Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Medicinal Chemistry 40 (2005) 582–589
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
www.elsevier.com/locate/ejmech

The cytotoxic activity of ursolic acid derivatives

Chao-Mei Ma [a,b], Shao-Qing Cai [a,*], Jing-Rong Cui [a], Rui-Qing Wang [a], Peng-Fei Tu [a], Masao Hattori [c], Mohsen Daneshtalab [b,*]

[a] *School of Pharmaceutical Sciences, Peking University, No. 38 Xueyuan Road, Haidian District, Beijing 100083, China*
[b] *School of Pharmacy, Memorial University of Newfoundland, St. John's, NL, Canada A1B 3V6*
[c] *Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan*

Received 2 November 2004; received in revised form 23 December 2004; accepted 7 January 2005

Available online 08 February 2005

Abstract

Ursolic acid and 2α-hydroxyursolic acid isolated from apple peels were found to show growth inhibitory activity against four tumor cell lines, HL-60, BGC, Bel-7402 and Hela. Structural modifications were performed on the C-3, C-28 and C-11 positions of ursolic acid and the cytotoxicity of the derivatives was evaluated. The SAR revealed that the triterpenes possessing two hydrogen-bond forming groups (an H-donor and a carbonyl group) at positions 3 and 28 exhibit cytotoxic activity. The configuration at C-3 was found to be important for the activity. Introduction of an amino group increased the cytotoxicity greatly. A 3β-amino derivative was 20 times more potent than the parent ursolic acid. The 28-aminoalkyl dimer compounds showed selective cytotoxicity.

Keywords: Apple peel; Triterpene; Ursolic acid derivative; Anti-tumor; Cytotoxic activity

1. Introduction

Triterpenes, especially, ursolic acid, oleanolic acid and betulinic acid exist abundantly in the plant kingdom. These triterpenes and their derivatives have been reported to have interesting bioactivity, such as anti-HIV [1–4], inhibition of HIV protease [5] and cytotoxicity to tumor cell lines [6–8]. Renewed interest in the cytotoxic activity of triterpenes has come from the findings that betulinic acid exhibited selective cytotoxicity against melanoma and had apoptosis induction properties [9]. Considerable structural modification has been performed on betulinic acid and potentially important derivatives, which may be developed as anti-tumor drugs, have been produced [10,11]. Ursolic acid and its esterified derivatives have also been reported to show significant cytotoxicity against some tumor cell lines [7,8]. Some oleanane and ursane triterpenoids with modified rings A and C have been reported to have high inhibitory activity against nitric oxide production. This suggests the potential of these compounds as cancer chemopreventive drugs, as excessive production of NO, which is closely related mechanistically to carcinogenesis can destroy functional normal tissues [12–16].

However, when compared to betulinic acid derivatives, ursolic acid derivatives have not been thoroughly explored for their cytotoxic activity. In order to find biologically more active derivatives of this naturally occurring triterpene, we selected apple peel extract as the source of these triterpenes. The present article describes the isolation and cytotoxicity of triterpenes from apple peels and the structural modification of ursolic acid.

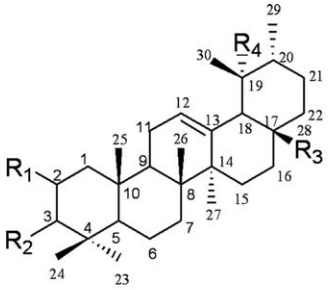
2. Chemistry

The ethyl acetate extract of the peel of apples (*Malus pumila* Mill) was subjected to column chromatography over Silica gel, ODS and HPLC to obtain six compounds. These compounds were identified as ursolic acid (**1**), 2α-hydroxyursolic acid (**2**), euscaphic acid (**3**), uvanol (**4**), 2α, 3α-dihydroxy-urs-12-en-28-oic acid (**5**), 2α, 3α-dihydroxy-olean-12-en-28-oic acid (**6**), by analyzing and comparing their spectral data with those reported in literature [17] (Fig. 1).

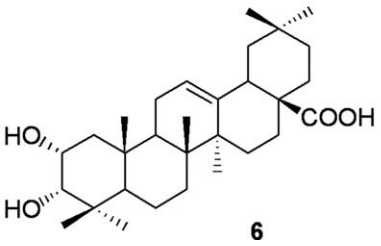
Compounds **1**–**4**, which were obtained in relatively large amounts, were tested for their cytotoxicity. Ursolic acid (**1**)

* Corresponding authors. Tel.: +1 709 777 6958; fax: +1 709 777 7044.
E-mail address: mohsen@pharm.mun.ca (M. Daneshtalab).

0223-5234/$ - see front matter © 2005 Elsevier SAS. All rights reserved.
doi:10.1016/j.ejmech.2005.01.001



	R_1	R_2	R_3	R_4
1	H	HO	COOH	H
2	HO	HO	COOH	H
3	HO	HO	COOH	OH
4	H	HO	CH_2OH	H
5	HO	HO	COOH	H



6

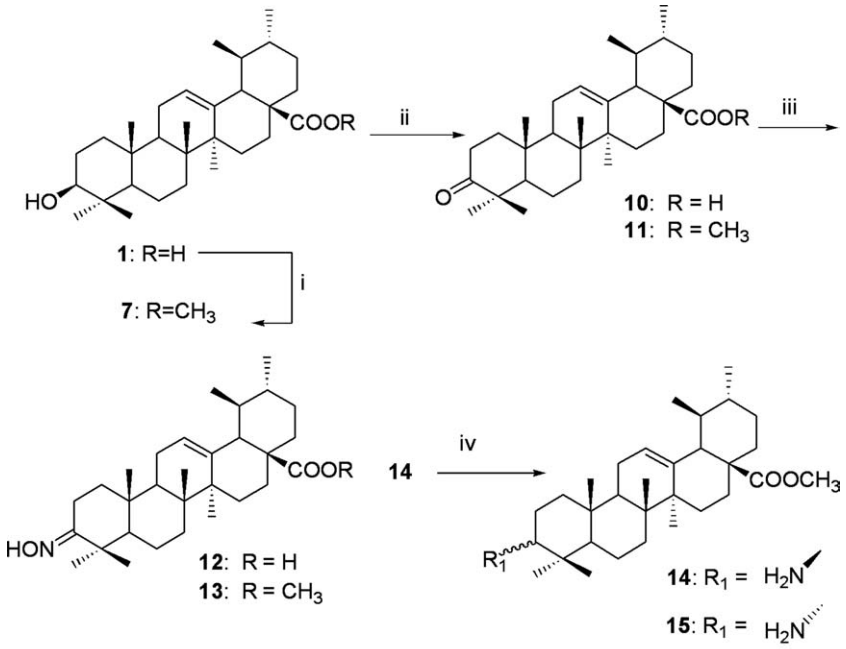
Fig. 1. Triterpenes isolated from apple peels.

and 2α-hydroxyursolic acid (**2**), with a β-hydroxyl group at C-3 and a carboxyl group at C-17, were found to show cytotoxicity against all the four tumor cell lines tested (HL-60, BGC, Bel-7402 and Hela). Compound **3**, whose structure is different from **2** due to the configuration of C-3 hydroxyl group, did not show cytotoxicity even at 100 μg/ml. Compound **4**, with a hydroxymethyl group at C-17, did not show cytotoxicity either. Ursolic acid (**1**) was obtained in the largest amount and thus was used as starting material for structure modification.

Ursolic acid and its methyl ester (obtained by treating ursolic acid with diazomethane) were oxidized with pyridinium chlorochromate (PCC) to give the 3-oxo compounds, **10** and **11**. Treatment of the 3-oxo compounds with NH_2OH in pyridine yielded the hydroxyimino compounds **12** and **13**. Reduction of **13** with $TiCl_3$ and $NaCNBH_3$ followed by column chromatography yielded two 3-amino compounds, **14** and **15** (Scheme 1). The large coupling constants of H-3 in the ^1H NMR of **14** [δ 2.38 (1H, dd, J = 12.0, 5.7 Hz)] compared with that of **15** [δ 2.67 (1H, br. s)] indicated that H-3 is α orientated in **14** and β orientated in **15** (i.e. compound **14** is 3β-amino compound and **15** is its 3α isomer) in Scheme 1.

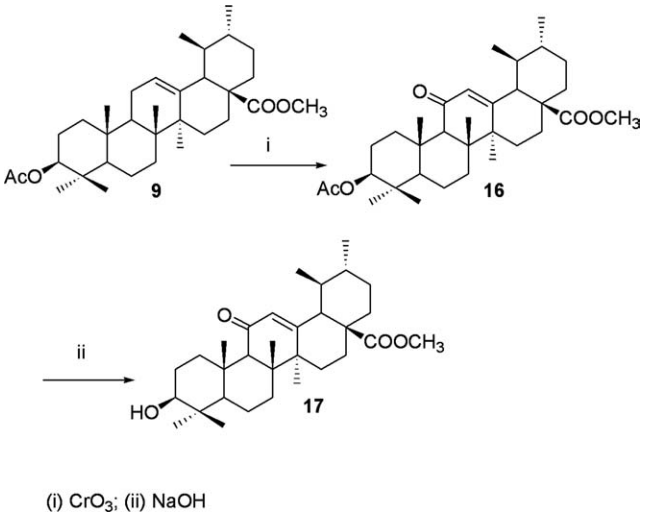
Acetyl ursolic acid methyl ester was refluxed with CrO_3 in acetic acid to give the 11-oxo compound, **16**. Alkaline hydrolysis of **16** yielded the 3-hydroxy-11-oxo derivative, **17**, as depicted in Scheme 2.

Compounds **19**, **21** and **23** were synthesized via the route outlined in Scheme 3. Ursolic acid was first converted to its acetyl ester, which was then treated with oxalyl chloride to give the corresponding acid chloride. Condensation of 1,9-diaminononane or diethylenetriamine with acetyl ursolic acid



(i) CH_2N_2; (ii) PCC; (iii) NH_2OH; (iv) $NaBH_3CN, TiCl_3$

Scheme 1. Synthesis of ursolic acid derivatives with different substituents at C-3.



(i) CrO_3; (ii) NaOH

Scheme 2. Synthesis of C-11 oxidized ursolic acid.

chloride followed by chromatography, produced compounds **19**, **21** and **23**. Saponification of these compounds yielded compounds **18**, **20** and **22**, respectively, in Scheme 3.

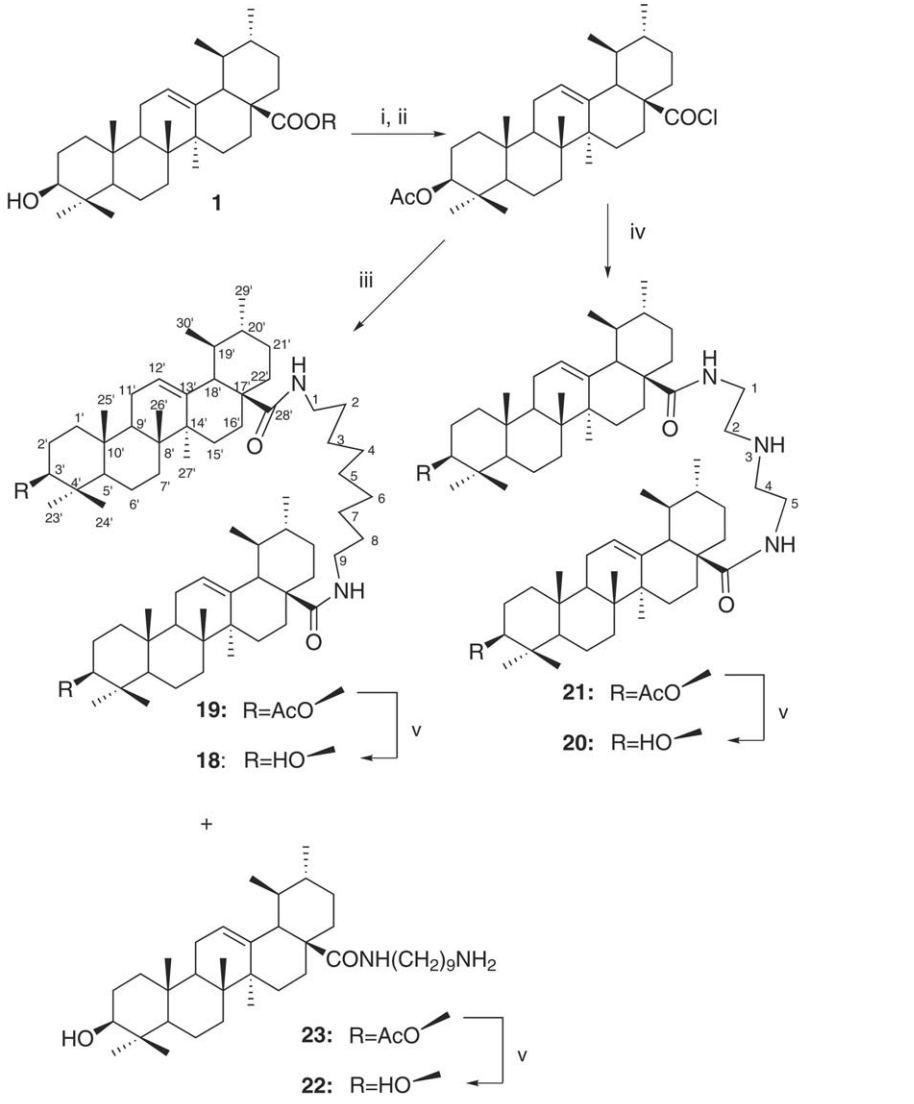
3. Biological activity

The cytotoxicity of all compounds was assessed against different cancer cell lines, as described in Section 6.

4. Results and discussion

The structures of the synthesized compounds are depicted in Fig. 2 and the corresponding in vitro cytotoxicity results are summarized in Table 1.

The ursolic acid methyl ester (**7**) showed similar activity as that of ursolic acid on all cell lines tested. The lack of cyto-



(i) $(AcO)_2O$, pyridine; (ii) $(COCl)_2$; (iii) $NH_2(CH_2)_9NH_2$; (iv) $NH_2(CH_2)_2NH(CH_2)_2NH_2$; (v) NaOH

Scheme 3. Synthesis of ursolic acid derivatives with substituents at C-17.

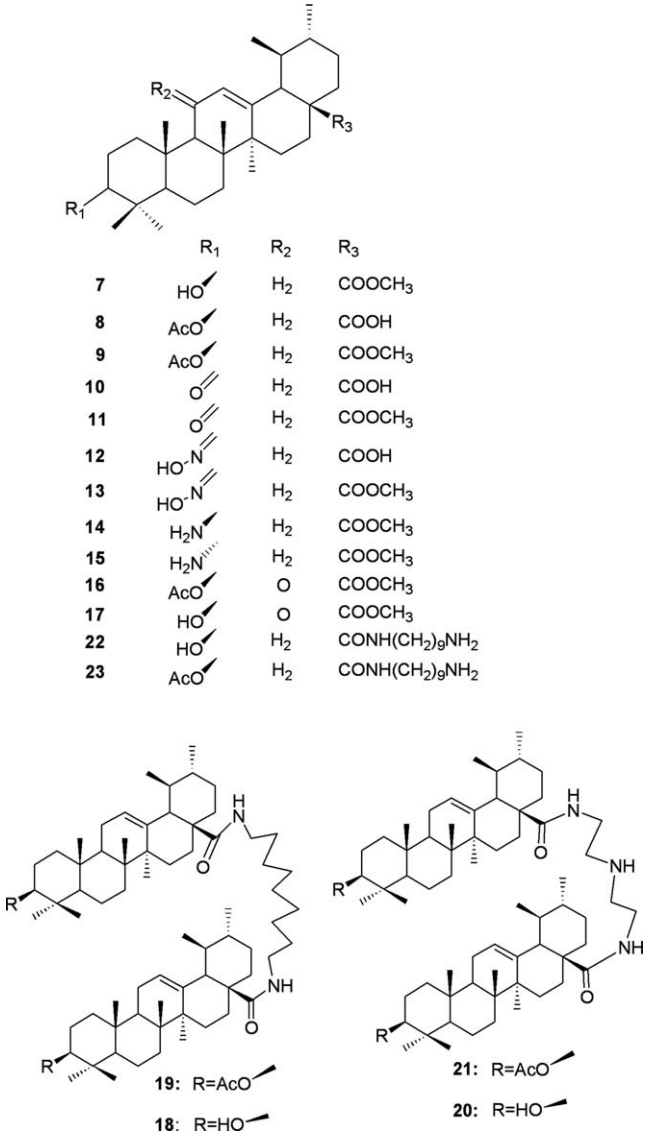


Fig. 2. Structures of ursolic acid derivatives for anti-tumor assay.

toxicity of compound **4**, with a hydroxyl group but no carbonyl group at C-17, suggests that a carbonyl group at position 17 is necessary for the cytotoxicity. Introduction of an additional oxo group to position 11 did not improve the cytotoxic activity (**16** vs. **9**), (**17** vs. **7**).

Among the C-3 modified derivatives, acetylursolic acid (**8**) was slightly less active than ursolic acid against BGC cell line. Esterification of both the 3-OH and 28-COOH of ursolic acid to yield compound **9** resulted in loss of cytotoxic activity. Compared to ursolic acid, the 3-oxo derivative (**10**) demonstrated more potent cytotoxicity against HL-60 and similar cytotoxicity against other cell lines tested. However, when the 28-COOH of the 3-oxo compound was methylated (**11**), the cytotoxicity once again was lost. The C-3 hydroxyimino derivatives (**12** and **13**) showed a cytotoxicity comparable to those of ursolic acid and ursolic acid methyl ester against all of the four cell lines. This evidence suggests that a hydrogen donor group at either position 3 and/or 28 is essential for the cytotoxic activity.

Table 1
Cytotoxicity of ursolic acid derivatives

Compound	ED_{50} (μg/ml)			
	HL-60	BGC	Bel-7402	Hela
1	72.0	53.7	45.0	49.4
2	49.0	55.9	54.8	55.0
3	> 100.0	> 100.0	> 100.0	> 100.0
4	> 100.0	> 100.0	> 100.0	> 100.0
5	nt	nt	nt	nt
6	nt	nt	nt	nt
7	54.8	54.1	41.5	51.8
8	55.9	100.0	58.4	50.0
9	> 100.0	> 100.0	> 100.0	> 100.0
10	19.5	60.0	58.5	76.5
11	> 100.0	> 100.0	> 100.0	> 100.0
12	53.3	53.3	38.1	50.1
13	88.3	71.2	58.0	64.3
14	2.0	2.5	1.7	2.4
15	79.5	> 100.0	39.6	55.2
16	>100.0	> 100.0	> 100.0	> 100.0
17	51.1	> 100.0	> 100.0	> 100.0
	HL-60	BGC	MDA-MB-435	
18	35.0	> 100.0	90.0	
19	> 100.0	> 100.0	> 100.0	
20	< 1.0[a]	> 100.0	> 100.0	
21	< 1.0[a]	> 100.0	> 100.0	
22	5.0	30.0	8.0	
23	5.0	35.0	95.0	

nt: not tested.

[a] Cytotoxicity only changed a little in the concentration range of 1–10 μg/ml.

Of the two 3-amino isomers, the 3β-form was 20 times more potent cytotoxic than the 3α-form (**14** vs. **15**), confirming that the configuration at C-3 is an important factor on the activity. The 3α-amino derivative exhibited somewhat selective cytotoxicity, showing similar cytotoxicity on HL-60, Bel-7402 and Hela cell lines but little toxicity against BGC cell line, compared with ursolic acid.

Significant improvement of cytotoxicity against HL-60 cell line was observed when amino alkyl groups were introduced to position 28 (**20**–**23**), whether or not the 3-OH group was free or acetylated. In the case of dimeric compounds with a secondary amino alky group (**20**, **21**), selective cytotoxicity was observed only against HL-60 cell lines. The dimeric compounds (**18**, **19**) with amide but no amino alkyl groups in position 28 showed only a little cytotoxicity, while the 3-acetylated analogue (**19**) was inactive.

It is interesting to note that the concentration dependent cytotoxicity patterns against HL-60 cell lines are quite similar among the dimers with a free secondary amino group (**20**, **21**). While the cytotoxicity of both compounds increases obviously when the concentration is increased from 10 to 100 μg/ml, there is only a little change in the pattern of cytotoxicity at concentrations between 1 and 10 μg/ml. In contrast, the cytotoxicity of compounds with a free primary amino group (**22**, **23**) drops sharply when concentration changes from 10 to 1 μg/ml. These phenomena may suggest that the mechanism of cytoxicity of the dimeric compounds (**20**, **21**) is different from that of the monomer compounds.

5. Conclusions

The above results indicate that apple peel can serve as a useful source of bioactive compounds, such as ursolic acid, which can be modified to more potent bioactive derivatives. Ursolic acid derivatives possessing two hydrogen-bond forming groups (an H-donor and a carbonyl group) at positions 3 and 28 exhibit cytotoxic activity. The configuration at C-3 was found to be important for the activity. Compounds with a β-orientated hydrogen-bond forming group at C-3 exhibit more potent cytotoxicity than the α-counterpart. Introduction of an amino-bearing group in either C-3 or C-28 position could greatly increase the cytotoxicity.

Ursolic acid has been reported to have significant cytotoxic activity [6–8,18]. A study on mechanism revealed that ursolic acid blocked the cell cycle progression in the G1 phase and that ursolic acid treatment resulted in the triggering of apoptosis as determined by a DNA fragmentation assay [18]. The present work has provided more potent cytotoxic derivatives of ursolic acid, such as the 3-amino and 28-aminoalkyl compounds. These compounds are now undergoing further mechanistic and pharmacologic studies for the possibility of development as anticancer drugs. The SARS described herein can serve as the basis for the synthesis of more potent usolic acid derivatives as anti-tumor drugs.

6. Experimental protocols

6.1. Extraction and Isolation

The apple peels were cut from the fruit of *M. pumila* Mill purchased in April, 2002 from the basement of the Lang Qiu Yuan Supermarket, Xue Yuan Road, Beijing, China.

The commercially obtained apple, (27 kg), was peeled to obtain the fresh apple peel. It was extracted with ethyl acetate by supersonication three times (2 h per time). After being concentrated in vacuo, the extract was defatted by petroleum ether (125 ml × 8) under supersonication and the white precipitate was collected to get the triterpene mixture (25 g). The triterpene mixture was separated by silica gel column chromatography, eluted with hexane–chloroform–acetone to get 43 fractions. Fr. 1–3 was purified with ODS eluted with H_2O–MeOH to get **4** (10 mg); Ursolic acid (**1**, 19 g) was obtained from Fr. 4–24. Fraction 25–34 was purified with ODS eluted with H_2O–MeOH and purified with HPLC to obtain **6** (2 mg) and **5** (3 mg); Fraction 35–40 was purified with ODS eluted with H_2O–MeOH to obtain **3** (200 mg); Fraction 41–43 was purified with ODS eluted with H_2O–MeOH and purified with HPLC to obtain compound **2** (30 mg). These compounds were identified by comparing their spectra data with the literature [17].

6.2. Chemistry

Optical rotation was measured with an AA-10R polarimeter manufactured by the Optical Activity Co. Ltd. NMR spectra were obtained on a Bruker AVANCE 500 MHz or on a JEOL JNM-AL 300 NMR spectrometer with TMS as internal standards. APCI and ESI mass spectra were measured on an Agilent 1100 series LC/MSD system. HRFAB-MS were measured on a JEOL JMS-700 apparatus with a resolution of 5000 and with *m*-nitrobenzyl alcohol as matrix. Silica gel used in column chromatography was provided by Tsingtao Marine Chemistry Co. Ltd. (200–300 mesh) or Sigma-Aldrich Com. (Silica gel, Davisil, 100–200 mesh). Aluminum oxide (neutral, –150 mesh) and octadecyl-functionalized silica gel (ODS) were obtained from Sigma-Aldrich. The Organic solvents for chromatography were obtained from Fisher Scientific. Chemicals for chemical synthesis were obtained from Sigma-Aldrich. Semi-preparative HPLC was carried out on a system Gold HPLC systems with an Agilent Zorbax SB-C18 column (9.4 × 250 mm) and methanol–water (80–100%) as mobile phase.

6.2.1. Methylation and acetylation of ursolic acid

Methyl ursolate (**7**) was prepared by treating ursolic acid with diazomethane in ether. Ursolic acid (**1**) was treated with acetic anhydride and pyridine (1:1) at room temperature overnight and worked up as usual to get acetyl ursolic acid (**8**). Compound **8** was treated with diazomethane get compound **9**.

6.2.2. 3-Oxo-urs-12-en-28-oic acid (*10*)

To a solution of ursolic acid (1.9 g, 4.1 mmol) in acetone–CH_2Cl_2 (10 ml) was added PCC (2.7 g, 12.5 mmol). After being stirred at room temperature for 8 h, the mixture was concentrated and partitioned with H_2O and $CHCl_3$. The $CHCl_3$ layer was concentrated and purified by silica gel column chromatography eluted with *n*-hexane–acetone (95:5) to give **10**. Yield 57%; amorphous powder; ^{1}H NMR ($CDCl_3$) δ 0.81 (3H, s, CH_3), 0.84 (3H, d, J = 6.6 Hz, CH_3), 0.93 (3H, d, J = 6.6 Hz, CH_3), 1.01 (3H, s, CH_3), 1.03 (3H, s, CH_3), 1.07 (6H, s, CH_3), 2.18 (1H, d, J = 12.0 Hz, H-18), 2.36 (1H, m, H_{2a}), 2.50 (1H, m, H_{2b}), 5.24 (1H, *t*-like, H-12); MS (EI): 454 $[M]^+$ (5), 408 (10), 248 (100), 203 (67), 133 (75).

6.2.3. 3-Oxo-urs-12-en-28-oic acid methyl ester (*11*)

Methyl ursolate was reacted in the same fashion with PCC as above to get compound **11**. Yield 90%; amorphous powder; ^{1}H NMR ($CDCl_3$) δ 0.80 (3H, s, CH_3), 0.87 (3H, d, J = 6.6 Hz, CH_3), 0.95 (3H, d, J = 6.6 Hz, CH_3), 1.04 (3H, s, CH_3), 1.05 (3H, s, CH_3), 1.08 (6H, s, CH_3), 2.25 (1H, d, J = 12.0 Hz, H-18), 2.38 (1H, m, H_{2a}), 2.55 (1H, m, H_{2b}), 3.63 (3H, s, CH_3O-28), 5.28 (1H, *t*-like, H-12); MS (EI): 468 $[M]^+$ (10), 453 (4), 408 (12), 393 (10), 262 (80), 203 (93), 133 (100).

6.2.4. 3-Hydroxyimino-urs-12-en-28-oic acid (*12*)

A solution of **10** (420 mg, 0.9 mmol) and hydroxylamine hydrochloride (145 mg, 2.5 mmol) in pyridine (2 ml) was heated for four hour at 50 °C. After cooling to room temperature, the reaction mixture was concentrated under vacuum to

dryness, then purified with RP-18 (MeOH, 90–100%) to obtain **12**. Yield 49%; colorless powder; ^{1}H NMR ($CDCl_3$) δ 0.82 (3H, s, CH_3), 0.88 (3H, d, J = 6.6 Hz, CH_3), 0.96 (3H, d, J = 6.6 Hz, CH_3), 1.02 (3H, s, CH_3), 1.05 (3H, s, CH_3), 1.08 (3H, s, CH_3), 1.16 (3H, s, CH_3), 2.22 (1H, d, J = 12.0 Hz, H-18),), 2.26 (1H, m, H-2a), 3.04 (1H, m, H-2b), 5.28 (1H, *t*-like, H-12); Negative APCI-MS: 468 (M^+-H, 100%).

*6.2.5. 3-Hydroxyimino-urs-12-en-28-oic acid methyl ester (**13**)*

A solution of **11** (366 mg, 0.78 mmol) and hydroxylamine hydrochloride (250 mg, 3.6 mmol) in pyridine (5 ml) was heated for 2 h at 50 °C. After cooling to room temperature, the reaction mixture was diluted with CH_2Cl_2 (75 ml) and washed with 10% HCl (3 × 50 ml). The CH_2Cl_2 layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The product was crystallized from diethyl ether and CH_2Cl_2 to obtain **13**. Yield 90%; colorless powder; ^{1}H NMR ($CDCl_3$) δ 0.80 (3H, s, CH_3), 0.87 (3H, d, J = 6.6 Hz, CH_3), 0.96 (3H, d, J = 6.6 Hz, CH_3), 1.02 (3H, s, CH_3), 1.04 (3H, s, CH_3), 1.06 (3H, s, CH_3), 1.17 (3H, s, CH_3), 2.18 (1H, m, H-2a), 2.25 (1H, d, J = 12.0 Hz, H-18), 3.03 (1H, m, H-2b), 3.62 (3H, s, CH_3O-28), 5.27 (1H, *t*, J = 3.0 Hz, H-12); MS (EI): 483 $[M]^+$ (4), 406 (7), 262 (87), 203 (100), 133 (96).

*6.2.6. 3β-Amino-urs-12-en-28-oic acid methyl ester (**14**) and 3α-amino-urs-12-en-28-oic acid methyl ester (**15**)*

Sodium cyanoborohydride (0.29 g, 9 mmol) was added to a methanol solution of **13** (200 mg, 0.41 mmol) and ammonium acetate (0.37 g, 6.8 mmol) under nitrogen atmosphere. The solution was cooled in ice water and 15% aqueous titanium trichloride (1.2 ml, 1.4 mmol) was added dropwise over 20 min. The mixture was stirred at room temperature for 12 h then adjusted with 2 N sodium hydroxide to pH 10. The aqueous solution was extracted with CH_2Cl_2, and the organic layer was washed with distilled water and concentrated to dryness. The crude products were subjected to neutral Al_2O_3 chromatography, and eluted with MeOH/$CHCl_3$ (0–20%) to give **14** (150 mg) and **15** (28 mg).

Compound **14**: yield 78%, amorphous powder $[\alpha]_D^{25}$ + 5.0° (c = 0.87, $CHCl_3$): ^{1}H NMR ($CDCl_3$) δ 0.71 (3H, s, CH_3), 0.79 (3H, d, J = 6.2 Hz, CH_3), 0.83 (3H, s, CH_3), 0.87 (3H, d, J = 6.0 Hz, CH_3), 0.92 (3H, s, CH_3), 1.00 (3H, s, CH_3), 1.18 (3H, s, CH_3), 2.16 (1H, d, J = 11.1 Hz, H-18), 2.38 (1H, dd, J = 12.0, 5.7 Hz, H-3), 3.54 (3H, s, CH_3O-28), 5.18 (1H, *t*-like, H-12); MS (EI): 469 $[M]^+$ (25), 454 (13), 413 (10), 411 (11), 262 (70), 203 (95), 133 (100).

Compound **15**: yield 15%, amorphous powder $[\alpha]_D^{25}$ + 7.1° (c = 0.39, $CHCl_3$): ^{1}H NMR ($CDCl_3$) δ 0.71 (3H, s, CH_3), 0.82 (3H, d, J = 6.2 Hz, CH_3), 0.86 (3H, s, CH_3), 0.90 (6H, s, 2 × CH_3), 0.91 (3H, d, J = 6.2 Hz, CH_3), 1.09 (3H, s, CH_3), 2.20 (1H, d, J = 11.1 Hz, H-18), 2.67 (1H, br, H-3), 3.58 (3H, s, CH_3O-28), 5.22 (1H, *t*-like, H-12); MS (EI): 469 $[M]^+$ (30), 454 (17), 413 (12), 411 (17), 262 (74), 203 (93), 133 (100).

*6.2.7. 3-Acetoxy-11-oxo-urs-12-en-28-oic acid methyl ester (**16**)*

Acetyl ursolic acid methyl ester (200 mg, 0.39 mmol) was refluxed with CrO_3 (50 mg, 0.5 mmol) in acetic acid (7 ml) for 2 h. The reaction mixture was partitioned with water and $CHCl_3$. The $CHCl_3$ layer was chromatographed on silica gel eluted with hexane–$CHCl_3$ to obtain the 11-oxo compound, **16**. Yield 60%; colorless powder; ^{1}H NMR ($CDCl_3$): δ 0.87 (9H, br, 3 × CH_3), 0.90 (3H, s, CH_3), 0.97 (3H, d, J = 6.3 Hz, CH_3), 1.15 (3H, s, CH_3), 1.29 (3H, s, CH_3), 2.05 (3H, s, –$OCOCH_3$), 2.32 (1H, s, H-9), 2.41 (1H, d, J = 11.1 Hz, H-18), 2.79 (1H, dt, J = 14.0, 3.5 Hz, H-1a), 3.63 (3H, s, $COOCH_3$), 4.51 (1H, dd, J = 4.8, 11.1 Hz, H-3), 5.60 (1H, br. H-12). EI-MS m/z 526 (M^+, 10%), 317 (75%), 189 (70%), 119 (70%), 43 (100%).

*6.2.8. 3-Hydroxy-11-oxo-urs-12-en-28-oic acid methyl ester (**17**)*

Compound **16** was treated with 4 N NaOH (1.2 ml) in CH_3OH–THF (1:1.5, 5 ml) at room temperature overnight. The solution was neutralized with 2 N HCl, removed the organic solvent and then extracted with EtOAc to get compound **17**. Yield 90%; colorless powder; ^{1}H NMR ($CDCl_3$): δ 0.75 (3H, s, CH_3), 0.82 (3H, d, J = 6.3 Hz, CH_3), 0.86 (3H, s, CH_3), 0.93 (3H, d, J = 6.3 Hz, CH_3), 0.95 (3H, s, CH_3), 1.07 (3H, s, CH_3), 1.23 (3H, s, CH_3), 2.26 (1H, s, H-9), 2.37 (1H, d, J = 11.1 Hz, H-18), 2.75 (1H, dt, J = 14.0, 3.5 Hz, H-1a), 3.18 (1H, dd, J = 5.8, 10.3 Hz, H-3), 3.57 (3H, s, $COOCH_3$), 5.59 (1H, br. H-12). EI-MS m/z 484 (M^+, 13%), 317 (74%), 189 (70%), 119 (78%), 43 (100%).

*6.2.9. General synthetic procedure for compounds **18–23***

Compounds **18–23** were synthesized via the route outlined in Scheme 3. To a solution of compound **8** (1.3–1.6 mmol) in 10 ml $CHCl_3$ was added oxalyl chloride (3 ml) and stirred at room temperature for 48 h. The mixture was concentrated to dryness under reduced pressure. Hexane (3 × 5 ml) was added to the residue, then concentrated to dryness.

To a CH_2Cl_2 (4 ml) solution of 1,9-diaminononane (1.28 mmol) or diethylenetriamine (1.7 mmol) was added the above acid chloride (1.27 mmol for diaminononane or 1.6 mmol for diethylenetriamine). The reaction mixture was stirred at room temperature overnight. The reaction mixture was concentrated and chromatographed on ODS to get compounds **19**, **21** and **23**.

Compounds **19**, **21** and **23** were treated with 4 N NaOH (1.2 ml) in CH_3OH–THF (1:1.5, 5 ml) at room temperature overnight. The solutions were neutralized with 2 N HCl, recovered the organic solvent and then extracted with EtOAc to get compounds **18**, **20** and **22**.

*6.2.9.1. N^1,N^9-Bis (3β-hydroxy-urs-12-en-28-oyl)-1,9-diaminononane (**18**).* Yield 96% from **19**; amorphous powder; ^{1}H NMR ($CDCl_3$): δ 0.80 (6H, s, CH_3), 0.88 (6H, d, J = 6.5 Hz CH_3), 0.92 (6H, d, J = 6.5 Hz, CH_3), 0.94 (6H, s, CH_3), 0.96 (6H, s, CH_3), 1.00 (6H, s, CH_3), 1.11 (6H, s, CH_3),

3.00 (2H, m, –CONHCHa–), 3.23 (2H, dd, J = 4.0, 11.0 Hz, H-3′), 3.31 (2H, m., –CONHCHb–), 5.31 (2H, br. H-12′), 5.91 (2H, t-like, –CONH–); Positive APCI-MS m/z 1036.8 (M + H^+, 10%), 282 (100); Positive HR-FAB-MS: 1035.8826 ([M + H]$^+$, $C_{69}H_{115}N_2O_4^+$; Calc. 1035.8862).

*6.2.9.2. N^1,N^9-Bis (3β-acetoxy-urs-12-en-28-oyl)-1,9-diaminononane (**19**).* Yield 26%; amorphous powder; ^{1}H NMR ($CDCl_3$): δ 0.79 (6H, s, CH_3), 0.88 (12H, br., CH_3), 0.93 (6H, d, J = 6.0 Hz, CH_3), 0.97 (12H, s, CH_3), 1.10 (6H, s, CH_3), 2.07 (6H, s, –OCOCH_3), 3.01 (2H, m, –CONHCHa–), 3.32 (2H, m., –CONHCHb–), 4.51 (2H, dd, J = 5.5, 10.0 Hz, H H-3′), 5.32 (2H, br. H-12′), 5.90 (2H, t-like, –CONH–); Negative APCI-MS m/z 1153.8 (M + Cl^-, 100%); Positive HR-FAB-MS: 1119.9033 ([M + H]$^+$, $C_{73}H_{119}N_2O_6^+$; Calc. 1119.9074).

*6.2.9.3. N^1,N^5-Bis (3β-hydroxy-urs-12-en-28-oyl)-diethylenetriamine (**20**).* Yield 95% from **21**; amorphous powder; ^{1}H NMR ($CDCl_3$): δ 0.78 (6H, s, CH_3), 0.89 (6H, d, J = 6.0 Hz, CH_3), 0.92 (12H, br., CH_3), 0.96 (6H, s, CH_3), 1.00 (6H, s, CH_3), 1.11 (6H, s, CH_3), 2.83 (4H, m. –CH_2NHCH_2–), 3.22 (4H, m, H-3′, overlapped with –CONHCHa–), 3.48 (2H, m, –CONHCHb–), 5.34 (2H, br., H-12′), 6.49 (2H, br. –CONH–); Positive API-MS m/z 981.0 (M + H^+, 100%), 1003.0 (M + Na^+, 70%); Positive HR-FAB-MS: 980.8181 ([M + H]$^+$, $C_{64}H_{106}N_3O_4^+$; Calc. 980.8189).

*6.2.9.4. N^1,N^5-Bis (3β-acetoxy-urs-12-en-28-oyl)-diethylenetriamine (**21**).* Yield 60%; amorphous powder; ^{1}H NMR ($CDCl_3$): δ 0.76 (6H, s, CH_3), 0.85 (6H, s, CH_3), 0.87 (6H, s, CH_3), 0.89 (6H, d, J = 6.0 Hz, CH_3), 0.94 (6H, d, J = 6.0 Hz, CH_3), 0.97 (6H, s, CH_3), 1.10 (6H, s, CH_3), 2.03 (6H, s, –OCOCH_3), 2.76 (4H, br. –CH_2NHCH_2–), 3.18 (2H, br., –CONHCHa–), 3.45 (2H, br., –CONHCHb–), 4.49 (2H, dd, J = 5.3, 10.3 Hz, H-3′), 5.30 (2H, br. H-12′), 6.31 (2H, br. –CONH–); Negative APCI-MS m/z 1098.7 (M + Cl^-, 100%); Positive HR-FAB-MS: 1064.8380 ([M + H]$^+$, $C_{68}H_{110}N_3O_6^+$; Calc. 1064.8400).

*6.2.9.5. N^1-(3β-hydroxy-urs-12-en-28-oyl)-1,9-diaminononane (**22**).* Yield 95% from **23**; amorphous powder; ^{1}H NMR ($CDCl_3$): δ 0.76 (3H, s, CH_3), 0.87 (3H, d, J = 6.0 Hz, CH_3), 0.92 (3H, d, J = 6.0 Hz, CH_3), 0.94 (3H, s, CH_3), 0.97 (3H, s., CH_3), 1.00 (3H, s., CH_3), 1.10 (3H, s, CH_3), 2.89 (2H, br. –NH_2), 2.99 (2H, m, –CH_2NH_2), 3.21 (1H, dd, J = 5.0, 10.5 Hz, H-3′), 3.29 (2H, m., –CONHCH_2), 5.31 (1H, br. H-12′), 5.93 (1H, br. –CONH–); Positive APCI-MS m/z 597.5 (M + H^+, 100%); Positive HR-FAB-MS: 597.5384 ([M + H]$^+$, $C_{39}H_{69}N_2O_2^+$; Calc. 597.5363).

*6.2.9.6. N^1-(3β-acetoxy-urs-12-en-28-oyl)-1,9-diaminononane (**23**).* Yield 44%; amorphous powder; ^{1}H NMR ($CDCl_3$): δ 0.76 (3H, s, CH_3), 0.81 (3H, d, J = 6.0 Hz, CH_3), 0.82 (3H, s, CH_3), 0.87 (3H, d, J = 6.0 Hz, CH_3), 0.93 (6H, s, CH_3), 1.05 (3H, s, CH_3), 2.05 (3H, s, –OCOCH_3), 2.70 (4H, br. –NH_2 and –CH_2NH_2), 2.99 (1H, m., –CONHCHa–), 3.29 (1H, m., –CONHCHb–), 4.47 (1H, dd, J = 5.5, 10.0 Hz, H-3′), 5.28 (1H, br. H-12′), 5.88 (1H, br. –CONH–); Positive HR-FAB-MS: 639.5471 ([M + H]$^+$, $C_{41}H_{71}N_2O_3^+$; Calc. 639.5468).

6.3. Cells and cytotoxicity assay

Cell lines. The cell lines used were: Human leukemia cancer cell line (HL-60); human gastric cancer cell line (BGC-823); human breast cancer cell line (MDA-MB-435); human cervical cell line (Hela), and human hepatocellular carcinoma cell line (Bel-7402).

The in vitro HL-60 tumor cell assay was carried out according to the procedure reported by Twentyman and Luscombe [19]; BGC-823, Bel-7402, and Hela tumor cell assays were carried out by a sulforhodamine B (SRB) assay method [20]. A cell suspension in the culture medium (4×10^4 cells per ml) was inoculated to each well of 96-well microtiter plate. One day after plating, a control plate at time zero was made. In the presence or absence of compounds, the cells were incubated for a further 48 h in a 5% CO_2 incubator. Cells were fixed with 50 μl of 20% trichloroacetic acid solution for 1 h at 4 °C and plates were washed five times with tap water and air-dried. A 50 μl of SRB solution (0.4 in 1% acetic acid) was added and the staining was done at room temperature for 30 min. The residual dye was washed out with 1% acetic acid and air-dried. To each well, Tris buffer solution (10 mM, pH 10.5) was added. Optical density (OD) was measured with a microtiter plate reader at 540 nm. Growth inhibition was calculated as follows:

% inhibition = [1 – (OD value for treated cells/OD value for untreated cells)] 100%

Acknowledgements

The authors are grateful to Dr. Sunao Takeda of Central Research Laboratories, SSP Co., Ltd. Japan, for measurement of HR-FAB-MS. The authors would also like to extend their appreciation to Dr. Norio Nakamura of Institute of Natural Medicine, Toyama Medical and Pharmaceutical University for his unfailing advice during the synthesis of triterpene derivatives.

References

[1] J.F. Mayaux, A. Bousseau, R. Pauwels, T. Huet, Y. Henin, N. Dereu, M. Evers, F. Soler, C. Poujade, E. De Clercq, J.B. Le Pecq, Proc. Natl. Acad. Sci. USA 91 (1994) 3564–3568.

[2] M. Evers, C. Poujade, F. Soler, Y. Ribeill, C. James, Y. Lelièvre, J.-C. Gueguen, D. Reisdorf, I. Morize, R. Pauwels, E.D. Clercq, Y. Hénin, A. Bousseau, J.-F. Mayaux, J.-B.L. Pecq, N. Dereu, J. Med. Chem. 39 (1996) 1056–1068.

[3] Y. Kashiwada, T. Nagao, A. Hashimoto, Y. Ikeshiro, H. Okabe, L.M. Cosentino, K.H. Lee, J. Nat. Prod. 63 (2000) 1619–1622.

[4] Y.-M. Zhu, J.-K. Shen, H.-K. Wang, L.-M. Cosentino, K.-H. Lee, Bioorg. Med. Chem. Lett. 11 (2001) 3115–3118.

[5] C.-M. Ma, N. Nakamura, M. Hattori, Chem. Pharm. Bull. (Tokyo) 47 (1999) 141–145.

[6] J. Liu, J. Ethnopharmacol. 49 (1995) 57–68.

[7] K.H. Lee, Y.M. Lin, T.S. Wu, D.C. Zhang, T. Yamagishi, T. Hayashi, I.H. Hall, J.J. Chang, R.Y. Wu, T.H. Yang, Planta Med. 54 (1988) 308–311.

[8] C.N. Lin, C.M. Lu, M.K. Cheng, K.H. Gan, S.J. Won, J. Nat. Prod. 53 (1990) 513–516.

[9] E. Pisha, H. Chai, I.S. Lee, T.E. Chagwedera, N.R. Farnsworth, G.A. Cordell, C.W. Beencher, H.H. Fong, A.D. Kinghorn, D.M. Brown, M.C. Wani, M.E. Wall, T.J. Hieken, T.K. Das Gupta, J.M. Pezzuto, Nat. Med. 1 (1995) 1046–1051.

[10] D.S.H.L. Kim, J.M. Pezzuto, E. Pisha, Bioorg. Med. Chem. Lett. 8 (1998) 1707–1712.

[11] J. Sarek, J. Klinot, P. Dzubak, E. Klinotova, V. Noskova, V. Krecek, G. Korinkova, J.O. Thomson, A. Janostakova, S. Wang, S. Parsons, P.M. Fischer, N.Z. Zhelev, M. Hajduch, J. Med. Chem. 46 (2003) 5402–5415.

[12] T. Honda, G.W. Gribble, N. Suh, H.J. Finlay, B.V. Rounds, L. Bore, F.G. Favaloro Jr., Y. Wang, M.B. Sporn, J. Med. Chem. 43 (2000) 1866–1877.

[13] T. Honda, B.V. Rounds, L. Bore, H.J. Finlay, F.G. Favaloro Jr., N. Suh, Y. Wang, M.B. Sporn, G.W. Gribble, J. Med. Chem. 43 (2000) 4233–4246.

[14] N. Suh, Y. Wang, T. Honda, G.W. Gribble, E. Dmitrovsky, W.F. Hickey, R.A. Maue, A.E. Place, D.M. Porter, M.J. Spinella, C.R. Williams, G. Wu, A.J. Dannenberg, K.C. Flanders, J.J. Letterio, D.J. Mangelsdorf, C.F. Nathan, L. Nguyen, W.W. Porter, R.F. Ren, A.B. Roberts, N.S. Roche, K. Subbaramaiah, M.B. Sporn, Cancer Res. 59 (1999) 336–341.

[15] T. Honda, H.J. Finlay, G.W. Gribble, N. Suh, M.B. Sporn, Bioorg. Med. Chem. Lett. 7 (1997) 1623–1628.

[16] H.J. Finlay, T. Honda, G.W. Gribble, D. Danielpour, N.E. Benoit, N. Suh, C. Williams, M.B. Sporn, Bioorg. Med. Chem. Lett. 7 (1997) 1769–1772.

[17] H. Kojima, H. Ogura, Phytochemistry 28 (1989) 1703–1710.

[18] Y.L. Hsu, P.L. Kuo, C.C. Lin, Life Sci. 75 (2004) 2303–2316.

[19] P.R. Twentyman, M. Luscombe, Br. J. Cancer 56 (1987) 279–285.

[20] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J.T. Warren, H. Bokesch, S. Kenney, M.R. Boyd, J. Natl. Cancer Inst. 82 (1990) 1107.