d, $J = 15.5$ Hz, 1H, –OCH_2CO–), 3.38–3.31 & 3.26–3.19 (m, 2H, –$NHCH_2CH_2$-$CH_2CH_2CH_2CH_2NH$–), 3.10 (d, $J = 6.3$ Hz, 2H, –$NHCH_2$–$CH_2CH_2CH_2CH_2CH_2NH$–), 2.85 (dd, $J_1 = 13.6$ Hz, $J_2 = 4.0$ Hz, 1H, H-18), 2.04 (s, 3H, CH_3CO–), 1.44 (s, 9H, –$C(CH_3)_3$), 1.15, 0.95, 0.94, 0.92, 0.86, 0.85, 0.68 (each s,3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 176.09, 170.95, 167.27, 155.96, 144.64, 122.18, 80.79, 79.04, 62.96, 55.26, 47.43, 46.98, 45.73, 41.83, 41.59, 40.38, 39.30, 38.99, 38.09, 37.66, 36.89, 33.68, 32.94, 32.50, 32.45, 30.64, 29.98, 29.55, 28.40, 28.01, 27.48, 26.89, 26.47, 26.31, 25.88, 23.56, 23.46, 23.38, 23.26, 21.24, 18.13, 16.91, 16.65, 15.31; IR (KBr) ν_{max} 3414 (NH), 2973, 2931 (CH), 1736, 1671 (C=O), 1253 (C–O–C) cm^{-1}. HR-MS calcd for $C_{45}H_{74}N_2O_7$ $[M+H]^+$ 755.5496, found 755.5503.

3β-Acetoxyolean-12-ene-28-oic acid [1-(6-amino-hexylamino)-1-oxo]ethyl ester (OA-5c)

The **OA-4c** (141.20 mg, 0.19 mmol) was reacted with TFA (0.56 mL, 7.49 mmol) according to the same procedure used in the preparation of **OA-5a**, to give **OA-5c** (58.2 mg, 79%) as a white solid: m.p.103.7–104.2 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.25 (d, $J = 5.4$ Hz, 1H, –CONH–), 5.30–5.29 (m, 1H, H-12), 4.63 (d, $J = 15.6$ Hz, 1H, –OCH_2CO–), 4.50–4.47 (m, 1H, H-3), 4.36 (d, $J = 15.6$ Hz, 1H, –OCH_2CO–), 3.38–3.31 (m, 2H, –$NHCH_2CH_2CH_2$-$CH_2CH_2CH_2NH_2$), 3.26–3.21 (m, 2H, –$NHCH_2CH_2CH_2$-$CH_2CH_2CH_2NH_2$), 2.86 (d, $J = 4.0$ Hz, 1H, H-18), 2.83–2.81 (m, 2H, –NH_2), 2.04 (s, 3H, CH_3CO–), 1.15, 0.94, 0.92, 0.91, 0.86, 0.85, 0.69 (each s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 176.15, 170.98, 167.53, 144.66, 122.20, 80.81, 62.89, 55.27, 47.44, 47.00, 45.74, 41.85, 41.59, 39.31, 38.92, 38.10, 37.67, 36.90, 33.69, 32.96, 32.52, 32.46, 30.65, 29.37, 28.02, 27.49, 26.27, 25.96, 25.88, 23.56, 23.47, 23.39, 23.27, 21.26, 18.14, 16.92, 16.66, 15.31; IR (KBr) ν_{max} 3437 (NH), 2940, 2862 (CH), 1735, 1672 (C=O), 1254 (C–O–C) cm^{-1}. HR-MS calcd for $C_{40}H_{66}N_2O_5$ $[M+H]^+$ 655.4972, found 655.4979.

3β-Acetoxyolean-12-ene-28-oic acid [1-(4-methyl-1-piperazinyl)-1-oxo]ethyl ester (OA-6)

The **OA-3** (100.0 mg, 0.2 mmol) was connected with *N*-methyl piperazine (0.1 mmL, 0.2 mmol) according to the same procedure used in the preparation of **OA-4a**, to give **OA-6** (93.0 mg, 81%) as a white solid: m.p.88.5–89.8 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.28(t, $J = 3.5$, 1H, H-12), 4.70(d, $J = 14.0$, 1H, –OCH_2CO–), 4.62(d, $J = 14.0$, 1H, –OCH_2CO–), 4.50–4.47(m, 1H, H-3), 2.87–2.89(m, 1H, H-18), 3.61–2.39 (m, 8H, piperazineH-2 × 2 & H-3 × 2 & H-5 × 2 & H-6 × 2), 2.30 (s, 3H, –N–CH_3), 2.04 (s, 3H, CH_3CO–), 1.13, 0.93, 0.92, 0.90, 0.86, 0.85, 0.84 (s, each 3H). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 177.06, 170.91, 165.18, 143.68, 122.30, 80.87, 60.90, 55.28, 54.79, 54.40, 47.52, 46.86, 45.90, 45.86, 44.38, 41.69, 41.27, 39.33, 38.10, 37.66, 36.91, 33.83, 33.03, 32.61, 32.32, 30.65, 29.01, 28.01, 27.62, 25.78, 23.66, 23.49, 23.04, 21.24, 18.20, 16.96, 16.65, 15.35; IR (KBr) ν_{max} 2939, 2869 (CH), 1735, 1681 (C=O), 1241 (C–O–C) cm^{-1}. HR-MS calcd for $C_{39}H_{62}N_2O_5$ $[M+H]^+$ 639.4659, found 639.4667.

3β-Acetoxyolean-12-ene-28-oic acid [1-(4-benzyl-1-piperazinyl)-1-oxo]ethyl ester (OA-7)

The **OA-3** (226.6 mg, 0.4 mmol) was connected with *N*-benzylpiperazine (70.50 mg, 0.4 mmol) according to the same procedure used in the preparation of **OA-4a**, to give **OA-7** (198.1 mg, 76%) as a white solid: m.p.81.5–82.3 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.73–7.19 (m, 5H, Ph-), 5.22 (t, $J = 3.4$, 1H, H-12), 4.64–4.55 (m, 2H, –OCH_2CO–), 4.43 (m, 1H, H-3), 3.55–2.38 (m, 8H, piperazineH-2 × 2 & H-3 × 2 & H-5 × 2 & H-6 × 2), 3.45 (s, 2H, $PhCH_2$–), 2.83 (dd, $J_1 = 13.7$, $J_2 = 4.2$, 1H, H-18), 1.98 (s, 3H, CH_3CO–), 1.07, 0.87, 0.86, 0.84, 0.80, 0.79, 0.66 (s, each 3H). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 177.07, 170.95, 165.14, 143.72, 137.51, 129.06, 128.34, 127.30, 122.31, 80.90, 62.84, 61.00, 55.30, 52.89, 52.55, 47.54, 46.88, 45.93, 44.66, 41.89, 41.70, 41.28, 39.35, 38.12, 37.68, 36.93, 33.85, 33.05, 32.63, 32.35, 30.57, 28.03, 27.64, 25.80, 23.69, 23.51, 23.40, 23.05, 21.26, 18.21, 16.98, 16.66, 15.36; IR (KBr) ν_{max} 2946, 2877 (CH), 1734, 1679 (C=O), 1254 (C–O–C) cm^{-1}. HR-MS calcd for $C_{45}H_{66}N_2O_5$ $[M+H]^+$ 715.4972, found 715.4977.

3β-Acetoxyolean-12-ene-28-oic acid [1-(1-piperazinyl)-1-oxo]ethyl ester (OA-8)

A solution of **OA-7** (160.0 mg, 0.2 mmol) dissolved in anhydrous ethanol (20 mL), adding 10% Pd/C (25.0 mg) under the stirring. Stirring in the hydrogen atmosphere for 24 h. Filtration and evaporation of solvent at reduced pressure gave light yellow solid, which was purified by silica gel chromatography with a gradient elution of methylene chloride-anhydrous methanol (10:1, v/v) to yield a white solid (102.0 mg, 73%), m.p.68.5–69.1 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.28 (t, $J = 3.5$, 1H, H-12), 4.72–4.62 (m, 2H, –OCH_2CO–), 4.49 (m, 1H, H-3), 3.63–2.41 (m, 8H, piperazineH-2 × 2 & H-3 × 2 & H-5 × 2 & H-6 × 2), 2.90 (dd, $J_1 = 13.9$, $J_2 = 4.4$, 1H, H-18), 2.05(s, 3H, CH_3CO–), 1.14, 0.93, 0.92, 0.88, 0.87, 0.86, 0.74(s, each 3H). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 177.06, 170.94, 165.11, 143.71, 122.30, 80.90, 60.97, 55.29, 52.72, 52.18, 47.54, 46.87, 45.92, 44.60, 41.81, 41.70,41.28, 39.34, 38.12, 37.67, 36.92, 33.85, 33.04, 32.63, 32.34, 30.66, 28.02, 27.63, 25.79, 23.68, 23.51,

23.40, 23.05, 21.25, 18.21, 16.97, 16.66, 15.35; IR (KBr) ν_{max} 2939, 2873 (CH), 1735, 1667 (C=O), 1241 (C–O–C) cm^{-1}. HR-MS calcd for $C_{38}H_{60}N_2O_5$ $[M+H]^+$ 625.4502, found 625.4507.

Synthesis and characterization of ursolic acid derivatives

3β-Acetoxyurs-12-ene-28-oic acid (UA-1), Yield: 72%, m.p.186.2–187.0 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.26 (t, $J = 3.5$ Hz, 1H, H-12), 4.49 (m, 1H, H-3), 2.18 (d, $J = 11.6$ Hz, 1H, H-18), 2.04 (s, 3H, CH_3CO–), 1.07, 0.96, 0.95, 0.88, 0.86, 0.85, 0.77 (s, each 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 184.35, 171.00, 143.60, 122.54, 80.93, 55.30, 47.55, 46.54, 45.83, 41.53, 40.90, 39.28, 38.06, 37.68, 36.99, 33.79, 33.04, 32.53, 32.44, 30.64, 28.03, 27.66, 25.89, 23.57, 23.51, 23.38, 22.85, 21.26, 18.16, 17.17, 16.64, 15.36; IR (KBr) ν_{max}: 2927, 2869 (CH), 1741, 1689 (C=O), 1241 (C–O–C) cm^{-1}. **Acetic acid 3β-(urs-12-ene-28 -chlorocarbonyl) ester (UA-2) and 2-(3β-Acetoxyurs-12-ene-28-acetoxy) acetic acid (UA-3)**, Yield: 29%, m.p.122.1–122.7 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.26 (t, $J = 3.5$, 1H, H-12), 4.58 (d, $J = 6.0$, 2H, –$\underline{CH_2}$COOH), 4.51–4.48 (m, 1H, H-3), 2.25 (d, $J = 11.3$, 1H, H-18), 2.05 (s, 3H, $\underline{CH_3}$CO–), 1.08, 0.96, 0.94, 0.87, 0.86, 0.85, 0.74 (s, each 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 176.71, 171.87, 171.07, 137.97, 125.75, 80.97, 59.86, 55.31, 52.78, 48.23, 47.50, 42.10, 39.57, 39.08, 38.79, 38.33, 37.68, 36.87, 36.42, 32.97, 30.62, 28.07, 27.97, 26.91, 24.24, 23.55, 23.44, 23.30, 21.27, 21.14, 18.20, 17.01, 16.72, 15.51; IR (KBr) ν_{max}: 2927, 2863 (CH), 1730 (C=O), 1247 (C–O–C) cm^{-1}. Procedures reference the OA ones.

3β-Acetoxyurs-12-ene-28-oic acid [1-(2-N-Boc-amino-ethylamino)-1-oxo]ethyl ester (UA-4a)

The **UA-3** (200.03 mg, 0.36 mmol) was connected with *N*-Boc-ethylenediamine (69.06 mg, 0.43 mmol) according to the same procedure used in the preparation of **OA-4a**, to give **UA-4a** (142.7 mg, 85%) as a white solid: m.p.119.2–119.8 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.69 (s, 1H, –CONH–), 5.31 (s, 1H, H-12), 4.87 (s, 1H, –N$\underline{H}$-Boc), 4.67 (d, $J = 15.7$ Hz, 1H, –OCH_2CO–), 4.50–4.47 (m, 1H, H-3), 4.31 (d, $J = 15.7$ Hz, 1H, –OCH_2CO–), 3.53–3.50 & 3.33–3.24 (m, 4H, –NH$\underline{CH_2CH_2}$NH–), 2.27 (d, $J = 11.0$ Hz, 1H, H-18), 2.04 (s, 3H, CH_3CO–), 1.44 (s, 9H,–$C(CH_3)_3$), 1.09, 0.95, 0.93, 0.88, 0.85, 0.83, 0.70 (each s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 176.03, 170.95, 168.14, 156.49, 139.23, 125.33, 80.84, 79.63, 62.78, 55.29, 52.84, 48.36, 47.39, 42.16, 40.35, 40.06, 39.50, 39.12, 38.76, 38.23, 37.66, 36.85, 36.57, 32.81, 30.53, 28.44, 28.33, 28.05, 27.68, 26.90, 24.33, 23.57, 23.50, 23.25, 21.25, 21.08, 18.14, 17.01, 16.97, 16.69, 15.46; IR (KBr) ν_{max} 3421 (NH), 2933, 2870 (CH), 1737, 1686 (C=O), 1246 (C–O–C) cm^{-1}. HR-MS calcd for $C_{41}H_{66}N_2O_7$ $[M+H]^+$ 721.4870, found 721.4877.

3β-Acetoxyurs-12-ene-28-oic acid [1-(2-amino-ethylamino)-1-oxo]ethyl ester (UA-5a)

The **UA-4a** (102.91 mg, 0.15 mmol) was reacted with TFA (0.44 mL, 5.89 mmol) according to the same procedure used in the preparation of **OA-5a**, to give **UA-5a** (63.4 mg, 72%) as a white solid: m.p.104.2–104.7 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.84 (d, $J = 5.3$ Hz, 1H, –CONH–), 5.28 (s, 1H, H-12), 4.63 (d, $J = 15.5$ Hz, 1H, –OCH_2CO–), 4.50–4.47 (m, 1H, H-3), 4.39 (d, $J = 15.5$ Hz, 1H, –OCH_2CO–), 3.66–3.50 (m, 2H, –NHC$\underline{H_2}CH_2NH_2$), 3.49–3.36 (m, 2H, –$NHCH_2\underline{CH_2}NH_2$), 2.97 (d, $J = 5.0$ Hz, 2H, –NH_2), 2.24 (d, $J = 11.2$ Hz, 1H, H-18), 2.04 (s, 3H, CH_3CO–), 1.09, 0.96, 0.93, 0.88, 0.86, 0.85, 0.70 (each s,3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 176.24, 170.96, 168.48, 139.03, 125.34, 80.83, 62.80, 55.29, 52.91, 48.36, 47.39, 42.15, 40.83, 40.18, 39.51, 39.09, 38.80, 38.27, 37.67, 36.85, 36.65, 32.82, 30.53, 28.06, 27.88, 26.90, 24.37, 23.54, 23.28, 21.26, 21.09, 18.15, 17.02, 16.95, 16.70, 15.47; IR (KBr) ν_{max} 3429 (NH), 2933, 2865 (CH), 1735, 1680 (C=O), 1246 (C–O–C) cm^{-1}. HR-MS calcd for $C_{36}H_{58}N_2O_5$ $[M+H]^+$ 599.4346, found 599.4353.

3β-Acetoxyurs-12-ene-28-oic acid [1-(4-N-Boc-amino-butylamino)-1-oxo]ethyl ester (UA- 4b)

The **UA-3** (250.11 mg, 0.36 mmol) was connected with *N*-Boc-1, 4-diaminobutane hydrochloride (119.81 mg, 0.54 mmol) according to the same procedure used in the preparation of **OA-4a**, to give **UA-4b** (232.5 mg, 71%) as a white solid: m.p.99.7–100.4 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.23 (s, 1H, –CONH–), 5.24 (s, 1H, H-12), 4.65 (d, $J = 15.5$ Hz, 1H, –OCH_2CO–), 4.59 (s, 1H, –N$\underline{H}$-Boc), 4.50–4.47 (m, 1H, H-3), 4.30 (d, $J = 15.5$ Hz, 1H, –OCH_2CO–), 3.39–3.34 & 3.27–3.20 (m, 2H, –NH$\underline{CH_2}$-CH_2CH_2 CH_2NH–), 3.13 (d, $J = 6.0$ Hz, 2H, –$NHCH_2$-$CH_2CH_2\underline{CH_2}$NH–), 2.21 (d, $J = 11.2$ Hz, 1H, H-18), 2.03 (s, 3H, CH_3CO–), 1.43 (s, 9H, –$C(CH_3)_3$), 1.09, 0.96, 0.92, 0.86, 0.85, 0.83, 0.69 (each s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 175.89, 170.94, 167.47, 155.96, 139.47, 125.14, 80.78, 79.17, 62.87, 55.25, 53.00, 48.36, 47.35, 42.18, 40.11, 39.48, 39.09, 38.81, 38.23, 37.64, 36.82, 36.71, 32.76, 30.47, 28.38, 28.03, 27.79, 27.49, 27.01, 24.42, 23.55, 23.48, 23.22, 21.24, 21.05, 18.10, 17.03, 16.94, 16.68, 15.42; IR (KBr) ν_{max} 3437 (NH), 2932, 2861 (CH), 1734, 1648 (C=O), 1246 (C–O–C) cm^{-1}. HR-MS calcd for $C_{43}H_{70}N_2O_7$ $[M+H]^+$ 727.5183, found 727.5191.

3β-Acetoxyurs-12-ene-28-oic acid [1-(4-amino-butylamino)-1-oxo]ethyl ester (UA-5b)

The **UA-4b** (121.60 mg, 0.17 mmol) was reacted with TFA (0.50 mL, 6.69 mmol) according to the same procedure used in the preparation of **OA-5a**, to give **UA-5b** (84.7 mg, 81%) as a white solid: m.p.106.3–106.6 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.42–6.40 (m, 1H, –CONH–), 5.25–5.24 (m, 1H, H-12), 4.66 (d, $J = 15.5$ Hz, 1H, –OCH_2CO–), 4.50–4.47 (m, 1H, H-3), 4.31 (d, $J = 15.5$ Hz, 1H, –OCH_2CO–), 3.58–3.52 (m, 2H, –NH$\underline{CH_2}CH_2$ $CH_2CH_2NH_2$), 3.37–3.35 & 3.26–3.24 (m, 2H, –NH$CH_2CH_2CH_2\underline{CH_2}NH_2$), 2.84 (s, 2H, –$NH_2$), 2.22 (d, $J = 11.2$ Hz, 1H, H-18), 2.04 (s, 3H, CH_3CO–), 1.09, 0.96, 0.92, 0.88, 0.86, 0.85, 0.70 (each s,3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 176.01, 170.95, 167.80, 139.45, 125.15, 80.79, 62.78, 55.26, 52.95, 48.37, 47.36, 42.19, 40.687, 39.50, 39.10, 38.80, 38.73, 38.25, 37.65, 36.83, 36.69, 32.79, 30.49, 28.19, 28.04, 27.82, 26.90, 24.40, 23.54, 23.49, 23.24, 21.25, 21.07, 18.12, 17.02, 16.96, 16.69, 15.43; IR (KBr) ν_{max} 3429 (NH), 2933, 2870 (CH), 1734, 1672 (C=O), 1247 (C–O–C) cm^{-1}. HR-MS calcd for $C_{38}H_{62}N_2O_5$ $[M+H]^+$627.4659, found 627.4667.

3β-Acetoxyurs-12-ene-28-oic acid [1-(6-N-Boc-amino-hexylamino)-1-oxo]ethyl ester (UA-4c)

The **UA-3** (300.00 mg, 0.54 mmol) was connected with *N*-Boc-1, 6-hexanediamine hydrochloride (163.4 mg, 0.65 mmol) according to the same procedure used in the preparation of **OA-4a**, to give **UA-4c** (273.5 mg, 67%) as a white solid: m.p.90.6–91.0 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.19 (s, 1H, –CONH–), 5.25–5.24 (m, 1H, H-12), 4.65 (d, $J = 15.5$ Hz, 1H, –OCH_2CO–), 4.50–4.47 (m, 2H, H-3 × 1 & –N$\underline{H}$-Boc × 1), 4.31 (d, $J = 15.5$ Hz, 1H, –OCH_2CO–), 3.38–3.31 & 3.26–3.19 (m, 2H, –NH$\underline{CH_2}CH_2$-$CH_2CH_2CH_2CH_2$NH-), 3.10 (d, $J = 6.4$ Hz, 2H, –NHCH_2-$CH_2CH_2CH_2$– $CH_2\underline{CH_2}$NH–), 2.22 (d, $J = 11.2$ Hz, 1H, H-18), 2.04 (s, 3H, CH_3CO–), 1.46 (s, 9H, –$C(CH_3)_3$), 1.09, 0.97, 0.92, 0.88, 0.86, 0.85, 0.70 (each s,3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 175.87, 170.94, 167.35, 155.96, 139.47, 125.06, 80.79, 79.05, 62.92, 55.26, 53.03, 48.37, 47.37, 42.19, 40.39, 39.50, 39.10, 38.98, 38.85, 38.26, 37.65, 36.84, 36.73, 32.76, 30.48, 29.99, 29.59, 28.40, 27.80, 26.89, 26.47, 26.31, 24.44, 23.57, 23.49, 23.23, 21.24, 21.06, 18.12, 17.05, 16.94, 16.70, 15.44; IR (KBr) ν_{max} 3398 (NH), 2941, 2862 (CH), 1737 (C=O), 1246 (C–O–C) cm^{-1}. HR-MS calcd for $C_{45}H_{74}N_2O_7$ $[M+H]^+$ 755.5496, found 755.5505.

3β-Acetoxyurs-12-ene-28-oic acid [1-(6-amino-hexylamino)-1-oxo]ethyl ester (UA-5c)

The **UA-4c** (135.60 mg, 0.17 mmol) was reacted with TFA (0.51 mL, 6.69 mmol) according to the same procedure used in the preparation of **OA-5a**, to give **UA-5c** (79.6 mg, 71%) as a white solid: m.p.92.5–92.9 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.24 (t, $J = 5.6$ Hz, 1H, –CONH–), 5.24 (d, $J = 3.3$ Hz, 1H, H-12), 4.65 (d, $J = 15.5$ Hz, 1H, –OCH_2-CO–), 4.50–4.47 (m, 1H, H-3), 4.31 (d, $J = 15.5$ Hz, 1H, –OCH_2CO–), 3.45–3.37 & 3.26–3.20 (m, 4H, –NH$\underline{CH_2}CH_2$-CH_2CH_2– $CH_2\underline{CH_2}NH_2$), 2.78 (s, 2H, –NH_2), 2.21 (d, $J = 11.1$ Hz, 1H, H-18), 2.04 (s, 3H, CH_3CO–), 1.09, 0.96, 0.92, 0.88, 0.85, 0.83, 0.70 (each s, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 175.91, 170.95, 167.46, 139.44, 125.17, 80.79, 62.86, 55.26, 53.01, 48.37, 47.37, 42.19, 39.50, 39.10, 38.97, 38.84, 38.26, 37.65, 36.84, 36.73, 32.77, 30.49, 29.66, 29.50, 28.04, 27.81, 26.42, 26.19, 24.43, 23.57, 23.49, 23.24, 21.25, 21.07, 18.12, 17.05, 16.95, 16.70, 15.44; IR (KBr) ν_{max} 3429 (NH), 2941, 2862 (CH), 1735, 1671 (C=O), 1253 (C–O–C) cm^{-1}. HR-MS calcd for $C_{40}H_{66}N_2O_5$ $[M+H]^+$655.4972, found 655.4977.

3β-Acetoxyurs-12-ene-28-oic acid [1-(4-methyl-1-piperazinyl)-1-oxo]ethyl ester (UA-6)

The **UA-3** (100.0 mg, 0.2 mmol) was connected with methyl piperazine (0.1 mL, 0.2 mmol) according to the same procedure used in the preparation of **OA-4a**, to give **UA-6** (84.63 mg, 74%) as a white solid: m.p.82.1–82.7 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.24 (t, $J = 3.4$, 1H, H-12), 4.67 (d, $J = 14.0$, 1H, –$OC\underline{H_2}$CO–), 4.61 (d, $J = 14.0$, 1H, –$OC\underline{H_2}$CO–), 4.51–4.48 (m, 1H, H-3), 3.61–2.39 (m, 8H, piperazineH-2 × 2 & H-3 × 2 & H-5 × 2 & H-6 × 2), 2.30 (s, 3H, –N–C$\underline{H_3}$), 2.27 (d, $J = 11.6$, 1H, H-18), 2.06 (s, 3H, C$\underline{H_3}$CO–), 1.08, 0.95, 0.94, 0.86, 0.85, 0.75(s, each 3H). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 176.85, 170.91, 165.21, 138.08, 125.50, 80.87, 60.84, 55.27, 54.78, 54.40, 52.73, 48.25, 47.46, 45.86, 44.38, 42.03, 41.58, 39.55, 39.06, 38.73, 38.28, 37.65, 36.84, 36.55, 32.90, 30.64, 28.04, 27.98, 24.19, 23.52, 23.44, 23.27, 21.24, 21.14, 18.18, 17.05, 17.00, 16.70, 15.48; IR (KBr) ν_{max} 2927, 2863 (CH), 1734 (C=O), 1247 (C–O–C) cm^{-1}. HR-MS calcd for $C_{39}H_{62}N_2O_5$ $[M+H]^+$639.4659, found 639.4664.

3β-Acetoxyurs-12-ene-28-oic acid [1-(4-benzyl-1-piperazinyl)-1-oxo]ethyl ester (UA-7)

The **UA-3** (600.0 mg, 1.1 mmol) was connected with benzylpiperazine (138.2 mg, 1.4 mmol) according to the same procedure used in the preparation of **OA-4a**, to give **UA-7** (249.4 mg, 64%) as a white solid: m.p.88.6–89.2 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.32–7.26 (m, 5H, Ph-), 5.24 (t, $J = 3.4$, 1H, H-12), 4.66 (d, $J = 14.0$, 1H, –$OC\underline{H_2}$CO–), 4.59 (d, $J = 14.0$, 1H, –$OC\underline{H_2}$CO–), 4.51–4.48 (m, 1H, H-3), 3.61–2.43 (m, 8H, piperazineH-2 × 2 & H-3 × 2 & H-5 × 2 & H-6 × 2), 3.50 (s, 2H, Ph$C\underline{H_2}$–), 2.27 (d, $J = 11.3$, 1H, H-18), 2.04 (s, 3H,

CH_3CO–), 1.42, 1.10, 1.08, 0.95, 0.93, 0.87, 0.85(s, each 3H). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 176.80, 170.86, 165.13, 138.07, 129.08, 128.32, 127.34, 125.47, 80.84, 62.74, 60.89, 55.24, 52.80, 52.69, 52.45, 48.22, 47.43, 44.55, 42.00, 41.74, 39.61, 39.04, 38.70, 38.25, 37.62, 36.82, 36.53, 32.87, 30.61, 28.02, 27.95, 26.85, 24.17, 23.50, 23.42, 23.24, 21.21, 21.12, 18.15, 17.03, 16.99, 16.67, 15.45; IR (KBr) ν_{max} 2919, 2869 (CH), 1734, 1671 (C=O), 1247 (C–O–C) cm^{-1}. HR-MS calcd for $C_{45}H_{66}N_2O_5$ $[M+H]^+$ 715.4972, found 715.4980.

3β-Acetoxyurs-12-ene-28-oic acid [1-(1-piperazinyl)-1-oxo]ethyl ester (UA-8)

The **UA-7** (100.0 mg, 0.2 mmol) was reacted with 10% Pd/C (25.0 mg) according to the same procedure used in the preparation of **OA-8**, to give **UA-8** (68.0 mg, 46%) as a white solid: m.p.83.1–84.3 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) *δ*: 5.17 (s, 1H, H-12), 4.60 (d, $J = 14.0$, 1H, –OCH_2O–), 4.54 (d, $J = 14.0$, 1H, –OCH_2O–), 4.44–4.41 (m, 1H, H-3), 3.55–2.34 (m, 8H, piperazineH-2 × 2 & H-3 × 2 & H-5 × 2 & H-6 × 2), 2.20 (d, $J = 11.3$, 1H, H-18), 1.97 (s, 3H, CH_3CO–), 1.03, 1.02, 1.00, 0.88, 0.87, 0.80, 0.68 (s, each 3H). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 176.90, 170.93, 165.19, 138.10, 125.52, 80.88, 60.84, 55.28, 52.74, 52.58, 52.23, 52.05, 48.28, 47.47, 42.04, 39.56, 39.07, 38.75, 38.29, 37.67, 36.86, 36.57, 32.91, 30.65, 28.05, 27.99, 24.21, 23.54, 23.45, 23.29, 21.25, 21.15, 18.19, 17.07, 17.02, 16.71, 15.49; IR (KBr) ν_{max} 2933, 2863 (CH), 1735, 1681 (C=O), 1247 (C–O–C) cm^{-1}. HR-MS calcd for $C_{38}H_{60}N_2O_5$ $[M+H]^+$625.4502, found 625.4509.

Biology

The antiproliferative activities of MCF-7 (human breast cancer cells), Hela (human cervical carcinoma cells) and A549 (human NSCLC cells) cells under different treatments of synthesized compounds were determined by the MTT colorimetric assay. Briefly, cells were cultured on RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in humidified atmosphere with 5% CO_2. For the cytotoxicity tests, cells were plated at a density of 1×10^4 cells per well in 96-well plates in complete medium and cultured for 24 h, and media replaced with RPMI-1640, 10% FBS with or without pentacyclic triterpenoids derivatives. Test compounds were dissolved in DMSO at final concentrations less than 0.1%, prior to addition to cell culture assays. Following incubation at 37 °C for 48 h, 10 μL MTT (5 mg/mL) was added to each well and incubated for 4 h. After careful removal of the medium, 150 μL DMSO was added to each well and shaken carefully. A microplate reader (Multiskan Ascen, Thermo Fisher Scientific, Waltham, MA) was employed to determine optical density (OD) at 570 nm. Dose response curves were plotted for the samples and the IC_{50} values were calculated as the concentrations of the test compounds resulting in 50% reduction of absorption compared with the control cells. The data represented the mean ± SD of three independent experiments in which each compound concentration was tested in three replicate wells.

Results and discussion

Synthesis and characterization

A series of eighteen novel **OA** and **UA** derivatives were designed and synthesized. For the preparation of OA and UA piperazine derivatives, synthesis of the compounds designed was quite straight-forward (Scheme 1). After protection of 3-OH by acetylation, OA and UA derivatives were esterified with 2-hydroxyacetic acid, and which were amidated with *N*-substituted piperazine in the presence of EDCI to obtain OA-6, -7, -8, and UA-6, -7, -8 in total yield of 12.6–22.6 and 13.9–29.4%, respectively. Similarly, OA-4, OA-5, UA-4, and UA-5 were prepared (Scheme 2). The compounds were characterized by IR, ^{1}H-NMR, ^{13}C-NMR and HR-MS. IR spectra showed typical absorption peaks for the functional groups present. ^{1}H-NMR and ^{13}C-NMR spectra were as expected. Compounds **OA-5a, OA-5b, OA-5c, OA-6, OA-8, UA-5a, UA-5b, UA-5c, UA-6,** and **UA-8** were characterized by GC-TOF HR-MS, while **OA-4a, OA-4b, OA-4c, OA-7, UA-4a, UA-4b, UA-4c** and **UA-7** were characterized by 6540 RRLC/Q-TOF HR-MS. The HR-MS measured values coincided with theoretical values. IR, ^{1}H-NMR, ^{13}C-NMR and HR-MS data of newly synthesized compounds are summarized in the "Chemistry" Section.

Anticancer activity

We determined the antiproliferative activity of the two pentacyclic triterpenoids (**OA** and **UA**) and 18 analogs against MCF-7 cell lines, and then tested the six most active compounds against HeLa and A549 cell lines. Gefitinib was used as the positive control and the results are summarized in Table 1.

Further analysis clearly revealed that strong antiproliferative activities were observed when various R groups were introduced at the C-28 position of **OA** and **UA**. Introduction of a piperazine moiety such as in **OA-6** (IC_{50} 64.08 ± 4.88 μM), **OA-8** (IC_{50} 40.27 ± 4.88 μM), **UA-6** (IC_{50} 61.25 ± 4.88 μM), and **UA-8** (IC_{50} 56.85 ± 4.88 μM) gave much stronger growth inhibitory effects than the lead compounds **OA** and **UA**, which had IC_{50} values > 100,

Table 1 IC_{50} Values of OA and UA Derivatives on MCF-7, Hela, and A549 Cells Proliferation

Sample	M/W	IC_{50}(μM) MCF-7	IC_{50}(μM) Hela	IC_{50}(μM) A549
Gefitinib	–	17.83 ± 7.85	15.40 ± 4.65	11.02 ± 3.27
OA	456.70	>100	>100	>100
OA-4a	698.49	>100	–	–
OA-4b	726.52	>100	–	–
OA-4c	754.55	59.27 ± 4.88	–	–
OA-5a	598.43	9.19 ± 0.82	8.56 ± 0.53	12.72 ± 0.79
OA-5b	626.47	7.48 ± 0.33	8.64 ± 0.88	9.87 ± 0.64
OA-5c	654.50	7.61 ± 0.33	6.29 ± 1.64	6.87 ± 0.78
OA-6	638.47	64.08 ± 4.88	–	–
OA-7	691.09	66.5 ± 4.88	–	–
OA-8	600.97	40.27 ± 4.88	–	–
UA	456.70	>100	>100	>100
UA-4a	698.49	>100	–	–
UA-4b	726.52	>100	–	–
UA-4c	754.55	>100	–	–
UA-5a	598.43	8.45 ± 0.26	8.37 ± 0.11	10.06 ± 1.39
UA-5b	626.47	9.21 ± 0.73	10.35 ± 2.72	15.14 ± 0.66
UA-5c	654.50	12.91 ± 0.71	17.21 ± 6.92	22.14 ± 2.33
UA-6	638.47	61.25 ± 4.88	–	–
UA-7	691.09	>100	–	–
UA-8	600.97	56.85 ± 4.88	–	–

nd Not determined

MCF-7 human breast cancer cells, Hela human cervical carcinoma cells, A549 human NSCLC cells, Gefitinib positive control for antiproliferative activity

Each data represents mean ± S.D. From three different experiments performed in triplicate

against MCF-7 cancer cells. However, their intermediates, **OA-7** and **UA-7**, displayed relatively weak cytotoxicity with IC_{50} values of 66.5 ± 4.88 μM and > 100 μM, respectively.

The derivatives containing ethylenediamine, butanediamine and hexanediamine moieties showed the strongest cytotoxic activity against three human cancer cell lines, followed by compounds **UA-5b** and **UA-5c**. In particular, IC_{50} values for **OA-5a, OA-5b, OA-5c** and **UA-5a** exhibited <10 μM and these compounds were twice as active against MCF-7 and HeLa cell lines (IC_{50} 9.19 ± 0.82/8.56 ± 0.53, 7.48 ± 0.33/8.64 ± 0.88, 7.61 ± 0.33/6.29 ± 1.64 and 8.45 ± 0.26/8.37 ± 0.11 μM, respectively) than positive control, gefitinib (IC_{50} 15.40 ± 4.65 μM). However, their intermediate products **OA-4a, OA-4b, UA-4a, UA-4b** and **UA-4c** displayed poor cytotoxicity against the cancer cell lines with IC_{50} values > 100 μM.

The pronounced influence of the incorporated diamines on antitumor effects could be explained by their capacity for formation of hydrogen bonds, improvement of water-solubility, and modified physicochemical properties.

Structure–activity relationships

The IC_{50} value for **OA-4a, OA-4b** and **OA-4c** were significantly higher than those for **OA-5a, OA-5b** and **OA-5c**. Also, **OA** and **UA** derivatives containing primary amine groups were more active than those containing secondary or tertiary amines. The antiproliferative activities of the secondary amine compounds were better than those of the tertiary amines. For example the IC_{50} value of **OA-7** and **UA-7** is higher than **OA-8** and **UA-8**. In summary, the order of antiproliferative activity in diamine compounds is primary amine > secondary amine > tertiary amine.

The antiproliferative activity against MCF-7 cell lines revealed a clear preference for activity when the R' group was an ethylenediamine, which might indicate that a more water-soluble catenulate diamine group at the C-28 position contributed to the potency of the target compounds. For example, the IC_{50} value of compound **OA-5a** was clearly lower than that of **OA-8**. In summary, introduction of a group with a catenulate structure may confer better antiproliferative activity in **OA** and **UA** derivatives, supported by the better antiproliferative activity compared to those containing a cyclic piperazine group.

Increasing carbon chain length, exemplified by **OA-5a, OA-5b** and **OA-5c**, had no significant impact on the antiproliferative activity of **OA** and **UA** derivatives.

Conclusion

A series of pentacyclic triterpenoid derivatives bearing diamine moieties were synthesized and investigated for their cytotoxic potential against MCF-7, HeLa and A549 cells. Most of the compounds showed significant antiproliferative activity compared to their parent compounds. In particular, OA-5c showed the highest inhibitory activity against three human cancer cell lines. Furthermore, OA-5a has the best prospects for development as a drug, since it has significant anticancer activity, is low-cost and is easily purified. Therefore, OA-5a is considered worthy of further investigation as a new drug candidate.

Acknowledgements This work was supported by the National Natural Science Foundation of China (Nos. 21102067).

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