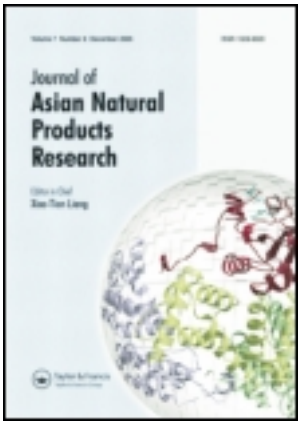

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Synthesis and antitumor activity evaluation of new asiatic acid derivatives

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Synthesis and antitumor activity evaluation of new asiatic acid derivatives

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Twelve novel asiatic acid (AA) derivatives were designed and synthesized. Their structures were confirmed using NMR, MS, and IR spectra. Their *in vitro* cytotoxicities on various cancer cell lines (HeLa, HepG2, BGC-823, and SKOV3) were evaluated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide assay. Most of the derivatives were found to have stronger cell growth inhibitory activity than AA. Among them, compounds **5**–**8** and **11** with substituted amide group at C-28 exhibited more potent cytotoxicity than AA, Gefitinib, and etoposide (positive control).

Keywords: asiatic acid derivatives; triterpenoid; synthesized; antitumor activity

1. Introduction

Asiatic acid (AA, 2α,3β,23-trihydroxyurs-12-ene-28-oic acid), a member of the ursane family of pentacyclic triterpenoids found in *Centalla asiatica*, can be easily prepared from hydrolysis of asiaticoside. AA has been traditionally used as a tonic in skin diseases and leprosy [1–3]. In addition, AA, like other triterpenes, has been reported to possess other biological effects including antitumor [4–6], anti-inflammation [7], hepatoprotective effect [8], anti-depression, and anti-Alzheimer's disease [9,10].

In previous studies, we have reported that some ursane triterpenoid derivatives such as boswellic acid and ursolic acid derivatives show strong biological activities [11–13]. Based on the reports that the acid moiety at C-17 and ester functionality at C-3 are essential for pharmacological activities of pentacyclic triterpenes [14,15], and a hydrogen donor group at either C-3 position and/or C-28 position of ursolic acid is essential for the cytotoxic activity [16]. A series of AA derivatives with substitution at positions of C-2, C-3, C-23, and C-28 of AA have been synthesized, and their cytotoxic activities have been evaluated *in vitro* on four cancer cell lines (HeLa, HepG2, SKOV3, and BGC-823). The results showed that acetylation of the C-2, C-3, C-23 alcohol, together with a substituted amino group at C-28, resulted in derivatives having stronger cell growth inhibitory activity than AA.

2. Results and discussion

2.1 Chemistry

AA was used as the lead compound, and the structure modification was done at the positions C-2, C-3, C-23, and C-28. The synthetic pathways are shown in Scheme 1. Compound **1** was prepared by reaction of AA with acetic anhydride in tetrahydrofuran

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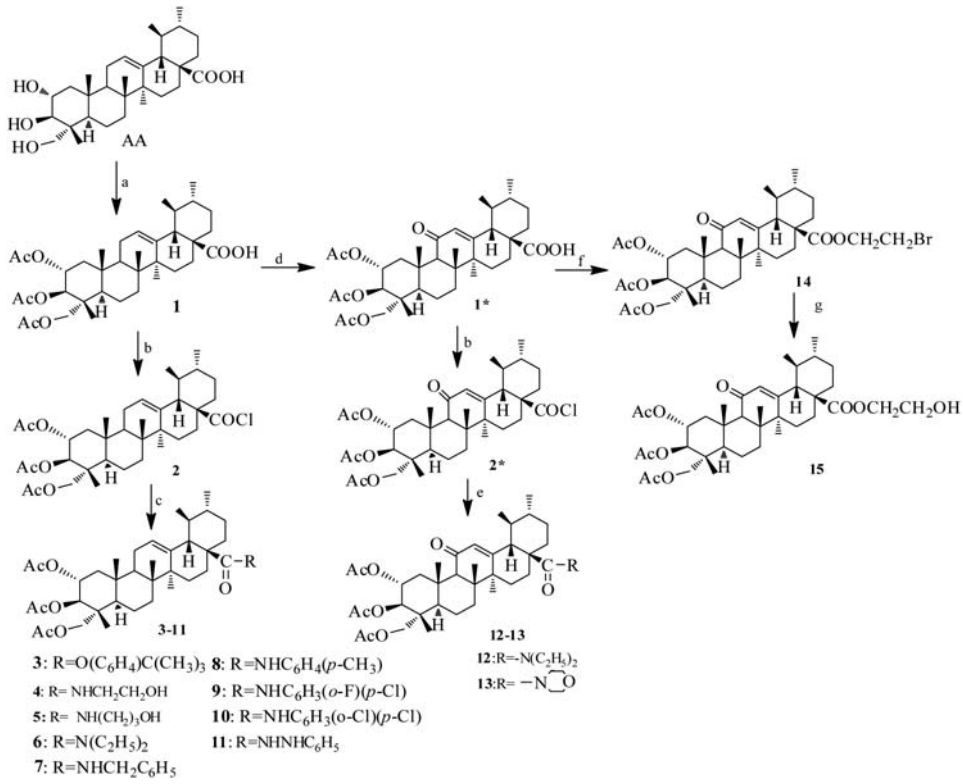

Scheme 1. Conditions and reagents: (a) Ac_2O, DMAP (cat.), THF, rt. (b) $(COCL)_2$, Et_3N, CH_2Cl_2, rt. (c) 4-Tert-butylphenol/4-nitrophenol/amines, Et_3N, CH_2Cl_2, rt. (d) $K_2Cr_2O_7$/AcOH, reflux 106°C. (e) $NH(C_2H_5)$/morpholine, Et_3N, rt. (f) $BrCH_2CH_2Br$, K_2CO_3, DMF, 0°C. (g) H_2O, K_2CO_3, DMF, rt.

(THF) in the presence of 4-dimethylaminopyridine in good yield, then it was treated with oxalyl chloride to give compound **2**. This intermediate was then condensed with the appropriate amino and phenol compounds in the presence of triethylamine to give the compounds **3**–**11**. Compound **1** was converted to α,β-unsaturated ketone **1*** by the oxidation with $K_2Cr_2O_7$ in the presence of acetic acid [17]. According to the same method for **3**–**11**, compounds **12** and **13** were synthesized from **2*** with amines. α,β-Unsaturated ketone **1*** with 1,2-dibromoethane in the presence of K_2CO_3 in DMF gave 2-bromoethyl ester, and with H_2O gave **15**. The target compounds were purified on a silica gel column with petroleum ether–ethyl acetate (or acetone) as eluents, and their structures were confirmed by NMR, MS, and IR spectra.

2.2 Biological activity

In this experiment, cells of HeLa, HepG2, and BGC-823 were the model chosen to determine the cytotoxic activity of AA, most of its derivatives, Gefitinib, and Vp-16 (a positive control). Antiproliferative effects were identified with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide. Each experiment was repeated at least three times. The results are summarized in Table 1.

As shown in Table 1, compounds **3**–**6** were tested in HeLa, BGC823, and SKOV3 three cell lines and showed better inhibitory activity than AA except for compound **3**. Compounds **5** and **6** presented more significant inhibition to HeLa and BGC-823 cell lines than Gefitinib and Vp-16 at a concentration of 10 μmol/l, while the inhibitory activity of compound **4** against

Table 1. Inhibitory activities of AA and derivatives on the HeLa, HepG2, BGC-823, and SKOV3 cells proliferation.

	Inhibition rate[a]				IC_{50} (μM)			
Compound	HeLa	HepG2	BGC823	SKOV3	HeLa	HepG2	BGC823	SKOV3
3	7.30 ± 0.36	nt	3.0 ± 0.23	10.3 ± 2.10	>10	nt	>10	>10
4	70.6 ± 2.1	nt	56.1 ± 1.3	42.1 ± 2.63	2.56 ± 0.63	nt	**4.44** ± 0.78	10.33 ± 1.22
5	80.8 ± 4.3	nt	76.7 ± 1.8	52.6 ± 1.5	**1.52** ± 0.55	nt	**2.65** ± 0.69	7.39 ± 0.77
6	74.8 ± 2.3	nt	87.1 ± 3.7	65.7 ± 1.8	**0.65** ± 0.32	nt	**0.12** ± 0.26	3.94 ± 0.61
7	79.1 ± 5.2	70.46 ± 3.6	84.3 ± 4.6	nt	1.77 ± 0.71	2.88 ± 0.25	1.45 ± 0.27	nt
8	73.4 ± 2.4	68.59 ± 7.3	82.3 ± 1.0	nt	2.28 ± 0.41	4.66 ± 1.26	2.39 ± 0.71	nt
9	62.7 ± 1.8	55.07 ± 3.8	53.7 ± 0.8	nt	5.45 ± 0.27	7.86 ± 1.57	8.13 ± 0.45	nt
10	48.2 ± 4.8	44.61 ± 6.8	38.9 ± 8.7	nt	16.66 ± 2.17	17.86 ± 17.86	11.8 ± 2.60	nt
11	71.8 ± 4.9	62.15 ± 6.8	87.1 ± 1.0	nt	1.14 ± 0.02	4.79 ± 3.07	1.51 ± 0.25	nt
12	76.7 ± 1.2	67.73 ± 12.2	71.5 ± 1.7	nt	3.32 ± 0.98	5.94 ± 2.03	4.54 ± 0.12	nt
13	37.4 ± 11.2	15.9 ± 3.6	21.9 ± 4.0	nt	26.62 ± 10.9	>10	>10	nt
15	70.3 ± 4.1	59.87 ± 7.1	53.0 ± 2.4	nt	4.78 ± 0.67	6.84 ± 1.43	8.75 ± 0.99	nt
AA	3.8 ± 3.6	17.8 ± 4.3	21.0 ± 3.7	nt	>10	>10	>10	nt
Gefitinib	42.6 ± 5.94	46.2 ± 7.93	46.0 ± 12.54	nt	17.12 ± 7.9	20.73 ± 16.36	19.27 ± 14.53	nt
Vp-16	69.7 ± 3.53	58.7 ± 5.97	60.1 ± 6.45	nt	2.37 ± 0.99	5.62 ± 2.11	5.01 ± 2.85	nt

nt, not tested.

[a] Inhibitory percentage of cells treated with each compound at a concentration of 10 μmol/l for 48 h.

both the cell lines was similar to that of Vp-16, higher than that of Gefitinib. It is evident that compounds **7**, **8**, and **11** exhibited good inhibitory activity on HeLa, HepG2, and BGC-823 cell lines higher than Gefitinib and Vp-16. Furthermore, IC_{50} values of compound **6** (0.65 ± 0.32 and 0.12 ± 0.26); compound **7** (1.77 ± 0.71, 2.88 ± 0.25, and 1.45 ± 0.27), and compound **11** (1.14 ± 0.02, 4.79 ± 3.07, and 1.51 ± 0.25) were significantly lower than Vp-16 and Gefitinib. Especially, compounds **6**, **7**, and **11** presented strong inhibition on the HeLa and BGC-823 cell lines. Among them, IC_{50} values of compounds **7** and **11** were approximate half of Vp-16 and 10% of Gefitinib on HeLa cells; IC_{50} value of compound **11** was one quarter of Gefitinib on HepG2 cells (Table 1).

The nearly identical cytotoxicity of compounds **4** and **15** (similar to Vp-16) suggested the unimportance of 11-oxo for biological activity. However, compound **3** with ester function was found to have relatively lower antitumor effect than other compounds. Modification of AA into amide group at C-28 position increased the antitumor activity such as in compounds **5**–**8** and **11**, and alkyl side chains at C-28 amide chain might be important to the inhibitory activity of tumor cell growth.

As demonstrated in Figure 1, HeLa and HepG2 cells were treated with each compound at different concentrations for 12 h. As shown in (C) and (E), exposure to 1 μM compounds **11** and **7** for 12 h induced HeLa cells fragmented, compared with the 0.1% DMSO (A). As shown in (D) and (F), exposure to 10 μM of compounds **11** and **7**, the fragmented cells were increased. The treatment of compound **11** (4 μM) exhibited similar cytotoxicity to Gefitinib (20 μM), as shown in (I) and (H).

3. Conclusion

In this paper, 12 AA derivatives with the modification of the functional groups of C-2, C-3, C-23, and C-28 were prepared, and their antitumor activities on HeLa, HepG2, BGC-823, and SKOV3 cell lines were evaluated. Among them, compounds **5**–**8** and **11** showed the most significant antiproliferative effects on tumor cells. As shown, our data suggested that most of AA conjugates with amino alcohol, aniline or aniline with electron-donating group substituted at C-28 such as compounds **4**, **5**, **8**, and **11** resulting in stronger cytotoxic activities than AA against the three cancer cell lines; AA conjugated with electron-withdrawing substituted group at C-28 such as compounds **9** and **10** might increase cytotoxic activity. It was suggested that 11-carbonyl group was not the necessary group of cytotoxic activity of AA derivatives according to the activities of compounds **6** and **12**. In addition, structure–activity relationship study of the modified compounds was carried out such that amino group-substituted C-28 might be important to the inhibitory activity of tumor cell growth.

4. Experimental

4.1 Material and methods

Melting points were determined in capillary tubes on a Buchi B-545 melting point apparatus produced by Broker Corporation (Flawil, Switzerland) and are uncorrected. NMR spectra were recorded on Bruker ARX-300 MHz spectrometers from Bruker Corporation (Ettlingen, Germany) and the solvent is $CDCl_3$, using trimethylsilyl as an internal standard. ESI-MS were recorded on Thermo-Finnigan LCQ equipment from Thermo Finnigan (San Francisco, CA, USA). HR-ESI-MS were recorded on micrOTOF-Q II equipment from BRUKER OPTICS (Ettlingen, Germany). Thin-layer chromatography was carried out with GF254, column chromatography with silica gel (200–300 mesh) obtained from Qingdao Marine Chemical Factory (Qingdao, China). The reagents were all of analytical grade or chemically pure.

Table 2. The ^{13}C NMR spectral data of some novel compounds **6**–**13**.

δ (ppm)	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
C_1	39.0	39.0	39.1	39.1	39.0	39.1	39.2	39.0
C_2	73.4	73.4	73.1	73.3	73.2	73.3	73.6	73.4
C_3	74.9	74.8	74.8	74.7	74.8	74.8	75.0	75.9
C_4	46.7	47.5	47.7	47.6	47.4	47.6	47.5	47.4
C_5	49.8	47.8	48.5	48.7	47.4	47.6	48.5	47.6
C_6	17.9	17.9	17.9	17.8	17.9	17.9	17.4	17.8
C_7	35.5	37.3	37.1	37.2	37.0	37.1	37.1	37.8
C_8	43.9	43.8	43.9	44.9	44.8	43.8	43.8	43.8
C_9	65.3	65.3	65.3	65.2	65.3	65.4	65.5	65.3
C_{10}	39.1	39.1	39.1	39.1	39.1	39.1	39.1	39.1
C_{11}	24.2	24.8	25.0	25.4	25.4	24.8	198.6	198.8
C_{12}	125.7	138.3	135.6	140.3	138.0	140.1	130.6	130.4
C_{13}	138.0	140.0	140.3	148.4	140.7	148.5	164.2	162.4
C_{14}	42.2	42.5	42.2	42.7	42.5	42.5	42.6	42.2
C_{15}	27.8	27.8	27.8	27.5	27.9	27.8	27.9	28.0
C_{16}	23.4	23.4	23.2	23.4	23.6	23.3	23.4	23.2
C_{17}	37.8	37.8	37.8	37.8	37.8	37.8	37.8	37.8
C_{18}	52.6	53.0	54.3	54.3	55.0	53.2	55.2	54.6
C_{19}	39.7	39.8	39.7	39.9	39.4	39.7	39.8	39.6
C_{20}	39.1	39.6	39.9	39.6	39.1	39.6	39.7	39.2
C_{21}	30.5	30.9	30.9	30.8	30.9	30.7	30.6	30.5
C_{22}	42.0	41.9	41.9	42.1	42.0	42.0	42.3	42.7
C_{23}	14.0	14.0	13.9	13.9	13.8	13.9	13.9	13.9
C_{24}	69.7	69.9	69.9	69.9	69.8	69.6	69.6	69.1
C_{25}	17.3	17.2	17.3	17.2	17.2	17.3	17.2	17.2
C_{26}	20.9	20.9	20.9	20.9	20.9	20.9	20.8	20.9
C_{27}	23.4	23.2	23.7	23.2	23.8	23.5	23.4	23.7
C_{28}	172.6	177.7	175.9	174.8	175.6	178.3	174.3	176.3
C_{29}	17.0	17.0	17.1	17.1	16.9	17.1	17.0	16.9
C_{30}	20.8	20.8	20.8	20.8	20.7	20.7	20.8	20.8
CH–N	47.6	47.6					44.1	48.6
	47.6						44.1	48.6
Ph		137.5	119.7	122.0	129.6	114.0		
		127.5	119.7	122.0	112.9	114.0		
		127.5	129.5	125.6	112.9	126.0		
		128.0	129.5	129.7	120.6	126.0		
		128.0	135.5	135.3	130.7	123.2		
		127.5	117.7	128.1	138.6	129.1		
CH$_3$–Ph			21.3					
CH$_3$**C**O—	170.9	170.8	170.8	170.8	170.6	170.8	170.3	170.8
	170.5	170.5	170.5	170.8	170.6	170.5	170.4	170.4
	170.4	170.4	170.4	170.8	170.6	170.4	170.4	170.4
CH$_3$CO—	21.1	21.2	21.2	21.2	21.6	21.2	21.2	21.0
	21.1	21.1	21.1	21.1	21.3	21.2	21.2	21.1
	21.1	21.1	21.1	21.1	21.0	21.0	21.3	21.2
N–CH$_2$**C**H$_3$	12.8						12.7	
	12.8						12.7	
CH$_2$—O—								66.2
								66.2

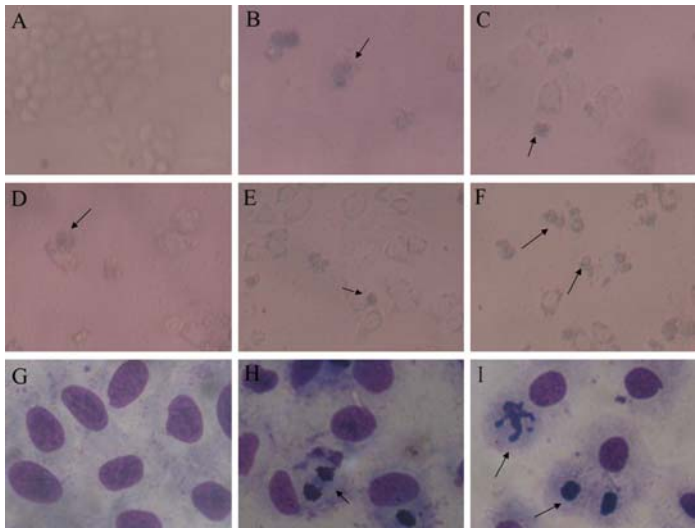

Figure 1. The morphological feature changes in tumor cells. HeLa cells were treated with: (A) 0.1% DMSO; (B) 10 μM of Vp-16; (C) 1 μM of compound **11**; (D) 10 μM of compound **11**; (E) 1 μmol/l of compound **7**; and (F) 10 μM of compound **7**, respectively; HepG2 cells were treated with: (G) 0.1% DMSO; (H) 20 μM of Gefitinib; (I) 4 μM of compound **11**, and then all HepG2 cells were stained with Giemsa.

4.2 Preparation of the compounds

*4.2.1 2α,3β,23-Triacetoxyurs-12-ene-28-oic acid (**1**)*

To a solution of AA (100 mg, 0.20 mmol) in THF (5 ml) was added acetic anhydride (567 mg, 6.00 mmol) and a small amount of 4-dimethylaminopyridine. The resultant mixture was stirred at room temperature for 2 h. Then, the solvent was removed by evaporation under reduced pressure, the resultant residue was added water and dispersed. Then, it was filtered and the filter cake was washed with water to pH 7. The crude product was purified by column chromatography (petroleum ether–acetic ether: 3/1) to give **1** as a white solid (105 mg, 85.50%); m.p. 150.4–152.0°C. IR ν_{max} (KBr, cm^{-1}): 3421, 2951, 2923, 1747, 1697, 1457, 1370, 1235, 1045; ^1H NMR (300 MHz, $CDCl_3$, δ ppm): 5.29 (t-like, 1H, H-12), 5.21–5.10 (m, 2H, H-2/3), 3.90 (d, 1H, $J = 12.0$ Hz, H-23), 3.61 (d, 1H, $J = 12.0$ Hz, H-23), 2.41 (1H, s, H-9), 2.19 (s, 3H, CH_3CO), 2.04 (s, 3H, CH_3CO), 1.96 (s, 3H, CH_3CO) (3 × CH_3CO), 1.21 (s, 3H, CH_3), 1.14 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.91 (s, 3H, CH_3) (4 × CH_3), 0.80 (d, 6H, $J = 12.0$ Hz, CH_3 × 2). ESI-MS: *m/z* 632.5 $[M + H_2O]^+$. HR-ESI-MS: *m/z* 615.3925 $[M + H]^+$ (calcd for $C_{36}H_{55}O_8$, 615.3923).

*4.2.2 4-(Tert-butyl) phenyl-2α,3β,23-triacetoxyurs-12-en-28-oate (**3**)*

To a solution of compound **1** (100 mg, 0.16 mmol) in dichloromethane (5 ml) was slowly added oxalyl chloride (82 mg, 0.64 mmol). The resultant mixture was stirred at room temperature for 24 h. Then, the solvent was removed by evaporation under reduced pressure, and cyclohexane (2 ml × 3) was added to the residue, concentrated to dryness to give **2**. Compound **2** (0.16 mmol) was dissolved in dichloromethane, then basified to pH 8–9 with triethylamine. Then, 4-tert-butyl-phenol (96 mg, 0.64 mmol) was added. The resultant mixture was stirred at room temperature for 3 h. After the solvent was removed by evaporation under reduced pressure, the resultant residue was dispersed in water, then acidified to pH 3–4 with dilute hydrochloric acid, then filtered and filter

cake was washed with water to pH 7. The residue was purified by column chromatography (petroleum ether–acetic ether: 8/1) to give **3** as a white solid (25 mg, 20.95%); m.p. 183.6–185.8°C. IR ν_{max} (KBr, cm^{-1}): 2912, 1735, 1630, 1611, 1509, 1390, 1360, 1120; ^1H NMR (300 MHz, $CDCl_3$, δ ppm): 7.39 (d, 1H, $J = 9.0$ Hz, Ar–H), 7.36 (d, 1H, $J = 9.0$ Hz, Ar–H), 6.95 (d, 1H, $J = 9.0$ Hz, Ar–H), 6.92 (d, 1H, $J = 9.0$ Hz, Ar–H), 5.34 (t-like, 1H, H-12), 5.20–5.09 (m, 2H, H-2/3), 3.88 (d, 1H, $J = 12.0$ Hz, H-23), 3.60 (d, 1H, $J = 12.0$ Hz, H-23), 2.40 (1H, s, H-9), 2.11 (s, 3H, CH_3CO), 2.04 (s, 3H, CH_3CO), 2.00 (s, 3H, CH_3CO) ($3 \times CH_3CO$), 1.31 (s, 9H, $CH_3 \times 3$), 1.21 (s, 3H, CH_3), 1.14 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.91 (s, 3H, CH_3) ($4 \times CH_3$), 0.80 (d, 6H, $J = 12.0$ Hz, $CH_3 \times 2$); ESI-MS: *m/z* 764.1 $[M + H_2O]^+$; HR-ESI-MS: *m/z* 747.4817 $[M + H]^+$ (calcd for $C_{46}H_{66}O_8$, 747.4819).

*4.2.3 N-[2α,3β,23-Triacetoxy-urs-12-ene-28-oyl]-2-amino-1-ethanol (**4**)*

According to the same method as for **3**, compound **4** was prepared from **1** (100 mg, 0.16 mmol) and 2-amino-1-ethanol (600 mg, 1.00 mmol) through the corresponding acyl chloride (**2**). The solid was purified by column chromatography (petroleum ether–acetic ether: 2.5/1) to give **4** as a white solid (60 mg, 55.89%); m.p. 149.0–151.0°C. IR ν_{max} (KBr, cm^{-1}): 3421, 2922, 1736, 1637, 1530, 1457, 1312, 1249; ^1H NMR (300 MHz, $CDCl_3$, δ ppm): 6.36 (s, 1H, NH), 5.35 (t-like, 1H, H-12), 5.18–5.07 (m, 2H, H-2/3), 3.86 (d, 1H, $J = 12.0$ Hz, H-23), 3.69 (t, 2H, $J = 6.0$ Hz, CH_2O), 3.59 (d, 1H, $J = 12.0$ Hz, H-23), 3.48–3.42 (m, 1H, NCH), 3.31–3.22 (m, 1H, NCH), 2.10 (s, 3H, CH_3CO), 2.04 (s, 3H, CH_3CO), 2.00 (s, 3H, CH_3CO) ($3 \times CH_3CO$), 1.50 (s, 3H, CH_3), 1.12 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.90 (s, 3H, CH_3) ($4 \times CH_3$), 0.85 (d, 6H, $J = 12.0$ Hz, $CH_3 \times 2$). ESI-MS: *m/z* 658.6 $[M + H]^+$. HR-ESI-MS: *m/z* 658.4399 $[M + H]^+$ (calcd for $C_{38}H_{59}NO_8$, 658.4393).

*4.2.4 N-[2α,3β,23-Triacetoxy-urs-12-ene-28-oyl]-3-aminopropanol (**5**)*

According to the same method for **3**, compound **5** was prepared from **1** (100 mg, 0.16 mmol) and 3-amino-1-propanol (600 mg, 8.10 mmol) through the corresponding acyl chloride (**2**). The solid was purified by column chromatography (petroleum ether–acetic ether: 2.5/1) to give **5** as a white solid (50 mg, 43.03%); m.p. 151.0–152.0°C. IR ν_{max} (KBr, cm^{-1}): 3418, 2946, 1736, 1633, 1526, 1454, 1373, 1247; ^1H NMR (300 MHz, $CDCl_3$, δ ppm): 6.21 (s, 1H, NH), 5.33 (t-like, 1H, H-12), 5.10–5.07 (m, 2H, H-2/3), **3.85** (d, 1H, $J = 12.0$ Hz, H-23), **3.59** (d, 1H, $J = 12.0$ Hz, H-23), 3.54 (t, 2H, $J = 6.0$ Hz, CH_2O), 3.47–3.38 (m, 1H, NCH), 3.19–3.08 (m, 1H, NCH), 2.70–2.59 (m, 2H, $NHCH_2CH_2$), **2.54** (t, 1H, $J = 12.0$ Hz, H-9), 2.09 (s, 3H, CH_3CO), 2.03 (s, 3H, CH_3CO), 1.99 (s, 3H, CH_3CO) ($3 \times CH_3CO$), 1.47 (s, 3H, CH_3), 1.10 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 0.90 (s, 3H, CH_3) ($4 \times CH_3$), 0.81 (d, 6H, $J = 12.0$ Hz, $CH_3 \times 2$). ESI-MS: *m/z*: 672.5 $[M + H]^+$. HR-ESI-MS: *m/z* 672.4522 $[M + H]^+$ (calcd for $C_{39}H_{61}NO_8$, 672.4521).

*4.2.5 N-(2α,3β,23-Triacetoxyurs-12-ene-28-oyl)diethyl-amine (**6**)*

According to the same method for **3**, compound **6** was prepared from **1** (100 mg, 0.16 mmol) and diethylamine (116 mg, 1.6 mmol) through the corresponding acyl chloride (**2**). The solid was purified by column chromatography (petroleum ether–acetic ether: 4/1) to give **6** as a white solid (38 mg, 35.50%); m.p. 118.2–120.0°C. IR ν_{max} (KBr, cm^{-1}): 2942, 1735, 1635, 1528, 1456, 1373, 1246; ^1H NMR (300 MHz, $CDCl_3$, δ ppm): 5.27 (t-like, 1H, H-12), 5.18–5.08 (m, 2H, H-2/3), 3.88 (d, 1H, $J = 12.0$ Hz, H-23), 3.58 (d, 1H, $J = 12.0$ Hz, H-23), 3.30–3.24 (m, 4H, N-$CH_2 \times 2$), 2.12 (s, 3H, CH_3CO), 2.10 (s, 3H,

CH_3CO), 1.99 (s, 3H, CH_3CO) ($3 \times CH_3CO$), 1.94–1.92 (m, 3H, $CH_3 \times 2$), 1.14 (s, 3H, CH_3), 1.11 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 0.90 (s, 3H, CH_3) ($4 \times CH_3$), 0.83 (d, 6H, $J = 12.0$ Hz, $CH_3 \times 2$); for ^{13}C NMR (300 MHz, $CDCl_3$, δ ppm) spectral data, see Table 1. ESI-MS: *m/z* 670.6 $[M + H]^+$. HR-ESI-MS: *m/z* 670.4723 $[M + H]^+$ (calcd for $C_{40}H_{63}N_1O_7$, 670.4721).

4.2.6 *N-(2α,3β,23-Triacetoxyurs-12-ene-28-oyl)benzylamine (7)*

According to the same method for **3**, compound **7** was prepared from **1** (100 mg, 0.16 mmol) and phenylamine (171 mg, 1.6 mmol) through the corresponding acyl chloride (**2**). The solid was purified by column chromatography (petroleum ether–acetic ether: 4/1) to give **7** as a white solid (61 mg, 54.23%); m.p. 137.9–139.7°C. IR ν_{max} (KBr, cm^{-1}): 3498, 2826, 1684, 1612, 1487, 1398, 1108; ^1H NMR (300 MHz, $CDCl_3$, δ ppm): 7.35–7.24 (m, 5H, Ar–H), 6.11 (s, 1H, NH), 5.23 (t-like, 1H, H-12), 5.16–5.07 (m, 2H, H-2/3), 4.53, 4.20 (d, 1H, $J = 12.0$ Hz NH*CH*$_2$), 3.86 (d, 1H, $J = 12.0$ Hz, H-23), 3.59 (d, 1H, $J = 12.0$ Hz, H-23), 2.09 (s, 3H, CH_3CO), 2.04 (s, 3H, CH_3CO), 1.99 (s, 3H, CH_3CO) ($3 \times CH_3CO$), 1.09 (s, 3H, CH_3), 1.08 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 0.90 (s, 3H, CH_3) ($4 \times CH_3$), 0.73 (d, 6H, $J = 12.0$ Hz, $CH_3 \times 2$); for ^{13}C NMR (300 MHz, $CDCl_3$, δ ppm) spectral data, see Table 1. ESI-MS: *m/z* 704.5 $[M + H]^+$; HR-ESI-MS: *m/z* 704.4523 $[M + H]^+$ (calcd for $C_{43}H_{61}N_1O_7$, 704.4521).

4.2.7 *N-(2α,3β,23-Triacetoxyurs-12-ene-28-oyl)*p-*toluidine (8)*

According to the same method for **3**, compound **8** was prepared from **1** (100 mg, 0.16 mmol) and *p*-toluidine (171 mg, 1.6 mmol) through the corresponding acyl chloride (**2**). The solid was purified by column chromatography (petroleum ether–acetic ether: 5/1) to give **8** as a white solid (62 mg, 53.08%); m.p. 255.0–257.5°C. IR ν_{max} (KBr, cm^{-1}): 3497, 2828, 1686, 1610, 1486, 1397, 1106; ^1H NMR (300 MHz, $CDCl_3$, δ ppm): 7.58 (s, 1H, NH), 7.34 (d, 2H, $J = 4.0$ Hz Ar–H), 7.11 (d, 2H, $J = 4.0$ Hz, Ar–H), 5.49 (t-like, 1H, H-12), 5.17–5.07 (m, 2H, H-2/3), 3.85 (d, 1H, $J = 12.0$ Hz, H-23), 3.59 (d, 1H, $J = 12.0$ Hz, H-23), 2.31 (s, 3H, Ar–*CH*$_3$), 2.09 (s, 3H, CH_3CO), 2.03 (s, 3H, CH_3CO), 2.00 (s, 3H, CH_3CO) ($3 \times CH_3CO$), 1.14 (s, 3H, CH_3), 1.07 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 0.92 (s, 3H, CH_3) ($4 \times CH_3$), 0.74 (d, 6H, $J = 9.0$ Hz, $CH_3 \times 2$); for ^{13}C NMR (300 MHz, $CDCl_3$, δ ppm) spectral data, see Table 1. ESI-MS: *m/z* 704.5 $[M + H]^+$; HR-ESI-MS: *m/z* 704.4519 $[M + H]^+$ (calcd for $C_{43}H_{61}N_1O_7$, 704.4521).

4.2.8 N-*(2α,3β,23-Triacetoxyurs-12-ene-28-oyl)-4-chloro-3-fluoroaniline (9)*

According to the same method as for **3**, compound **9** was prepared from **1** (100 mg, 0.16 mmol) and 4-chloro-3-fluoroaniline (144 mg, 1.6 mmol) through the corresponding acyl chloride (**2**). The solid was purified by column chromatography (petroleum ether–acetic ether: 5/1) to give **9** as a white solid (80 mg, 67.48%); m.p. 159.8–161.4°C. IR ν_{max} (KBr, cm^{-1}): 3496, 2827, 1686, 1610, 1486, 1397, 1103; ^1H NMR (300 MHz, $CDCl_3$, δ ppm): 7.74 (s, 1H, Ar–H), 7.62 (s, 1H, NH), 7.18–7.07 (m, 2H, Ar–H), 5.49 (t-like, 1H, H-12), 5.17–5.07 (m, 2H, H-2/3), 3.85 (d, 1H, $J = 12.0$ Hz, H-23), 3.59 (d, 1H, $J = 12.0$ Hz, H-23), 2.09 (s, 3H, CH_3CO), 2.03 (s, 3H, CH_3CO), 2.00 (s, 3H, CH_3CO) ($3 \times CH_3CO$), 1.14 (s, 3H, CH_3), 1.08 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.92 (s, 3H, CH_3) ($4 \times CH_3$), 0.71 (d, 6H, $J = 9.0$ Hz, $CH_3 \times 2$); for ^{13}C NMR (300 MHz, $CDCl_3$, δ ppm) spectral data, see Table 1. ESI-MS: *m/z* 742.4 $[M + H]^+$. HR-ESI-MS: *m/z* 764.3102 $[M + Na]^+$ (calcd for $C_{42}H_{57}Cl_1F_1N_1O_7$, 764.3100).

4.2.9 *N-(2α,3β,23-Triacetoxyurs-12-ene-28-oyl)-3,4-di-chloroaniline (**10**)*

According to the same method for **3**, compound **10** was prepared from **1** (100 mg, 0.16 mmol) and 3,4-dichloroaniline (230 mg, 1.6 mmol) through the corresponding acyl chloride (**2**). The solid was purified by column chromatography (petroleum ether–acetic ether: 5/1) to give **10** as a white solid (86 mg, 71.00%); m.p. 129.6–131.1°C. IR ν_{max} (KBr, cm^{-1}): 3492, 2825, 1685, 1613, 1486, 1390, 1105; ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 7.80 (s, 1H, Ar–H), 7.67 (s, 1H, NH), 7.35 (d, 1H, $J = 9.0$ Hz, Ar–H), 7.21 (d, 1H, $J = 9.0$ Hz, Ar–H), 5.50 (t-like, 1H, H-12), 5.17–5.07 (m, 2H, H-2/3), 3.85 (d, 1H, $J = 12.0$ Hz, H-23), 3.59 (d, 1H, $J = 12.0$ Hz, H-23), 2.09 (s, 3H, CH_3CO), 2.03 (s, 3H, CH_3CO), 2.00 (s, 3H, CH_3CO) ($3 \times CH_3CO$), 1.14 (s, 3H, CH_3), 1.08 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.92 (s, 3H, CH_3) ($4 \times CH_3$), 0.70 (d, 6H, $J = 9.0$ Hz, $CH_3 \times 2$); for ^{13}C NMR (300 MHz, $CDCl_3$, δ ppm) spectral data, see Table 1. ESI-MS: *m/z* 758.4 $[M + H]^+$; HR-ESI-MS: *m/z* 780.3410 $[M + Na]^+$ (calcd for $C_{42}H_{57}Cl_2F_1N_1O_7$, 780.3408).

4.2.10 *N-(2α,3β,23-Triacetoxyurs-12-ene-28-oyl)-phenylhydrazine (**11**)*

According to the same method for **3**, compound **11** was prepared from **1** (100 mg, 0.16 mmol) and phenylhydrazine (69 mg, 0.64 mmol) through the corresponding acyl chloride (**2**). The solid was purified by column chromatography (petroleum ether–acetic ether: 3/1) to give **11** as a white solid (49 mg, 43.50%); m.p. 186.6–188.2°C. IR ν_{max} (KBr, cm^{-1}): 3492, 2825, 1685, 1613, 1486, 1390, 1105; ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 7.69 (s, 1H, NH), 7.25–6.85 (m, 5H, Ar–H), 5.45 (t-like, 1H, H-12), 5.19–5.09 (m, 2H, H-2/3), 3.87 (d, 1H, $J = 12.0$ Hz, H-23), 3.61 (d, 1H, $J = 12.0$ Hz, H-23), 2.10 (s, 3H, CH_3CO), 2.04 (s, 3H, CH_3CO), 2.00 (s, 3H, CH_3CO) ($3 \times CH_3CO$), 1.27 (s, 3H, CH_3), 1.13 (s, 3H, CH_3), 1.12 (s, 3H, CH_3), 1.00 (s, 3H, CH_3) ($4 \times CH_3$), 0.81 (d, 6H, $J = 9.0$ Hz, $CH_3 \times 2$); for ^{13}C NMR (300 MHz, $CDCl_3$, δ ppm) spectral data, see Table 1. ESI-MS: *m/z* 705.5 $[M + H]^+$; HR-ESI-MS: *m/z* 727.4299 $[M + Na]^+$ (calcd for $C_{42}H_{60}N_2O_7$, 727.4293).

4.2.11 *2α,3β,23-Triacetoxyurs-11-oxo-12-ene-28-oic acid (**1***)*

A solution of **1** (50.0 mg, 0.08 mmol) and $K_2Cr_2O_7 \cdot 2H_2O$ (75 mg, 0.25 mmol) in 10 ml of acetic acid was refluxed for 5 h. The mixture was cooled to 20°C and diluted with H_2O (5 ml), then extracted with CH_2Cl_2 (3×5 ml). The organic phase was neutralized with saturated $NaHCO_3$ solution to pH 7–8, washed with water ($5 \text{ ml} \times 2$), and saturated NaCl solution (5 ml). The organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure yielded light green solid, which was purified by silica gel chromatography (petroleum ether–ethyl acetate: 2/1) to yield a white solid (41.6 mg, 81.1%). m.p. 288.4–289.6°C. IR ν_{max} (KBr, cm^{-1}): 3446, 1745, 1661, 922, 803, 744; ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 5.37 (s, 1H, H-12), 5.28–5.19 (m, 2H, H-2/3), 3.90 (d, 1H, $J = 12.0$ Hz, H-23), 3.61 (d, 1H, $J = 12.0$ Hz, H-23), 2.41 (1H, s, H-9), 2.11 (s, 3H, CH_3CO), 2.04 (s, 3H, CH_3CO), 1.96 (s, 3H, CH_3CO) ($3 \times CH_3CO$), 1.37 (s, 3H, CH_3), 1.28 (s, 3H, CH_3), 1.21 (s, 3H, CH_3), 0.97 (s, 3H, CH_3) ($4 \times CH_3$), 0.80 (d, 6H, $J = 12.0$ Hz, $CH_3 \times 2$). ESI-MS: *m/z* 629.5 $[M + H]^+$; HR-ESI-MS: *m/z* 629.3723 $[M + H]^+$ (calcd for $C_{37}H_{55}NO_9$, 629.3721).

4.2.12 *N-[2α,3β,23-Triacetoxy-urs-11-oxo-12-ene-28-oyl]-diethylamine (**12**)*

Compound **1*** (54.5 mg, 0.09 mmol) was allowed to react with diethylamine (25.3 mg, 0.35 mmol) by using general procedure to get compound **12** (22.4 mg, 56.28%). The reaction mixture was stirred at room temperature for 4 h. The residue was purified

by column chromatography (petroleum ether–ethyl acetate: 3/2). m.p. 237.3–240.0°C; IR ν_{max} (KBr, cm^{-1}): 2971, 2930, 2872, 1744, 1651, 1614, 1473, 1390, 1234, 1035; ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 5.67 (t-like, 1H, H-12), 5.34–5.26 (m, 1H, H-2), 5.05 (d, 1H, $J = 12.0$ Hz, H-3), 3.85 (d, 1H, $J = 12.0$ Hz, H-23), 3.57 (d, 1H, $J = 12.0$ Hz, H-23), 3.28–3.22 (m, 4H, N–$CH_2 \times 2$), 2.36 (s, 1H, H-9), 2.09 (s, 3H, CH_3CO), 2.01 (s, 3H, CH_3CO), 1.95 (s, 3H, CH_3CO) ($3 \times CH_3CO$), 1.90–1.88 (m, 6H, $CH_3 \times 2$), 1.29 (s, 3H, CH_3), 1.28 (s, 3H, CH_3), 1.12 (s, 3H, CH_3), 1.00 (s, 3H, CH_3) ($4 \times CH_3$), 0.87 (d, 6H, $J = 6.0$ Hz, $CH_3 \times 2$); for ^{13}C NMR (300 MHz, $CDCl_3$, δ ppm) spectral data, see Table 1. ESI-MS: *m/z* 684.5 $[M + H]^+$; HR-ESI-MS: *m/z* 684.4567 $[M + H]^+$ (calcd for $C_{40}H_{61}N_1O_8$, 684.4566).

4.2.13 *N-[2α,3β,23-Triacetoxy-urs-11-oxo-12-ene-28-oyl]-morpholine (**13**)*

Compound **1*** (100.00 mg, 0.14 mmol) was allowed to react with morpholine (49.0 mg, 0.56 mmol) by using general procedure to get compound **13** (38.0 mg, 36.61%). The reaction mixture was stirred at room temperature for 5 h. The residue was purified by column chromatography (petroleum ether–ethyl acetate: 2.8/1). m.p. 138.8–140.7°C; IR ν_{max} (KBr, cm^{-1}): 2955, 2872, 1746, 1661, 1620, 1460, 1369, 798, 757; ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 5.63 (s, 1H, H-12), 5.30–5.27 (m, 1H, H-2), 5.09 (d, 1H, $J = 12.0$ Hz, H-3), 3.85 (d, 1H, $J = 12.0$ Hz, H-23), 3.64–3.57 (m, 8H, $N(CH_2CH_2)_2O$), 3.57 (d, 1H, $J = 12.0$ Hz, H-23), 2.10 (s, 3H, CH_3CO), 2.02 (s, 3H, CH_3CO), 1.95 (s, 3H, CH_3CO) ($3 \times CH_3CO$), 0.98 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.89 (s, 3H, CH_3), 0.87 (s, 3H, CH_3) ($4 \times CH_3$), 0.78 (d, 6H, $J = 9.0$ Hz, $CH_3 \times 2$); for ^{13}C NMR (300 MHz, $CDCl_3$, δ ppm) spectral data see Table 1. ESI-MS: *m/z* 730.5 $[M + Na]^+$; HR-ESI-MS: *m/z* 730.4525 $[M + Na]^+$ (calcd for $C_{40}H_{59}N_1O_9$, 730.4526).

4.2.14 *2-Bromoethyl-2α,3β,23-triacetoxy-11-oxo-urs-12-en-28-oate (**14**)*

Compound **1*** (100 mg, 0.16 mmol) and potassium carbonate (0.045 g, 0.32 mmol) were dissolved in DMF (4 ml), and then 1,2-dibromoethane (0.060 g, 0.32 mmol) was added and the solution was stirred at 0°C for 2–3 h. The reaction mixture was filtered, and the cake was washed with ethyl acetate (4×15 ml). The organic phase was neutralized by 1N HCl (2×10 ml), washed with saturated sodium chloride solution, and dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave the title compound as a white solid (81 mg, 68.94%). m.p. 234.7–236.9°C. IR ν_{max} (KBr, cm^{-1}): 2932, 2873, 1740, 1659, 1045, 563; ESI-MS: *m/z* 814.3 $[M + 2K]^+$. HR-ESI-MS: calcd for $C_{38}H_{55}Br_1O_9$ H^+ $[M + H]^+$: 735.3123, found 735.3122.

4.2.15 *2-(Hydroxyl)ethyl-2α,3β,23-triacetoxy-11-oxo-urs-12-en-28-oate (**15**)*

A solution of **14** (100 mg, 0.14 mmol) in 10 ml of 95% alcohol was refluxed, then the solution was cooled to room temperature and filtered. The residue was successively washed with ethyl acetate. The organic phase was washed with saturated sodium bicarbonate and saturated sodium chloride solution, and dried over anhydrous magnesium sulfate. Filtration and evaporation of solvent at reduced pressure gave the title compound as a white solid (47 mg, 49.89%) (petroleum ether–ethyl acetate: 4/1), m.p. 129.7–132.6°C. IR ν_{max} (KBr, cm^{-1}): 3556, 2949, 2875, 1741, 1718, 1648, 1035, 901, 799, 745; ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 5.37 (s, 1H, H-12), 5.29–5.18 (m, 2H, H-2/3), 4.22–4.15 (m, 4H, —OCH_2-CH_2O—), 3.90 (d, 1H, $J = 12.0$ Hz, H-23), 3.61 (d, 1H, $J = 12.0$ Hz, H-23), 2.41 (1H, s, H-9), 2.11 (s, 3H, CH_3CO), 2.04 (s, 3H, CH_3CO), 1.96 (s, 3H, CH_3CO) ($3 \times CH_3CO$), 1.37 (s, 3H, CH_3), 1.28 (s, 3H, CH_3), 1.21 (s, 3H, CH_3), 0.97 (s, 3H, CH_3) ($4 \times CH_3$), 0.80 (d, 6H, $J = 12.0$ Hz,

$CH_3 \times 2$). ESI-MS: *m/z* 673.5 $[M + H]^+$; HR-ESI-MS: *m/z* 673.4519 $[M + H]^+$ (calcd for $C_{38}H_{56}O_{10}$, 673.4521).

4.3 Biological activity assays

Tumor cells (200 μl per well) were cultured in 96-well plates in Roswell Park Memorial Institute (RPMI) 1640 with 10% fetal calf serum under 5% CO_2 and 37°C. After 24 h, the appropriate test compound was added with different indicated concentrations of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} mol/l, respectively, for another 72-h incubation. Then, dimethyl-2-thiazolyl-2,5-diphenyl-2*H*-tetrazolium-bromide was added, and absorbance was measured on an ELISA reader at a wavelength of 490 nm. Each test was carried out three times. The concentration of compounds which gives 50% growth inhibition corresponds to the IC_{50}. The results are summarized in Table 1.

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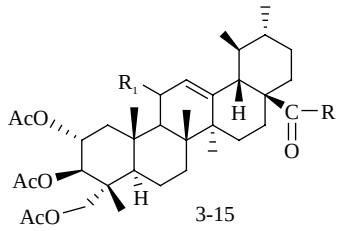

3-15

R_1 = H, or = O

The synthesis of AA derivatives with cytotoxic activity against various cancer cell lines (HeLa, HepG2, BGC-823, and SKOV3) is reported.

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Synthesis and antitumor activity evaluation of new asiatic acid derivatives

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