ORIGINAL ARTICLE

A C G publications

Rec. Nat. Prod. 7:4 (2013) 254-265

records of natural products

Synthesis and Antitumor Activity of 17-carboxylic acid Modified Amide Derivatives of 23-hydroxy betulinic acid

Yi Bi[1], Jin-Yi Xu[2*], Fei Sun[2], Xiao-Ming Wu[3*], Wen-Cai Ye[4] , Yi-Jun Sun[5], Qing-Guo Meng[1] and Wen-Wen Huang[1]

[1]*School of pharmacy, Yantai University, Yantai, Shandong 264005, China;*
[2]*Department of Medicinal Chemistry, China Pharmaceutical University, Nanjing, Jiangsu 210009, China ;*
[3]*Center for Drug Discovery, College of Pharmacy, China ;Pharmaceuticcl University, Nanjing, Jiangsu 210009, China;*
[4] *Department of Phytochemistry, China ;Pharmaceutical University, Nanjing, Jiangsu 210009, China;*
[5]*Drug Screening Center, Nanjing KeyGen Biotech. Co. Ltd., Nanjing, Jiangsu 210012, China.*

(Received August 8, 2012; Revised February 1, 2013; Accepted February 4, 2013)

Abstract: A novel series of 17-carboxylic acid modified amide derivatives of 23-hydroxy betulinic acid (**1**) were prepared and tested in vitro against five cell lines: A549 (human lung carcinoma), BEL-7402 (human hepatoma), SF-763 (human cerebroma), B16 (mice melanoma) and HL-60 (human leukaemia). Within this series of compounds, **4a** (IC_{50}=21.08 μM in SF-763, IC_{50}=21.63 μM in HL-60), **4b** (IC_{50}=28.45 μM in HL-60,IC_{50}=29.32 μM in BEL-7402) and **6g** (IC_{50}=26.09 μM in BEL-7402, IC_{50}=22.65 μM in HL-60) have the more potent cytotoxic activity than lead compound **1**. The preliminary structure-activity relationship analysis of the C-28 amide derivatives is also discussed.

Keywords: 23-hydroxy betulinic acid; amide derivatives; structure modification; antitumor activity; structure-activity relationship.

1. Introduction

23-Hydroxy betulinic acid (**1**) and betulinic acid (**2**) have recently attracted much attention due to their antitumor activity in different cell lines in pentacyclic triterpenes kingdom [1-2]. Although many other biological activities have been reported such as antitumor, antiviral, antioxidant and so on, most research focus on their antitumor activity and have synthesized plenty of derivatives especially betulinic acid. As a good lead compound, betulinic acid showed potent antitumor activity in a series of cell lines and the mechanism of which might be related to the proliferation, migration, cell cycle and apoptosis of tumor cells [3-10]. 23-Hydroxy betulinic acid has the similar chemical structure to

* Corresponding author: E-Mail: jinyixu@china.com (J.-Y.Xu); xmwu@cpu.edu.cn (X.-M.Wu); Phone: +86-25-83271445(J.-Y.Xu); +86-25-83242366 (X.-M.Wu) *Fax:* +86-25-83302827

betulinic acid, so we can use the experience of modification of betulinic acid to design and synthesize new 23-hydroxy betulinic acid derivatives.

In our previous study, several 23-hydroxy betulinic acid derivatives have showed more potent anti-tumor activity than betulinic acid and 23-hydroxy betulinic acid in different cell lines in vitro, especially compound **3** has the most potent cytotoxic activity. Preliminary structure-activity relationship displayed that the polarity and length of the chain on C-28 had an important impact on the antitumor activity [11,12]. These results motivated us to design and synthesis novel derivatives modified on 17-carboxylic acid moiety of 23-hydroxy betulinic acid.

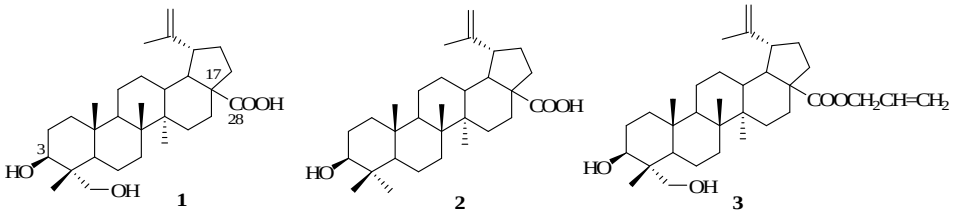

Figure 1. Structure of 23-hydroxy betulinic acid (1), betulinic acid (2) and the derivative of betulinic acid (3)

In this paper, we report a series of new 17-carboxylic acid modified amide derivatives of 23-hydroxy betulinic acid and their antitumor activity. The preliminary structure-activity relationship is also discussed.

2. Materials and Methods

2.1. General

Melting points were obtained on a MEL-TEMP II melting-point apparatus and are uncorrected. IR were determined on the Nicolet Impact 410 or Bruker FT-IR TENSOR27 instrument.1H-NMR spectra were recorded on a BRUKER-ACF-300 or BRUKER-ACF-500 instrument (chemical shifts are expressed as d values relative to TMS as internal standard). ESI were recorded on an HP 1100 LC/MSD spectrometer. HR-MS were obtained using a Agilent QTOF 6520 instrument.

2.2. Synthesis

*2.2.1 General procedure for synthesis of **4a-f***

Ac_2O (0.75mL, 7.5mmol) was added to a solution of 23-hydroxy betulinic acid (600.0mg, 1.25mmol) in dry pyridine(25mL). The mixture was stirred for a night at room temperature. After adding EtOAc (20mL), the mixture was washed with 9% HCl (30mL×3) and brine (30mL×3), dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by crystallization in EtOAc to afford the desired ester compound as yellow powder (680.0mg, 98%). $(COCl)_2$ (0.1mL) was added to a solution of ester compound (500.0mg，0.90mmol) in dry CH_2Cl_2 (10mL). The mixture was stirred for 4h at room temperature, evaporated to dryness and soluted in CH_2Cl_2 (10mL). The solution was added dropwise to the mixture of corresponding $H_2NCHR^1COOCH_3$.HCl (2 equiv) and DMAP (85.0mg, 0.696mmol) in CH_2Cl_2 (20mL) and stirred for 8h at the room temperature and concentrated to dryness. The mixture was diluted with EtOAc (30mL) and filtered. The filtrate was washed with 9% HCl(20mL×2), H_2O(20mL×2), brine(20mL×2), dried

over anhydrous Na_2SO_4, filtered, and concentrated to dryness. The residue was purified by column chromatography on silica gel to afford compound **4a-f**.

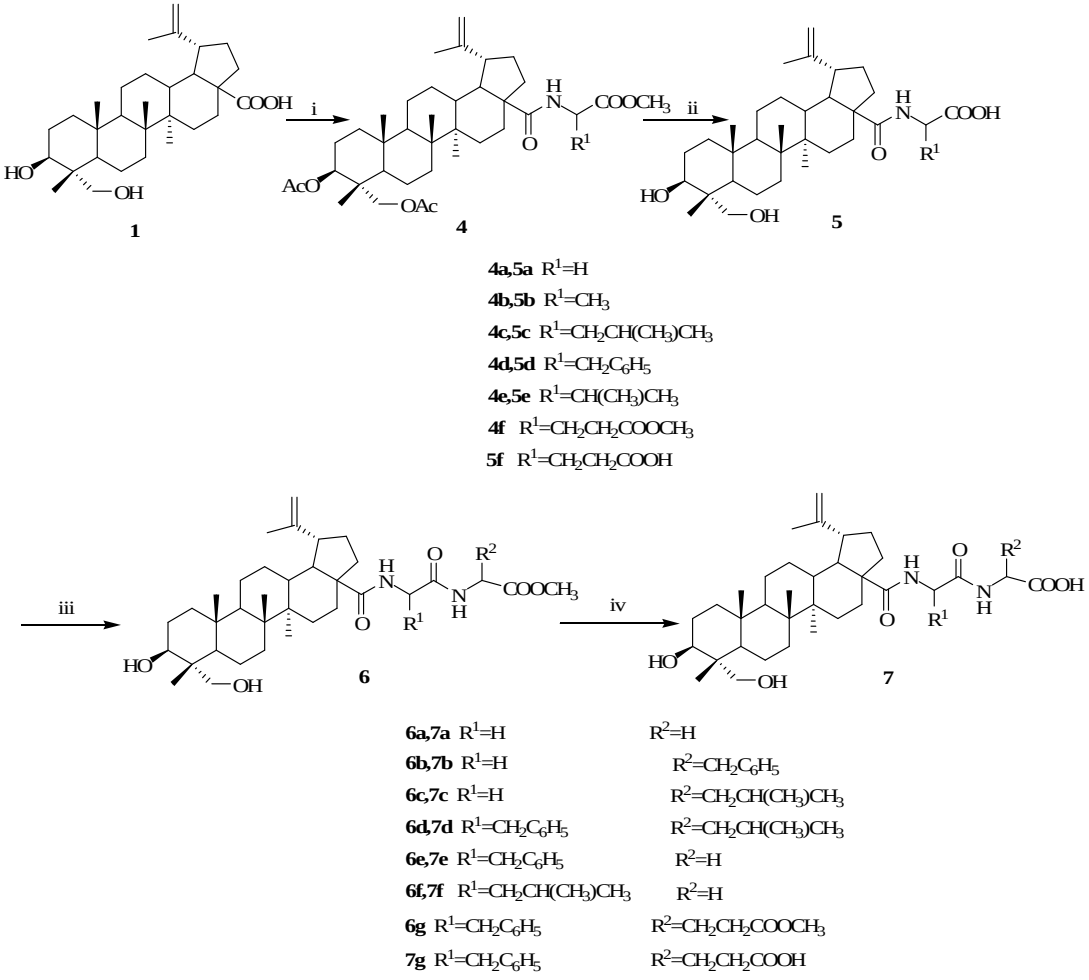

Figure 2. Synthesis of 23-hydroxy betulinic acid amide derivatives

Reagents and conditions: (i) Ac_2O, pyridine/rt/12h, then $(COCl)_2$, CH_2Cl_2/rt/4h, then $H_2NCHR^1COOCH_3 \cdot HCl$, DMAP/rt/8h; (ii) 4N NaOH, CH_3OH, THF/reflux/4h; (iii) $H_2NCHR^2COOCH_3 \cdot HCl$, EDC, HOBT/rt/8h; (iv) 4N NaOH, CH_3OH, THF/reflex/4h.

*Methyl N-[3β,23-diacetoxylup-20(29)-en-28-oyl]-glycinate (**4a**)*: $H_2NCH_2COOCH_3$.HCl (217.0mg, 1.740mmol), column chromatography with petroleum ether/acetone = 5:1（v:v). Yield: 338.0mg (62%) as a white solid, mp 109-111°C. IR (KBr, cm^{-1}) ν 3431, 2938, 2868, 1740, 1636, 1445, 1374, 1245, 1202, 1037, 427. ^{1}H-NMR ($CDCl_3$, 500MHz) δ 0.80, 0.86, 0.93, 0.96, 1.69 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 2.01, 2.06 (s, 3H each, 3 and 23-$OCOCH_3$), 3.09 (m, 1H, 19-CH), 3.68, 3.84 (2H, dd, J_A=J_B=11.6 Hz, J_{AB}=78.1 Hz, 23-CH_2), 3.76 (s, 3H, 17- $CONHCH_2COOCH_3$), 4.00 (t, 2H, 17-$CONHCH_2COOCH_3$), 4.59, 4.73 (d, 2H, *J*=68.3 Hz, 29=CH_2), 4.75 (m, 1H, 3-CH), 6.05 (t, 1H, 28-CONH). MS (EI): *m/z* $[M+H]^+$ 628.3，$[M+Na]^+$ 650.4，$[M+K]^+$ 666.3. HR-MS (ESI, M+H) *m/z*: calcd for $C_{37}H_{57}NO_7$ 628.4213, found 628.4217.

*Methyl N-[3β,23-diacetoxylup-20(29)-en-28-oyl]-L-alaninate (**4b**)*: L-$H_2NCH(CH_3)COOCH_3$.HCl (233.0mg, 1.740mmol), column chromatography with petroleum ether/acetone = 5:1（v:v). Yield: 302.0mg (55%) as a white solid, mp 180-182°C. IR (KBr, cm^{-1}) ν 3454, 3413, 2946, 2872, 2359, 1744, 1666, 1495, 1371, 1240, 1038, 887. ^{1}H-NMR ($CDCl_3$, 300MHz) δ 0.80, 0.87, 0.91, 0.96, 1.69 (s,

3H each, 24, 25, 26, 27and 30-CH_3), 2.01, 2.06 (s, 3H each, 3 and 23-$OCOCH_3$), 3.10 (m, 1H, 19-CH), 3.68, 3.84 (2H, dd, J_A=J_B=11.6 Hz, J_{AB}=48.6 Hz, 23-CH_2), 3.75 (s, 3H, 28-$CONHCH(CH_3)COOCH_3$), 4.56 (m, 1H, 28- $CONHCH(CH_3)COOCH_3$), 4.59, 4.72 (d, 2H, *J*=38.8 Hz, 29=CH_2), 4.74 (m, 1H, 3-CH), 6.10 (d, 1H, *J*=7.06, 28-CONH). MS (EI): *m/z* $[M+H]^+$ 642.3, $[M+Na]^+$ 664.3, $[M+K]^+$ 680.3. HR-MS (ESI, M+H) *m/z*: calcd for $C_{38}H_{59}NO_7$ 642.4370, found 642.4374.

*Methyl N-[3β,23-diacetoxylup-20(29)-en-28-oyl]-L-leucinate (**4c**)*:
L-$H_2NCH(CH_2CHCH_3CH_3)COOCH_3$.HCl (315.0mg, 1.740mmol), column chromatography with petroleum ether/acetone = 5:1(v:v). Yield: 315.0mg (53%) as a white solid, mp 108-111°C IR (KBr, cm^{-1}) ν 3417, 2953, 2870, 1743, 1664, 1510, 1469, 1371, 1246, 1163, 1037, 882. ^{1}H-NMR ($CDCl_3$, 300MHz) δ 0.80, 0.88, 0.91, 0.97, 1.67 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 2.01, 2.06 (s, 3H each, 3 and 23-$OCOCH_3$), 3.10 (m, 1H, 19-CH), 3.68, 3.84 (2H, dd, J_A=J_B=11.7 Hz, J_{AB}=47.0 Hz, 23-CH_2), 3.72 (s, 3H, 28-$CONHCH(CH_2CHCH_3CH_3)COOCH_3$), 4.61 (m, 1H, 28-CONH $CH(CH_2CHCH_3CH_3)$ $COOCH_3$), 4.59, 4.72 (d, 2H, *J*=38.6 Hz, 29=CH_2), 4.76 (m, 1H, 3-CH), 5.86 (d, 1H, *J*=8.3 Hz, 28-CONH). MS (EI): *m/z* $[M+H]^+$ 684.5, $[M+Na]^+$ 706.5, $[M+K]^+$ 722.4. HR-MS (ESI, M+H) *m/z*: calcd for $C_{41}H_{65}NO_7$ 684.4839, found 684.4835.

*Methyl N-[3β,23-diacetoxylup-20(29)-en-28-oyl]-L-phenylalaninate (**4d**)*:
L-$H_2NCH(CH_2C_6H_5)COOCH_3$.HCl (374.0mg, 1.740mmol), column chromatography with petroleum ether/acetone = 5:1（v:v). Yield: 408.0mg (67%) as a white solid, mp 98-100°C. IR (KBr, cm^{-1}) ν 3446, 3066, 2948, 2869, 1740, 1663, 1503, 1449, 1370, 1246, 1037, 882, 700. ^{1}H-NMR ($CDCl_3$, 300MHz) δ 0.80, 0.86, 0.89, 0.93, 1.69 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 2.01, 2.05 (s, 3H each, 3 and 23-$OCOCH_3$), 3.03 (m, 2H, 28-$CONHCH(CH_2C_6H_5)$ $COOCH_3$), 3.14 (m, 1H, 19-CH), 3.67, 3.86 (2H, dd, J_A=J_B=11.6 Hz, J_{AB}=56.8 Hz, 23-CH_2), 3.72 (s, 3H, 28-$CONHCH(CH_2C_6H_5)COOCH_3$), 4.57, 4.69 (d, 2H, *J*=36.8 Hz, 29=CH_2), 4.75 (m, 2H, 3-CH and 28-$CONHCH(CH_2C_6H_5)COOCH_3$), 5.86 (d, 1H, *J*=7.7 Hz, 28-CONH), 7.21 (m, 5H, 28- $CONHCH(CH_2C_6H_5)COOCH_3$). MS (EI): *m/z* $[M+H]^+$ 718.4. HR-MS (ESI, M+H) *m/z*: calcd for $C_{44}H_{63}NO_7$ 718.4683, found 718.4679.

*Methyl N-[3β,23-diacetoxylup-20(29)-en-28-oyl]-L-valinate (**4e**)*
L-$H_2NCH(CHCH_3CH_3)COOCH_3$.HCl (291.0mg, 1.740mmol), column chromatography with petroleum ether/acetone = 5:1(v:v). Yield: 297.0mg (51%) as a white solid, mp 108-110°C. IR (KBr, cm^{-1}) ν 3447, 2954, 2870, 1741, 1667, 1501, 1468, 1371, 1245, 1151, 1038, 882. ^{1}H-NMR ($CDCl_3$, 300MHz) δ 0.80, 0.87, 0.92, 0.97, 1.67 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 2.01, 2.06 (s, 3H each, 3 and 23-$OCOCH_3$), 3.10 (m, 1H, 19-CH), 3.68, 3.83 (2H, dd, J_A=J_B=11.3 Hz, J_{AB}=45.8 Hz, 23-CH_2), 3.72 (s, 3H, 28-$CONHCH(CHCH_3CH_3)COOCH_3$), 4.57 (m, 1H, 28-CONH $CH(CHCH_3CH_3)COOCH_3$), 4.59, 4.72 (d, 2H, *J*=38.00 Hz, 29=CH_2), 4.76 (m, 1H, 3-CH), 6.04 (d, 1H, *J*=8.3 Hz, 28-CONH). MS (EI): *m/z* $[M+H]^+$ 670.4, $[M+Na]^+$ 692.4. HR-MS (ESI, M+H) *m/z*: calcd for $C_{40}H_{63}NO_7$ 670.4683, found 670.4685.

*Dimethyl N-[3β,23-diacetoxylup-20(29)-en-28-oyl]-L-glutamate (**4f**)*
L-$H_2NCH(CH_2CH_2COOCH_3)COOCH_3$.HCl (367.0mg, 1.740mmol), column chromatography with petroleum ether/acetone = 3:1(v:v). Yield: 335.0mg (54%) as a white solid, mp 82-84°C. IR (KBr, cm^{-1}) ν 3410, 2949, 2870, 1741, 1666, 1509, 1444, 1372, 1246, 1037, 883. ^{1}H-NMR ($CDCl_3$, 300MHz) δ 0.81, 0.88, 0.92, 0.97, 1.68 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 2.01, 2.06 (s, 3H each, 3 and 23-$OCOCH_3$), 3.07 (m, 1H, 19-CH), 3.72 (m, 7H, one of 23-CH_2 and 28-$CONHCH(CH_2CH_2COOCH_3)COOCH_3$), 3.85 (1H, d， *J*=11.7 Hz, 23-CH_2), 3.72 (s, 3H, 28-$CONHCH(CH_2CH_2COOCH_3)COOCH_3$), 4.56 (m, 1H, 28-$CONHCH(CH_2CH_2COOCH_3)COOCH_3$), 4.59, 4.72 (d, 2H, *J*=39.6 Hz, 29=CH_2), 4.74 (m, 1H, 3-CH), 6.28 (d, 1H，*J*=7.6 Hz, 28-CONH). MS (EI): *m/z* $[M+H]^+$ 714.6, $[M+Na]^+$ 736.6, $[M+K]^+$ 752.6. HR-MS (ESI, M+H) *m/z*: calcd for $C_{41}H_{63}NO_9$ 714.4581, found 714.4586.

*2.2.2. General procedure for synthesis of **5a-f***

Compound **4** (0.30mmol) was dissolved in CH_3OH (4mL) and THF (10mL). 4N NaOH (4mL) was added to the solution and refluxed for 4h. 9% HCl was added to the mixture until the white solid appear, filtered and the insoluble substance was washed with H_2O. The residue was dried to obtain the corresponding **5a-f**.

*N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-glycine (**5a**)*: **4a** (188.0mg, 0.30mmol). Yield: 133.0mg (84%) as a white solid, mp 213-215°C. IR (KBr, cm^{-1}) ν 3426, 3070, 2941, 2868, 1737, 1640, 1450, 1384, 1243, 1195, 1044, 880. ^{1}H-NMR (DMSO, 300MHz) δ 0.52, 0.77, 0.83, 0.91, 1.62 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 2.97 (m, 1H, 19-CH), 3.06, 3.31 (2H, dd, J_A=J_B=10.6 Hz, J_{AB}=75.9 Hz, 23-CH_2), 3.55 (m, 5H, 3-CH, 3-OH, 23-OH, 28-CONHCH_2COOH), 4.53, 4.64 (d, 2H, *J*=30.9 Hz, 29=CH_2), 7.68 (t, 1H, 28-CONHCH_2COOH). MS (EI): *m/z* $[M-H]^-$ 528.3. HR-MS (ESI, M+H) *m/z*: calcd for $C_{32}H_{51}NO_5$ 530.3845, found 530.3840.

*N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-L-alanine (**5b**)*: **4b** (192.0mg, 0.30mmol). Yield: 132.0mg (81%) as a white solid, mp 179-181°C. IR (KBr, cm^{-1}) ν 3424, 2942, 2869, 1722, 1636, 1513, 1451, 1384, 1298, 1196, 1043, 884. ^{1}H-NMR (DMSO, 300MHz) δ 0.51, 0.77, 0.83, 0.91, 1.62 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 3.10 (m, 2H, 19-CH and one of 23-CH_2), 3.39 (m, 2H, 3-CH and one of 23-CH_2), 4.13 (m, 2H, 3-OH, 28-CONHCH(CH_3)COOH), 4.32 (t, 1H, 23-OH), 4.52, 4.63 (d, 2H, *J*=33.9 Hz, 29=CH_2), 7.68 (d, 1H, *J*=7.2 Hz, 28-CONHCH(CH_3)COOH), 12.26 (s, 1H, 28-CONHCH(CH_3)COOH). MS (EI): *m/z* $[M-H]^-$ 542.3. HR-MS (ESI, M+H) *m/z*: calcd for $C_{33}H_{53}NO_5$ 544.4002, found 544.4006.

*N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-L-leucine (**5c**)*: **4c** (205.0mg, 0.30mmol). Yield: 132.0mg (75%) as a white solid, mp 198-200°C. IR (KBr, cm^{-1}) ν 3447, 2949, 2872, 1720, 1638, 1524, 1446, 1383, 1196, 1045, 882. ^{1}H-NMR (DMSO, 300MHz) δ 0.50, 0.77, 0.82, 0.90, 1.62 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 3.00 (m, 2H, 19-CH and one of 23-CH_2), 3.39 (m, 2H, 3-CH and one of 23-CH_2), 4.10 (d, 1H, *J*=4.9 Hz, 3-OH), 4.24 (m, 1H, 28-CONHCH($CH_2CHCH_3CH_3$)COOH), 4.34 (t, 1H, 23-OH), 4.52, 4.63 (d, 2H, *J*=33.4 Hz, 29=CH_2), 7.66 (d, 1H, *J*=8.2 Hz, 28-CONHCH($CH_2CHCH_3CH_3$)COOH), 12.25 (s, 1H, 28-CONHCH($CH_2CHCH_3CH_3$)COOH). MS (EI): *m/z* $[M+H]^+$ 586.4, $[M+Na]^+$ 608.4. HR-MS (ESI, M+H) *m/z*: calcd for $C_{36}H_{59}NO_5$ 586.4471, found 586.4468.

*N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-L-phenylalanine (**5d**)*: **4d** (215.0mg, 0.30mmol). Yield: 152.0mg (82%) as a white solid, mp 180-182°C. IR (KBr, cm^{-1}) ν 3425, 2942, 2869, 1718, 1639, 1522, 1451, 1386, 1199, 1044, 880, 699. ^{1}H-NMR (DMSO, 300MHz) δ 0.47, 0.52, 0.72, 0.81, 1.59 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 2.98 (m, 4H, 19-CH and one of 23-CH_2 and 28-CONHCH($CH_2C_6H_5$)COOH), 3.36 (m, 2H, 3-CH and one of 23-CH_2), 4.08 (d, 1H, *J*=4.8 Hz, 3-OH), 4.31 (t, 1H, 23-OH), 4.41 (m, 1H, 28-CONHCH($CH_2C_6H_5$)COOH), 4.49, 4.60 (d, 2H, *J*=30.8 Hz, 29=CH_2), 7.19 (m, 5H, 28-CONHCH($CH_2C_6H_5$)COOH), 7.69 (d, 1H, *J*=8.8 Hz, 28-CONHCH($CH_2C_6H_5$)COOH), 12.48(s, 1H, 28-CONHCH($CH_2C_6H_5$)COOH). MS (EI): *m/z* $[M+H]^+$ 620.4, $[M+Na]^+$ 642.4. HR-MS (ESI, M+H) *m/z*: calcd for $C_{39}H_{57}NO_5$ 620.4315, found 620.4319.

*N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-L-valine (**5e**)*: **4e** (201.0mg, 0.30mmol). Yield: 140.0mg (82%) as a white solid, mp 175-177°C. IR (KBr, cm^{-1}) ν 3429, 3177, 2943, 2870, 1727, 1640, 1500, 1460, 1384, 1196, 1043, 882. ^{1}H-NMR (DMSO, 300MHz) δ0.50, 0.77, 0.82, 0.90, 1.62 (s, 3H each, 24, 25, 26, 27 and 30-CH_3), 2.98 (m, 2H, 19-CH and one of 23-CH_2), 3.41 (m, 2H, 3-CH and one of 23-CH_2), 4.06 (m, 2H, 3-OH and 28-CONHCH($CHCH_3CH_3$)COOH), 4.35 (t, 1H, 23-OH), 4.52, 4.63 (d, 2H, *J*=32.9 Hz, 29=CH_2), 7.40 (d, 1H, *J*=8.4 Hz, 28-CONHCH($CHCH_3CH_3$)COOH),

12.35 (s, 1H, 28-CONHCH(CHCH$_3$CH$_3$)COOH). MS (EI): *m/z* [M+H]$^+$ 572.3, [M+Na]$^+$ 594.3. HR-MS (ESI, M+H) *m/z*: calcd for $C_{35}H_{57}NO_5$ 572.4315, found 572.4313.

*N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-L-glutamic acid (**5f**)*: **4f** (214.0mg, 0.30mmol). Yield: 128.0mg (71%) as a white solid, mp 188-190°C. IR (KBr, cm^{-1}) ν 3447, 2944, 2870, 1717, 1640, 1512, 1450, 1386, 1198, 1041, 884. ^{1}H-NMR (DMSO, 300MHz) δ0.51, 0.77, 0.83, 0.91, 1.62 (s, 3H each, 24, 25, 26, 27 and 30-CH$_3$), 2.98 (m, 2H, 19-CH and one of 23-CH$_2$), 3.40 (m, 2H, 3-CH and one of 23-CH$_2$), 4.14 (m, 2H, 3-OH and 28-CONHCH(CH$_2$CH$_2$COOH)
COOH), 4.33 (t, 1H, 23-OH), 4.52, 4.63 (d, 2H, *J*=33.4 Hz, 29=CH$_2$), 7.66 (d, 1H, *J*=7.7 Hz, 28-CONHCH(CH$_2$CH$_2$COOH)COOH), 12.19 (s, 2H, 28-CONHCH(CH$_2$CH$_2$COOH)COOH). MS (EI): *m/z* [M+H]$^+$ 602.4, [M+Na]$^+$ 624.4, [M+K]$^+$ 640.3. HR-MS (ESI, M+H) *m/z*: calcd for $C_{36}H_{57}NO_7$ 616.4213, found 616.4211.

*2.2.3. General procedure for synthesis of **6a-g***

Corresponding compound **5** (0.20mmol) in CH_2Cl_2 (10mL) was added to $H_2NCHR^2COOCH_3$.HCl (1.2equiv) in CH_2Cl_2 (10mL) in the absence of EDC (0.40mmol) and HOBT (0.50mmol). The mixture was stirred for 8h at the room temperature and washed with H_2O (20mL×2), brine (20mL×2), dried over anhydrous Na_2SO_4, filtered, and concentrated to dryness. The residue was purified by column chromatography on silica gel to afford compound **6a-g**.

*Methyl N'-[N-[3β, 23-dihydroxylup-20(29)-en -28-oyl]-2-aminoethanoyl]-glucinate (**6a**)*: **5a** (106.0mg, 0.20mmol), $H_2NCH_2COOCH_3$.HCl (30.0mg, 0.24mmol). column chromatography with CH_2Cl_2/acetone = 2:1（v:v). Yield: 96.0mg (80%) as a white solid, mp213-215°C. IR (KBr, cm^{-1}) ν 3417, 3072, 2943, 2868, 1749, 1642, 1526, 1444, 1376, 1211, 1043, 883, 567. ^{1}H-NMR ($CDCl_3$, 300MHz) δ 0.69, 0.85, 0.88, 0.96, 1.68 (s, 3H each, 24, 25, 26, 27 and 30-CH$_3$), 3.08 (m, 1H, 19-CH), 3.40, 3.70 (2H, dd, J_A=J_B=10.3 Hz, J_{AB}=89.0 Hz, 23-CH$_2$), 3.61 (m, 1H, 3-CH), 3.75 (s, 3H, 28-CONHCH$_2$CONHCH$_2$COOCH$_3$), 4.00 (m, 4H, 28-CONHCH$_2$CONHCH$_2$COOCH$_3$ and 28-CONHCH$_2$CONHCH$_2$COOCH$_3$), 4.59, 4.73 (d, 2H, *J*=40.9 Hz, 29=CH$_2$), 6.33 (t, 1H, 28-CONHCH$_2$CONHCH$_2$COOCH$_3$), 6.66 (t, 1H, 28-CONHCH$_2$CONHCH$_2$COOCH$_3$). MS (EI): *m/z* [M+H]$^+$ 601.3, [M+Na]$^+$ 623.4. HR-MS (ESI, M+H) *m/z*: calcd for $C_{35}H_{56}N_2O_6$ 601.4217, found 601.4214.

*Methyl N'-[N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-2-aminoethanoyl]-L-phenylalaninate (**6b**)*: **5a** (106.0mg, 0.20mmol), $H_2NCH(CH_2C_6H_5)COOCH_3$.HCl (52.0mg, 0.24mmol). column chromatography with petroleum ether /acetone =1:1(v:v). Yield: 105.0mg (76%) as a white solid, mp182-184°C. IR (KBr, cm^{-1}) ν 3474, 3419, 2953, 2866, 1747, 1638, 1502, 1452, 1383, 1045, 882, 699, 616. ^{1}H-NMR ($CDCl_3$, 300MHz) δ 0.68, 0.85, 0.88, 0.95, 1.67 (s, 3H each, 24, 25, 26, 27and 30-CH$_3$), 3.12 (m, 3H, 19-CH and 28-CONHCH$_2$CONHCH(CH$_2$C$_6$H$_5$)COOCH$_3$), 3.40 (1H, d, *J*=10.33, one of 23-CH$_2$), 3.65 (m, 2H, 3-CH and one of 23-CH$_2$), 3.72 (s, 3H, 28-CONHCH$_2$CONHCH(CH$_2$
C$_6$H$_5$)COOCH$_3$), 3.89 (m, 2H, 28- CONHCH$_2$CONHCH(CH$_2$C$_6$H$_5$)COOCH$_3$), 4.59, 4.72 (d, 2H, *J*=41.1 Hz, 29=CH$_2$), 4.85 (m, 1H, 28-CONHCH$_2$CONHCH(CH$_2$C$_6$H$_5$)COOCH$_3$), 6.17 (t, 1H, 28-CONHCH$_2$CONHCH(CH$_2$C$_6$H$_5$)COOCH$_3$), 6.27 (d, 1H, *J*=7.5 Hz, 28-CONHCH$_2$CONHCH(CH$_2$
C$_6$H$_5$)COOCH$_3$), 7.19 (m, 5H, 28-CONHCH$_2$CONHCH(CH$_2$C$_6$H$_5$)COOCH$_3$). MS (EI): *m/z* [M+H]$^+$ 691.3, [M+Na]$^+$ 713.3, [M+K]$^+$ 729.3. HR-MS (ESI, M+H) *m/z*: calcd for $C_{42}H_{62}N_2O_6$ 691.4686, found 691.4683.

*Methyl N'-[N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-2-aminoethanoyl]-L-leucinate (**6c**)*: **5a** (106.0mg, 0.20mmol), $H_2NCH(CH_2CHCH_3CH_3)C_6H_5COOCH_3$.HCl (40.0mg, 0.24mmol). column chromatography with petroleum ether /acetone =1:1（v:v). Yield: 96.0mg (73%) as a white solid,

mp144-146°C. IR (KBr, cm^{-1}) ν 3416, 3075, 2951, 2869, 1746, 1638, 1517, 1467, 1385, 1204, 1153, 1046, 882. ^{1}H-NMR (DMSO, 300MHz) δ 0.50, 0.60, 0.77, 0.88, 1.62 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 3.07 (m, 2H, 19-CH and one of 23-CH_2), 3.42 (2H, m, 3-CH and one of 23-CH_2), 3.61 (s, 3H, 28-$CONHCH_2CONHCH(CH_2CHCH_3CH_3)COOCH_3$), 3.69 (m, 2H, 28-$CONHCH_2CONH$ $CH(CH_2CHCH_3CH_3)COOCH_3$), 4.13 (t, 1H, 23-OH), 4.30 (m, 2H, 3-OH and 28-$CONHCH_2CONH$ $CH(CH_2CHCH_3CH_3)COOCH_3$), 4.52, 4.64 (d, 2H, *J*=34.3 Hz, 29=CH_2), 7.75 (t, 1H, 28-$CONHCH_2$ $CONHCH(CH_2CHCH_3CH_3)COOCH_3$), 7.98 (d, 1H, *J*=7.6 Hz, 28-$ONHCH_2CONHCH(CH_2CHCH_3$ $CH_3)COOCH_3$). MS (EI): *m/z* $[M+Na]^+$ 679.3, $[M+K]^+$ 695.3. HR-MS (ESI, M+H) *m/z*: calcd for $C_{39}H_{64}N_2O_6$ 657.4843, found 657.4847.

*Methyl N'-[N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-2-amino-2-benzyl-ethanoyl]-L-leucinate (**6d**)*: **5d** (124.0mg, 0.20mmol), $H_2NCH(CH_2CHCH_3CH_3)C_6H_5COOCH_3$.HCl (40.0mg, 0.24mmol). column chromatography with petroleum ether /EtOAc =1:2 (v:v). Yield: 103.0mg (69%) as a white solid, mp146-148°C. IR (KBr, cm^{-1}) ν 3411, 3068, 2950, 2869, 1745, 1665, 1497, 1469, 1369, 1202, 1149, 1046, 879, 695. ^{1}H-NMR (DMSO, 300MHz) δ 0.44, 0.51, 0.72, 0.79, 1.58 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 2.95 (m, 4H, 19-CH and one of 23-CH_2 and 28-$CONHCH(CH_2C_6H_5)CONHCH$ $(CH_2CHCH_3CH_3)COOCH_3$), 3.37 (2H, m, 3-CH and one of 23-CH_2), 3.62 (s, 3H, 28-$CONHCH(CH_2C_6H_5)CONHCH(CH_2CHCH_3CH_3)COOCH_3$), 4.04 (br, 1H, 3-OH), 4.33 (m, 2H, 23-OH and 28-$CONHCH(CH_2C_6H_5)CONHCH(CH_2CHCH_3CH_3)COOCH_3$), 4.49 (m, 1H, 28-$CONHCH(CH_2C_6H_5)CONHCH(CH_2CHCH_3CH_3)COOCH_3$), 4.49, 4.58 (d, 2H, *J*=27.7 Hz, 29=CH_2), 7.22 (m, 5H, 28-$CONHCH(CH_2C_6H_5)CONHCH(CH_2CHCH_3CH_3)COOCH_3$), 7.63 (d, 1H, *J*=8.5 Hz, 28-$CONHCH(CH_2C_6H_5)CONHCH(CH_2CHCH_3CH_3)COOCH_3$), 7.98 (d, 1H, *J*=7.9 Hz, 28-$CONHCH$ $(CH_2C_6H_5)CONHCH(CH_2CHCH_3CH_3)COOCH_3$). MS (EI): *m/z* $[M+Na]^+$ 769.5. HR-MS (ESI, M+H) *m/z*: calcd for $C_{46}H_{70}N_2O_6$ 747.5312, found 747.5314.

*Methyl N'-[N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-2-amino-2-benzyl-ethanoyl]-glucinate (**6e**)*: **5d** (124.0mg, 0.20mmol), $H_2NCH_2COOCH_3$.HCl (30.0mg, 0.24mmol). column chromatography with petroleum ether / acetone =1:1（v:v). Yield: 106.0mg (77%) as a white solid, mp135-137°C. IR (KBr, cm^{-1}) ν 3424, 2943, 2867, 1751, 1664, 1512, 1497, 1452, 1374, 1206, 1046, 882, 702. ^{1}H-NMR (DMSO, 300MHz) δ 0.42, 0.51, 0.72, 0.78, 1.58 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 2.86 (m, 2H, 28-$CONHCH(CH_2C_6H_5)CONHCH_2COOCH_3$), 3.03 (m, 2H, 19-H and one of 23- CH_2), 3.36 (m, 2H, 3-CH and one of 23-CH_2), 3.63 (s, 3H, 28-$CONHCH(CH_2C_6H_5)CONHCH_2COOCH_3$), 3.87 (m, 2H, 28-$CONHCH(CH_2C_6H_5)CONHCH_2COOCH_3$), 4.08 (d, 1H, *J*=4.8 Hz, 3-OH), 4.31 (t, 1H, 23-OH), 4.52 (m, 1H, 28-$CONHCH(CH_2C_6H_5)CONHCH_2COOCH_3$), 4.49, 4.60 (d, 2H,, *J*=32.8 Hz, 29=CH_2), 7.20 (m,, 5H, 28-$CONHCH(CH_2C_6H_5)CONHCH_2COOCH_3$), 7.65 (d, 1H, *J*=8.3 Hz, 28-$CONHCH(CH_2C_6H_5)CONHCH_2COOCH_3$), 8.11 (t, 1H, 28-$CONHCH(CH_2C_6H_5)CONHCH_2COO$ CH_3). MS (EI): *m/z* $[M+H]^+$ 691.3, $[M+Na]^+$ 713.4, $[M+K]^+$ 729.4. HR-MS (ESI, M+H) *m/z*: calcd for $C_{42}H_{62}N_2O_6$ 691.4686, found 691.4683.

*Methyl N'-[N-[3β, 23 -dihydroxylup- 20(29)- en- 28 -oyl]- 2- amino -2-(2' -isobutyl)- ethanoyl]-glucinate (**6f**)*: **5c** (117.0mg, 0.20mmol), $H_2NCH_2COOCH_3$.HCl (30.0mg, 0.24mmol). column chromatography with petroleum ether / acetone =3:2 (v:v). Yield: 93.0mg (71%) as a white solid, mp160-162°C. IR (KBr, cm^{-1}) ν 3415, 2950, 2873, 1756, 1636, 1520, 1466, 1383, 1207, 1045, 886.^{1}H-NMR (DMSO, 300MHz) δ 0.50, 0.77, 0.82, 0.85, 1.62 (s, 3H each, 24, 25,26, 27and 30-CH_3), 3.02 (m, 2H, 19-CH and one of 23-CH_2), 3.40 (2H, m, 3-CH and one of 23-CH_2),3.60 (s, 3H, 28-$CONHCH(CH_2CHCH_3CH_3)CONHCH_2COOCH_3$), 3.83 (m, 2H, 28-CONHCH (CH_2CH CH_3 $CH_3)CONHCH_2COOCH_3$), 4.10 (d, 1H, *J*=4.8 Hz, 3-OH), 4.34 (m, 2H, 23-OH and 28-$CONHCH(CH_2CHCH_3CH_3)CONHCH_2COOCH_3$), 4.52, 4.64 (d, 2H, *J*=34.9 Hz, 29=CH_2), 7.54 (d, 1H, *J*=8.5 Hz, 28- $CONHCH(CH_2CHCH_3CH_3)CONHCH_2COOCH_3$), 8.00 (t, 1H, 28- CONHCH $(CH_2CHCH_3CH_3)CONHCH_2COOCH_3$). MS (EI): *m/z* $[M+H]^+$ 657.5, $[M+Na]^+$ 679.5, $[M+K]^+$ 695.5. HR-MS (ESI, M+H) *m/z*: calcd for $C_{39}H_{64}N_2O_6$ 657.4843, found 657.4848.

*Dimethyl N'-[N-[3β, 23- dihydroxylup- 20(29) -en- 28 -oyl]- 2- amino- 2- benzyl-ethanoyl]-L-glutamate (**6g**)*: **5d** (124.0mg, 0.20mmol), $H_2NCH(CH_2CH_2COOCH_3)COOCH_3$.HCl (51.0mg, 0.24mmol). column chromatography with petroleum ether / acetone =2:1(v:v). Yield: 104.0mg (63%) as a white solid, mp138-140°C. IR (KBr, cm^{-1}) ν 3424, 2923, 2852, 1741, 1632, 1554, 1447, 1376, 1217, 1168, 1048, 737. ^{1}H-NMR (DMSO, 300MHz) δ 0.52, 0.78, 0.81, 0.84, 0.98 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 2.96 (m, 4H, 28-CONHCH($CH_2C_6H_5$)CONHCH($CH_2CH_2COOCH_3$)$COOCH_3$ and 19-H and one of 23-CH_2), 3.37 (m, 2H, 3-CH and one of 23-CH_2), 3.58, 3.64 (s, 3H each, 28-CONHCH($CH_2C_6H_5$)CONHCH($CH_2CH_2COOCH_3$)$COOCH_3$), 4.12 (d, 1H, *J*=4.6 Hz, 3-OH), 4.36 (m, 4H, 28-CONHCH($CH_2C_6H_5$)CONHCH($CH_2CH_2COOCH_3$)$COOCH_3$ and 29=CH_2), 7.19 (m, 6H, 28-CONHCH($CH_2C_6H_5$)CONHCH($CH_2CH_2COOCH_3$)$COOCH_3$), 8.28 (d, 1H, *J*=7.3 Hz, 28-CONHCH($CH_2C_6H_5$)CONHCH($CH_2CH_2COOCH_3$)$COOCH_3$). MS (EI): *m/z* $[M+H]^+$ 777.6, $[M+Na]^+$ 799.6, $[M+K]^+$ 815.6. HR-MS (ESI, M+H) *m/z*: calcd for $C_{46}H_{68}N_2O_8$ 777.5054, found 777.5051.

*2.2.4. General procedure for synthesis of **7a-g***

Compound **6** (0.10mmol) was disolved in CH_3OH (4mL) and THF (4mL). 4N NaOH (2mL) was added to the solution and refluxed for 4h. 9% HCl was added to the mixture until the white solid appear, filtered and the insoluble substance was washed with H_2O. The residue was dried to obtain the corresponding **7a-f**.

*N'-[N-[3β, 23-dihydroxylup-20(29)-en -28-oyl]-2-aminoethanoyl]-glucine (**7a**)*: **6a** (60.0mg, 0.10mmol). Yield: 50.0mg (85%) as a white solid, mp 255°C (dec). IR (KBr, cm^{-1}) ν 3416, 2929, 2867, 1730, 1640, 1530, 1450, 1383, 1197, 1044, 882, 622. ^{1}H-NMR (DMSO, 500MHz) δ 0.51, 0.77, 0.82, 0.87, 1.62 (s, 3H each, 24, 25, 26, 27 and 30-CH_3), 2.97 (m, 1H, 19-CH), 3.05, 3.70 (d, 1H, *J*=8.9 Hz, one of 23-CH_2), 3.40 (m, 2H, 3-CH and one of 23-CH_2), 3.67 (m, 4H, 28-$CONHCH_2CONHCH_2COOH$), 4.10 (d, 1H, *J*=89.0 Hz, 3-OH), 4.33 (t, 1H, 23-OH), 4.52, 4.64 (d, 2H, *J*=58.8 Hz, 29=CH_2), 6.77 (t, 2H, 28- $CONHCH_2CONHCH_2COOH$).MS (EI): *m/z* $[M+H]^+$ 587.4, $[M+Na]^+$ 609.5. HR-MS (ESI, M+H) *m/z*: calcd for $C_{34}H_{54}N_2O_6$ 587.4060, found 587.4065.

*N'-[N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-2-aminoethanoyl]-L-phenylalanine (**7b**)*: **6b** (69.0mg, 0.10mmol). Yield: 55.0mg (82%) as a white solid, mp 158-160°C. IR (KBr, cm^{-1}) ν 3401, 2924, 2854, 1731, 1644, 1585, 1447, 1334, 1125, 1048, 879, 611. ^{1}H-NMR (DMSO, 300MHz) δ 0.51, 0.76, 0.81, 0.86, 1.62 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 2.95 (m, 4H, 19-CH and 28-$CONHCH_2CONHCH(CH_2C_6H_5)COOCH_3$ and one of 23-CH_2), 3.36 (m, 2H, 3-CH and one of 23-CH_2), 3.63 (d, 2H, *J*=5.7 Hz, 28-$CONHCH_2CONHCH(CH_2C_6H_5)COOH$), 4.09 (d, 1H, *J*=5.2 Hz, 3-OH), 4.32 (t, 1H, 23-OH), 4.43 (m, 1H, 28-$CONHCH_2CONHCH(CH_2C_6H_5)COOH$), 4.52, 4.64 (d, 2H, *J*=34.9 Hz, 29=CH_2), 7.24 (m, 5H, 28-$CONHCH_2CONHCH(CH_2C_6H_5)COOH$), 7.69 (t, 1H, 28-$CONHCH_2CONHCH(CH_2C_6H_5)COOH$), 7.89 (d, 1H, *J*=7.8 Hz, 28-$CONHCH_2CONHCH(CH_2C_6H_5)$ COOH). MS (EI): *m/z* $[M+H]^+$ 677.4, $[M+Na]^+$ 699.5. HR-MS (ESI, M+H) *m/z*: calcd for $C_{41}H_{60}N_2O_6$ 677.4530, found 677.4533.

*N'-[N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-2-aminoethanoyl]-L-leucine (**7c**)*: **6c** (66.0mg, 0.10mmol). Yield: 54.0mg (84%) as a white solid, mp 242-244°C. IR (KBr, cm^{-1}) ν 3415, 2923, 2869, 1724, 1638, 1519, 1467, 1385, 1271, 1156, 1045, 882, 612. ^{1}H-NMR (DMSO, 300MHz) δ 0.51, 0.77, 0.82, 0.88, 1.62 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 2.95 (m, 1H, 19-CH), 3.06 (d, 1H, *J*=9.2 Hz, one of 23-CH_2), 3.41 (m, 2H, 3-CH and one of 23-CH_2), 3.65 (m, 2H, 28-$CONHCH_2CONHCH(CH_2CHCH_3CH_3)COOH$), 4.12 (m, 2H, 23-OH and 28-$CONHCH_2CONHCH$ $(CH_2CHCH_3CH_3)COOH$), 4.32 (t, 1H, 3-OH), 4.53, 4.64 (d, 2H, *J*=32.7 Hz, 29=CH_2), 7.72 (m, 2H, 28-$CONHCH_2CONHCH(CH_2CHCH_3CH_3)COOH$ and 28- $CONHCH_2CONHCH(CH_2CHCH_3CH_3)$ COOH). MS (EI): *m/z* $[M-H]^-$ 641.5. HR-MS (ESI, M+H) *m/z*: calcd for $C_{38}H_{62}N_2O_6$ 643.4686, found 643.4681.

*N'-[N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-2-amino-2-benzyl-ethanoyl]-L-leucine (**7d**)*:**6d** (75.0mg, 0.10mmol). Yield: 58.0mg (79%) as a white solid, mp 213-215°C. IR (KBr, cm^{-1}) ν 3417, 2951, 2868, 1715, 1639, 1519, 1467, 1383, 1200, 1153, 1045, 882, 699. ^{1}H-NMR (DMSO, 300MHz) δ 0.42, 0.52, 0.72, 0.78, 1.58 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 2.88 (m, 4H, 19-CH and one of 23-CH_2 and 28-CONHCH($CH_2C_6H_5$)CONHCH($CH_2CHCH_3CH_3$)COOH), 3.40 (2H, m, 3-CH and one of 23-CH_2), 4.09 (d, 1H, 3-OH), 4.28 (m, 2H, 23-OH and 28-CONHCH($CH_2C_6H_5$)CONH CH($CH_2CHCH_3CH_3$)COOH), 4.49 (m, 1H, 28-CONHCH($CH_2C_6H_5$)CONHCH($CH_2CHCH_3CH_3$) COOH), 4.49, 4.58 (d, 2H, *J*=29.1 Hz, 29=CH_2), 7.20 (m, 5H, 28-CONHCH($CH_2C_6H_5$)CONHCH($CH_2CHCH_3CH_3$)COOH), 7.67 (d, 1H, *J*=8.6 Hz, 28-CONHCH(CH_2 C_6H_5)CONHCH($CH_2CHCH_3CH_3$)COOH), 7.76 (d, 1H, *J*=7.8 Hz, 28-CONHCH($CH_2C_6H_5$)CONHCH ($CH_2CHCH_3CH_3$)COOH). MS (EI): *m/z* $[M-H]^-$ 731.5. HR-MS (ESI, M+H) *m/z*: calcd for $C_{45}H_{68}N_2O_6$ 733.5156, found 733.5159.

*N'-[N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-2-amino-2-benzyl-ethanoyl]-Glucine (**7e**)*: **6e** (69.0mg, 0.10mmol). Yield: 41.0mg (61%) as a white solid, mp 223-225°C. IR (KBr, cm^{-1}) ν 3415, 2940, 2867, 1714, 1638, 1521, 1448, 1384, 1204, 1042, 886, 616. ^{1}H-NMR (DMSO, 300MHz) δ 0.41, 0.51, 0.75, 0.86, 1.58 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 2.83 (m, 2H, 28-CONHCH($CH_2C_6H_5$)CONHCH$_2$COOH), 3.03 (m, 2H, 19-H and one of 23-CH_2), 3.36 (m, 2H, 3-CH and one of 23-CH_2), 3.79 (m, 2H, 28-CONHCH($CH_2C_6H_5$)CONHCH$_2$COOH), 4.09 (d, 1H, *J*=4.9 Hz, 3-OH), 4.32 (t, 1H, 23-OH), 4.48 (m, 1H, 28-CONHCH($CH_2C_6H_5$)CONHCH$_2$COOH), 4.48, 4.59 (d, 2H, *J*=32.4 Hz, 29=CH_2), 7.20 (m, 5H, 28-CONHCH($CH_2C_6H_5$)CONHCH$_2$COOH), 7.67 (d, 1H, *J*=8.6 Hz, 28-CONHCH($CH_2C_6H_5$)CONHCH$_2$COOH), 7.92 (t, 1H, 28-CONHCH($CH_2C_6H_5$)CONH CH_2COOH). MS (EI): *m/z* $[M-H]^-$ 675.5. HR-MS (ESI, M+H) *m/z*: calcd for $C_{41}H_{60}N_2O_6$ 677.4530, found 677.4535.

*N'-[N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-2-amino-2-(2'-isobutyl)-ethanoyl]-glucine (**7f**)*: **6f** (66.0mg, 0.10mmol). Yield: 46.0mg (72%) as a white solid, mp 188-190°C. IR (KBr, cm^{-1}) ν 3429, 2949, 2866, 1708, 1636, 1530, 1447, 1384, 1226, 1042, 886, 620. ^{1}H-NMR (DMSO, 500MHz) δ 0.50, 0.77, 0.83, 0.87, 1.62 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 3.02 (m, 2H, 19-CH and one of 23-CH_2), 3.40 (2H, m, 3-CH and one of 23-CH_2), 3.73 (m, 2H, 28-CONHCH($CH_2CHCH_3CH_3$)CONH CH_2COOH), 4.09 (d, 1H, *J*=4.8 Hz, 3-OH), 4.34 (m, 2H, 23-OH and 28-CONHCH($CH_2CHCH_3CH_3$) CONHCH$_2$COOH), 4.52, 4.64 (d, 2H, *J*=58.8 Hz, 29=CH_2), 7.54 (d, 1H, *J*=8.5 Hz, 28-CONHCH ($CH_2CHCH_3CH_3$)CONHCH$_2$COOH), 7.80 (t, 1H, 28-CONHCH($CH_2CHCH_3CH_3$)CONHCH$_2$COOH). MS (EI): *m/z* $[M+H]^+$ 643.5, $[M+Na]^+$ 665.4, $[M-H]^-$ 641.4. HR-MS (ESI, M+H) *m/z*: calcd for $C_{38}H_{62}N_2O_6$ 643.4686, found 643.4684.

*N'-[N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-2-amino-2-benzyl-ethanoyl]-L-glutamic acid (**7g**)*: **6g** (78.0mg, 0.10mmol). Yield: 50.0mg (67%) as a white solid, mp 218-220°C. IR (KBr, cm^{-1}) ν 3415, 2927, 2859, 1721, 1636, 1514, 1454, 1384, 1266, 1131, 1040, 613. ^{1}H-NMR (DMSO, 300MHz) δ 0.52, 0.78, 0.80, 0.84, 0.97 (s, 3H each, 24, 25, 26, 27and 30-CH_3), 2.96 (m, 4H, 28-CONHCH($CH_2C_6H_5$)CONHCH(CH_2CH_2COOH)COOH and 19-H and one of 23- CH_2), 3.32 (m, 2H, 3-CH and one of 23-CH_2), 4.22 (m, 5H, 23-OH and 28-CONHCH($CH_2C_6H_5$)CONHCH(CH_2CH_2CO OH)COOH and 3-OH and 23-OH and one of 29=CH_2), 5.04 (s, 1H, one of 29=CH_2), 7.20 (m, 5H, 28-CONHCH($CH_2C_6H_5$)CONHCH(CH_2CH_2COOH)COOH), 7.70 (d, 1H, *J*=4.3 Hz, 28-CONHCH(CH_2 C_6H_5)CONHCH(CH_2CH_2COOH)COOH), 8.06 (d, 1H, *J*=7.9 Hz, 28-CONHCH($CH_2C_6H_5$)CONHCH (CH_2CH_2COOH)COOH). MS (EI): *m/z* $[M+H]^+$ 749.5, $[M-H]^-$ 747.5. HR-MS (ESI, M+H) *m/z*: calcd for $C_{44}H_{64}N_2O_8$ 749.4741, found 749.4745.

2.3 Cytotoxic activity in vitro[11]

The cytotoxic activity in vitro was measured using the MTT assay. The tumor cell line panel consisted of A549, BEL-7402, SF763, B16 and HL-60 (final concentration in the growth medium was $2\sim4\times10^4$/mL). MTT solution (20μL/well) was added after cells were treated with drug for 48 h, and cells were incubated for a further 4 h at 37℃ . The purple formazan crystals were dissolved in 150μl DMSO. After 5 min, the plates were read on an automated microplate spectrophotometer at 570 nm. Assays were performed in triplicate in three independent experiments. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated. In all of these experiments, three replicate wells were used to determine each point.

3. Results and Discussion

As shown in Figure **2**, treatment of **1** with Ac_2O and then $(COCl)_2$ in CH_2Cl_2 and then $H_2NCHR^1COOCH_3\cdot HCl$ in the presence of DMAP produced compounds **4a-f** in 51-67% yields. Hydrolysis of **4a-f** with 4N NaOH in THF and CH_3OH furnished derivatives **5a-f** (71-84%)，among which, **5a**, **5c** and **5d** were then treated with $H_2NCHR_2COOCH_3\cdot HCl$ in the presence of EDC and HOBT to give corresponding amides **6a-g** in the yields of 63-80%. Finally, alkaline catalyzed hydrolysis of the resulted amides gave C-17 amide derivatives **7a-g** in the yields of 61-85%.[13-14]

The cytotoxic activity of 23-hydroxy betulinic acid, betulinic acid and all derivatives in vitro was determined by the MTT cytotoxicity assay, and the result is summarized in Table 1. Many different cell lines were used: A549 (human lung carcinoma), BEL-7402 (human hepatoma), SF-763 (human cerebroma), B16 (mice melanoma), HL-60 (human leukaemia). The MTT assay results showed that most of the 23-hydroxy betulinic acid derivatives had better cytotoxic activities against the tested cells than betulinic acid and 23-hydroxy betulinic acid.

Compounds **4a-f** displayed moderate cytotoxic activities against all cell lines espect **4a** and **4b**.

Compounds **5a-f** showed no cytotoxicity, despite the carboxylic acid substitution at the end of C-28 side chain. The reason maybe that the polarity of the compounds is too strong and affect the penetration of them into cells.

In the series of **6a-g**, only **6g** revealed potent cytotoxicity against all cell lines with IC50 values ranging from 22.65 to 31.97 μM. In A549 and HL-60 cell lines, **6a-f** had better activity than betulinic acid and 23-hydroxy betulinic acid. In SF-763, compounds **6b**, **6e**, **6f** exhibited better activity than betulinic acid and 23-hydroxy betulinic acid. Compounds **6a**, **6c**, **6d** showed better activity than 23-hydroxy betulinic acid but less potent than betulinic acid. In BEL-7402 and B-16, **6a-f** had better activity than 23-hydroxy betulinic acid but weaker than betulinic acid.

Compounds **7a-g** showed almost the same cytotoxic activity as 23-hydroxy betulinic acid. The end of the C-28 side chain in **7a-g** was carboxylic acid, which maybe affect the activity of derivatives due to the large polarity.

4. Conclusions

In summary, a series of novel C-28 amide derivatives modified on 17-carboxylic acid of 23-hydroxy betulinic acid were obtained and tested for their cytotoxic activities against five human tumor cell lines in vitro. Most of the amide derivatives showed moderate potent cytotoxic activities on all the tested cells except for compounds **5a-f.** The compounds **4a**, **4b** and **6g** have the most potent cytotoxic activity. The terminal group and branched chains on the C-28 side chain maybe have a major impact on their antitumor activity.

The further structure modification and SAR studies of 23-hydroxy betulinic acid derivatives are in progress in our laboratory and the results will be reported in due course[15].

Table1. The cytotoxicity data of 23-hydroxy betulinic acid and its derivatives [IC50 (μmol/L) ±SD]

Compound	Cell line				
	A549	**BEL-7402**	**SF-763**	**B16**	**HL-60**
HBA	81.36±3.54	89.81±7.32	90.09±8.31	75.64±8.55	80.54±9.13
BA	89.62±11.23	52.51±2.55	78.89±9.24	50.09±7.32	76.77±10.58
4a	39.02±10.71	28.46±5.78	21.08±7.56	45.13±7.07	21.63±6.52
4b	41.64±9.68	29.32±4.51	33.55±9.10	59.80±14.45	28.45±9.49
4c	70.09±9.16	35.08±8.22	59.98±5.44	71.17±9.35	59.75±10.37
4d	80.50±14.02	54.61±6.74	80.82±6.03	69.06±5.87	87.74±8.23
4e	68.56±4.25	43.80±3.51	79.89±9.24	71.54±8.32	56.05±12.01
4f	57.90±12.45	40.61±2.22	54.35±8.41	70.09±6.67	39.79±10.02
5a	>100	>100	>100	>100	>100
5b	>100	>100	>100	>100	>100
5c	>100	>100	>100	>100	>100
5d	>100	>100	>100	>100	>100
5e	>100	>100	>100	>100	>100
5f	>100	>100	>100	>100	>100
6a	72.89±10.83	56.90±4.67	85.14±16.42	74.43±9.46	73.10±12.07
6b	56.63±2.79	68.81±10.07	59.88±9.67	67.48±13.06	68.12±7.06
6c	69.50±6.78	55.79±9.34	82.54±11.75	71.58±9.06	76.54±8.89
6d	73.03±16.35	58.53±8.40	81.94±10.51	77.32±10.24	75.31±14.23
6e	53.80±8.45	60.32±7.87	56.43±6.30	60.08±14.51	53.58±3.86
6f	45.41±10.91	51.74±8.32	40.52±7.88	56.86±13.02	44.30±7.53
6g	28.75±12.53	26.09±4.40	28.43±8.19	31.97±10.32	22.65±3.49
7a	83.12±6.24	79.91±15.05	78.11±15.06	89.53±9.39	84.03±4.02
7b	88.68±17.87	80.57±10.32	84.71±9.80	91.26±9.02	79.06±18.10
7c	81.92±1.24	87.85±24.05	92.14±15.04	90.03±11.02	85.73±8.72
7d	84.15±16.57	94.37±8.29	82.69±6.54	86.63±13.76	79.37±22.01
7e	78.66±11.30	100.15±9.46	90.56±13.04	95.53±14.12	86.08±10.70
7f	86.31±10.16	103.17±7.52	95.08±14.09	98.08±16.32	84.07±5.74
7g	71.01±18.57	89.08±7.34	93.60±12.18	91.35±12.07	86.71±13.02

BA: betulinic acid, HBA: 23-hydroxy betulic acid
Data is mean of three experiments.

Acknowledgments

This study was supported by grant from National Natural Science Fund (No. 30472083 and No. 81273377), Key fund of Ministry of Education of China (No. 108069) and Taishan Scholar Project to Fenghua Fu for financial assistance.

References

[1] E. Pisha, H. Chai, I-S Lee, T. E. Chagwedera, N. R. Farnsworth, G. A. Cordell, C. W. W. Beecher, H. H. S. Fong, A. D. Kinghorn, D. M. Brown, M. C. Wani, M. E. Wall, T. J. Hieken, T. K. D. Gupta and J. M. Pezzuto (1995). Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis, *Nat. Med*. **1**, 1046-1051.

[2] W. C. Ye, N. N. Ji, S. X. Zhao, J. H. Liu, T. Ye, M.A. McKervey and P. Stevenson (1996). Triterpenoids from Pulsatilla chinensis, *Phytochemistry*. **42**, 799-802.

[3] V. Zuco, R. Supino, S. C. Righetti, L. Cleris, E. Marchesi, C. Gambacorti-Passerini and F. Formelli (2002). Selective cytotoxicity of betulinic acid on tumor cell lines, but not on normal cells, *Cancer Letters*. **175**, 17–25.

[4] R. C. Santos, J. A. R. Salvador, R. Cortés, G. Pachón, S. Marín and M. Cascante (2011). New betulinic acid derivatives induce potent and selective antiproliferative activity through cell cycle arrest at the S phase and caspase dependent apoptosis in human cancer cells, *Biochimie*. **93**, 1065-1075.

[5] I. Baglin, A. C. Mitaine-Offer, M. Nour, K. Tan, C. Cave and M. A. Lacaille-Dubois (2003). A review of natural and modified betulinic, ursolic and echinocystic acid derivatives as potential antitumor and anti-HIV agents, *Mini-Rev Med Chem*. **3**, 525-539.

[6] S. Fulda and K. M. Debatin (2005). Sensitization for Anticancer Drug-Induced Apoptosis by Betulinic Acid, *Neoplasia*. **7**, 162-170.

[7] Y. Perumal and S. Dharmarajan (2005). Betulinic Acid and Its Derivatives: A Review on their Biological Properties, *Curr Med Chem*. **12**, 657-666.

[8] R. Mukherjee, M. Jaggi, P. Rajendran, M. J. A. Siddiqui, S. K. Srivastava, A. Vardhanb and A. C. Burman (2004). Betulinic acid and its derivatives as anti-angiogenic agents, *Bioorg Med Chem Lett*. **14**, 2181-2184.

[9] H. J. Finlay, T. Honda and G. W. Gribble (2002). Synthesis of novel [3,2-b]indole fused oleanolic acids as potential inhibitors of cell proliferation, *ARKIVOC*. **xii**, 38-46.

[10] Z. N. Ji, W. C. Ye, G. G. Liu and W. L. W. Hsiao (2002). 23-Hydroxybetulinic acid mediated apoptosis is accompanied by decreases in bcl-2 expression and telomerase activity in HL-60 cells, *Life Sci.* **72**, 1-9.

[11] Y. Bi, J.Y. Xu, X. M. Wu, W. C. Ye, S. T. Yuan and L.Y. Zhang (2007). Synthesis and cytotoxic activity of 17-carboxylic acid modified 23-hydroxy betulinic acid ester derivatives, *Bioorg Med Chem Lett*, **17**, 1475–1478.

[12] J. P. Zhou, D. Li, X. M. Wu, W. C. Ye and L. Y. Zhang (2007). Synthesis and antitumor activity of derivatives of 23-hydroxybetulinic acid, *Chin Chem Lett*. **18**, 1195-1198.

[13] I. C. Sun, C. H. Chen, Y. Kashiwada, J. H. Wu, H. K. Wang and K. H. Lee (2002). Anti-AIDS Agents 49 Synthesis, Anti-HIV, and Anti-Fusion Activities of IC9564 Analogues Based on Betulinic Acid, *J. Med. Chem.* **45**, 4271-4275.

[14] H. J. Jeong, H. B. Chai, S. Y. Park and D. S. H. L. Kim (1999). Preparation of amino acid conjugates of betulinic acid with activity against human melanoma, *Bioorg Med Chem Lett*. **9**, 1201-1204.

[15] X. M. Wu, J. Y. Xu, Y. Bi, J. P. Zhou, L. Y. Zhang, S. T. Yuan, D. Li and P. Xu (2009). Synthesis method, application and pretaration of 23-hydroxy betulinic acid derivatives, CHN 200610040277, In Chinese.