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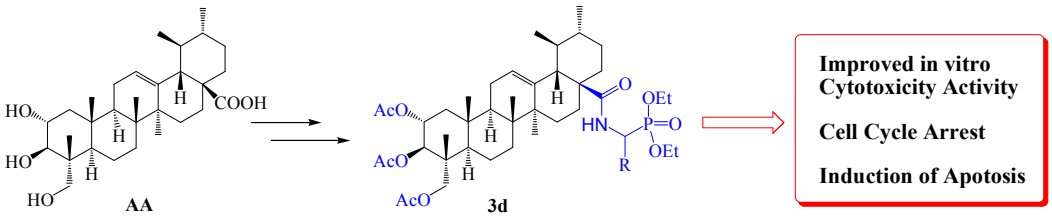
Graphical abstract

Synthesis, antiproliferative and apoptosis-inducing effects of novel asiatic acid derivatives containing α-aminophosphonates

Ri-Zhen Huang, Cai-Yi Wang, Jian-Fei Li, Gui-Yang Yao, Ying-Ming Pan, Man-Yi Ye, Heng-Shan Wang, Ye Zhang



A series of novel asiatic acid derivatives containing α-aminophosphonates was designed and synthesized as antitumor agents. Representative compound **3d** blocked the T24 cell cycle at G_1/S phase by the p53-dependent pathway and induced apoptosis through mitochondrial pathway.

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Synthesis, antiproliferative and apoptosis-inducing effects of novel asiatic acid derivatives containing α-aminophosphonates

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Abstract

A series of novel asiatic acid (**AA**) derivatives containing α-aminophosphonate were designed and synthesized as antitumor agents. In vitro antitumor activities of these compounds against five cancer cell lines (A549, Hct-116, T24, Spca-2 and SK-OV-3 cell) and a normal cell line (HUVEC cell) were evaluated, employing standard MTT assay. Antitumor activities screening result indicated that many target compounds displayed moderate to high levels of antitumor activities compared with **AA**, 5-fluorouracil (5-FU) and cisplatin, and exhibited much lower cytotoxicy against normal cell than 5-FU and cisplatin. In addition, the mechanism of representative compound **3d** was preliminarily investigated by AO/EB staining, Hoechst 33258 staining, JC-1 mitochondrial membrane potential staining, flow cytometry and western blot. Compound **3d** inducing apoptosis involved intracellular Ca^{2+} production, the loss of mitochondrial membrane potential and intracellular reactive oxygen species (ROS) production. Western blot analysis also demonstrated that compound **3d** treatment triggered the mitochondrial apoptotic pathway, indicating by changing Bax/Bcl-2 ratios, cytochrome *c* release, and caspase-9 activation. Moreover, the cell cycle analysis showed that compound **3d** may confine T24 cells in G_1/S phase mainly through the p53-dependent pathway. Together, these results implied a critical role of ROS, caspase-9 and p53 in compound **3d**-inducing G_1/S arrest and apoptosis of T24 cells.

Keywords: Asiatic acid; α-Aminophosphonate; Cytotoxicity; Apoptosis; Cell cycle

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1. Introduction

Natural products are widely used to treat human disease and have contributed to drug discovery for at least a century [1−3]. Terpenes, which are produced by some plants used in traditional medicine, have shown anticancer activity against various cancer cell lines *in vitro* [4,5]. The pentacyclic triterpenes are a class of terpenes with known biological and pharmacological properties, including anti-inflammatory, antimicrobial, anti-HIV, antioxidant, hepatoprotective, and analgesic activity [6−12]. Several naturally occurring pentacyclic triterpenes and their derivatives can induce apoptosis in a wide variety of cancer cells, such as hepatoma, lung carcinoma, neuroblastoma and colorectal carcinoma [13−15]. However, the disadvantage of using many of these triterpenoids include toxicity and large differences of the effective concentrations for various cellular effects [16]. Because of their promising biological properties, modification of pentacyclic triterpenes to obtain agents with improved antitumor activity is of interest to bioorganic chemists.

Asiatic acid (**AA**, 2α,3α,23-trihydroxyurs-12-ene-28-oic acid) is a pentacyclic triterpene acid isolated from *Centalla asiatica*, a herb used for several thousand years in Chinese traditional medicine [16]. It has been reported to have a wide range of pharmacological activities and to offer potential chemoprotection against cancer [17−20]. **AA** can induce apoptosis in human hepatoma cells by enhancing intracellular calcium release and up-regulation of p53 expression [21]. **AA** has also been shown to induce apoptosis in SK-MEL-2 human melanoma cells on increase of intracellular reactive oxygen species (ROS) and enhanced expression of Bax, but not Bcl-2, protein [22]. Structure–activity relationship studies have shown that the modification of C-2, C-3, C-23, and C-28 of **AA** can improve its biological activities [23,24]. Furthermore, we have reported that addition of an aniline group to **AA** at C-28 enhanced its anticancer activity and induced apoptosis in human hepatoma cells resulting from production of ROS and increased expression of Bcl-2 and its downstream proteins [25]. These results support continuing investigation of the antitumor potential of **AA** derivatives, but the numbers of compounds that have been synthesized and evaluated is still limited. Consequently, considerable effort is being applied to the design and synthesis of novel **AA** derivatives to screen as antitumor drugs.

The addition of α-aminophosphonates (APAs) to some pharmacal core increases antitumor activity, and many APA derivatives have been shown to strongly inhibit the growth of human tumours [26−31]. Pharmacological studies have revealed that aminophosphonate derivatives can induce apoptosis through a mitochondria-dependent pathway [32] without significant toxicity to normal cells, and we have confirmed that the addition of APAs to some potential pharmacal cores led to improved mitochondria-dependent antitumor activity [26–28]. One would expect that adding APA moieties to an **AA** skeleton to lead to improved antitumor activity and multiple apoptotic pathways. However, to the best of our knowledge, the synthesis and antitumor activity of **AA** derivatives including APA moieties at C-28 have not been reported. Therefore, in the present work, we introduced APA functional groups at C-28 of the **AA** skeleton and evaluated the

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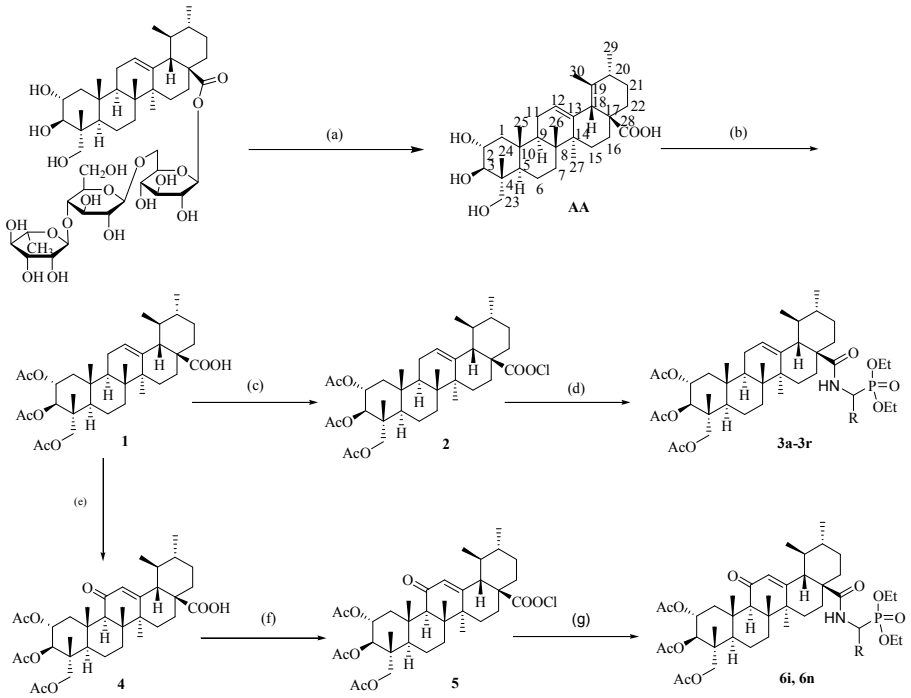
in vitro cytotoxicity of the resulting compounds. We also investigated the mechanism of apoptosis induced by compound **3d**.

2. Results and discussion

2.1. Chemistry



Scheme 1. General synthetic route for compound **3**−**6**. Reagents and conditions: (a) (i) NaOH, MeOH, reflux; (ii) 2 mol/L HCl, MeOH (b) Ac_2O, pyridine; (c) $COCl_2$, CH_2Cl_2; (d) amine, apas, CH_2Cl_2; (e) K2CrO7, AcOH; (f) $COCl_2$, CH_2Cl_2; (g) amine, apas, CH_2Cl_2.

AA derivatives bearing APA structures (compounds **3** and **6**) were synthesized as shown in Scheme 1. The asiaticoside was isolated from the leaf of *Centella asiatica (L.) Urba* as previously described [13], characterized by ^{1}H nuclear magnetic resonance (NMR) and mass spectroscopy and treated with NaOH in MeOH to yield the **AA** sodium salt. The **AA** sodium salt solution was acidified in an ice-water bath to give **AA**. **AA** was acetylated to yield 2α,3β,23-triacetoxyurs-12-ene-28-oic acid (**1**). **AA** chloride was obtained by the condensation of compound **1** with oxalyl chloride in the presence of triethylamine, and this intermediate was then reacted with a variety of substituted α-aminophosphonates [28] to produce the desired compounds **3a**−**3r**. 2α,3β,23-triacetoxyurs-11-oxo-12-ene-28-oic (**4**) was synthesized by the treatment of **1** in the presence of glacial acetic acid as previously described [33]. **AA** chloride was obtained by the condensation of compound **4** with oxalyl chloride in the presence of triethylamine, and this intermediate was then reacted with a variety of substituted α-aminophosphonates to give the desired compounds **6i** and **6n**. All the new compounds were confirmed by spectroscopic methods, including ^{1}H NMR, ^{13}C NMR and high resolution mass spectroscopy.

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2.2 Biological Activity

2.2.1 Cytotoxicity Test

The *in vitro* cytoxicity of the synthesized compounds was evaluated by methylthiazoltetrazolium (MTT) assay in a panel of five human cancer cell lines including A549 adenocarcinomic human alveolar basal epithelial cells, SK-OV-3 human ovarian cancer cells, Spca-2 human lung cancer cells, T24 human bladder carcinoma cells and Hct-116 human colon tumour cells. The commercial anticancer drugs 5-fluorouracil (5-FU) and cisplatin were positive controls. The assay results are shown in Table 1.

As shown in Table 1, the IC_{50} values of most of the conjugates were lower than that of **AA** and 5-FU, indicating that the introduction of an APA moiety in the **AA** skeleton should lead to improved cytotoxicity. Many of the target compounds, shown in bold in Table 1, had better anticancer activity against the six cell lines than cisplatin, but all had lower cytotoxicity against human umbilical vein endothelial cells (HUVEC) than cisplatin and 5-FU, which indicated that they were good candidate for anti-tumour drugs.

In most cases, halogen substituents in the 2- and 4-position of the benzene group of APA moieties led, as in in **3d** and **3f**, to significant increase in potency compared with other analogues. Compounds possessing a chloro group in the 2- and 4-position (**3d** and **3f**) had increased potency against T24 cells, with IC_{50} values of 6.59 and 8.93 μM, respectively. **3e,** with a chloro group in the 3-position had an IC_{50} of 13.92 μM in the same cell line. However, introduction of a naphthalene substituent led, in **3p** and **3q**, to reduced potency, particularly against the A549 lung cancer cell line, with IC_{50} values of 54.79 μM and 46.33 μM, respectively. Compound **3l,** with a methoxy substituent in the para position of the benzene ring of its APA moiety, also had significant activity against SK-OV-3 cells, with an IC_{50} of 10.26 μM. Some of the compounds had strong selective potency against Hct-116 colon cancer cells and Spca-2 lung cancer cells, with IC_{50} values of 11.07 μM and 10.10 μM, respectively. The nearly identical cytotoxicity of compounds **6i** and **6n** compared with compounds **3i** and **3n**, suggests the lack of importance of 11-oxo for biological activity.

The introduction of chloro as a highly electronegative centre seemed to improve the biological properties of these compounds. Comparison with activities against normal HUVEC (Table 1) revealed that the anti-proliferative activity of some compounds against cancer cells was much higher than the activity against normal cells.

Table 1. IC_{50} values (μM) of asiatic acid and its derivatives **3** and **6** towards five selected tumor cell lines and normal cell lines for 48 h.

Compounds	IC_{50}^{a}(μM)					
	A549	Hct-116	T24	Spca-2	SK-OV-3	HUVEC

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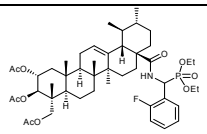
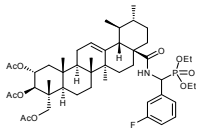
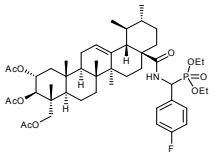
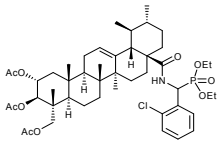
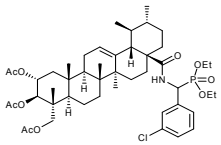
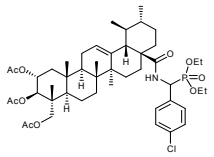
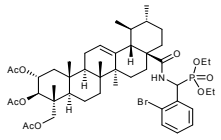
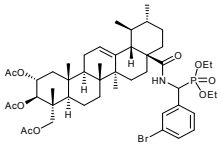
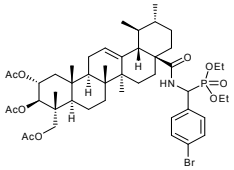
 **3a**	21.77±1.12	25.22±1.45	**16.19±2.91**	**10.10±4.01**	31.26±0.31	>100
 **3b**	27.15±0.77	28.74±0.23	32.03±1.00	45.78±2.11	34.64±0.51	>100
 **3c**	20.18±1.19	18.19±2.32	**20.78±2.12**	29.73±1.54	25.24±0.15	>100
 **3d**	14.89±2.31	26.09±2.11	**6.59±0.16**	**15.01±1.95**	27.52±0.67	>100
 **3e**	29.80±2.52	28.01±1.03	**13.92±0.92**	18.02±0.99	35.47±1.96	>100
 **3f**	15.70±0.46	17.09±2.51	**8.93±1.26**	16.06±2.62	18.60±0.14	>100
 **3g**	**12.27±1.20**	24.91±2.54	**14.88±1.98**	22.61±1.97	26.95±0.84	>100
 **3h**	26.28±0.68	15.20±1.56	**20.24±1.63**	24.13±2.19	21.17±0.36	>100
 **3i**	17.59±1.70	16.97±1.49	**17.93±0.77**	27.76±0.97	18.07±0.47	>100

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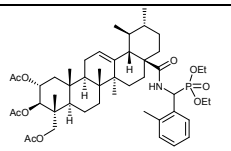
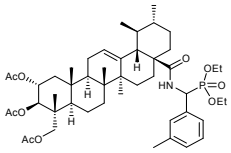
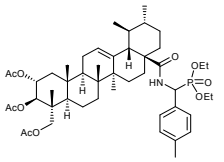
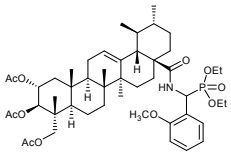
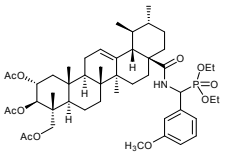
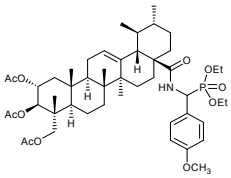
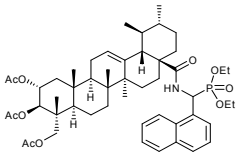
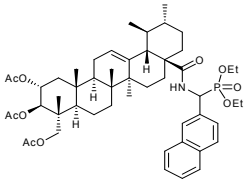
 **3j**	25.99±0.52	37.87±2.54	31.93±3.34	26.98±0.77	22.63±1.72	>100
 **3k**	27.71±0.55	32.64±1.20	**25.24±0.55**	23.80±1.09	19.37±0.53	>100
 **3l**	**12.36±4.42**	28.54±1.36	**17.83±4.54**	20.28±1.07	**10.26±0.69**	>100
 **3m**	47.31±1.12	11.07±2.96	**24.11±0.66**	31.43±2.09	40.13±0.32	>100
 **3n**	35.73±1.34	27.42±0.97	**16.65±1.85**	22.75±1.26	42.29±0.79	>100
 **3o**	45.57±0.45	25.09±3.55	**18.11±0.33**	27.70±1.98	38.20±0.83	>100
 **3p**	54.79±2.27	41.02±1.29	43.80±1.03	46.14±1.23	40.14±0.12	>100
 **3q**	46.33±2.08	38.97±0.61	49.10±0.11	48.72±0.54	46.22±0.27	>100

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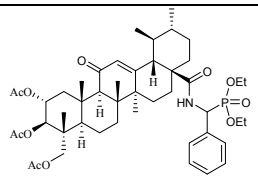
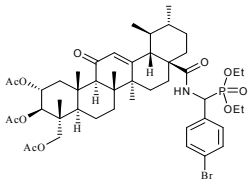
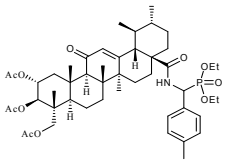
 **3r**	37.92±1.18	22.98±0.90	**28.92±0.53**	39.39±2.35	30.87±0.18	>100
 **6i**	23.35±1.52	18.85±1.69	**13.80±1.90**	16.76±1.48	19.70±0.11	>100
 **6n**	27.12±3.01	28.94±0.78	**21.89±1.65**	25.11±0.99	20.12±1.13	>100
AA	37.81±2.16	29.35±1.39	33.72±1.25	39.44±0.87	35.65±0.98	>100
5-FU	34.47±1.90	23.50±2.34	40.14±2.14	44.34±1.90	27.23±0.16	56.00±4.0
Cisplatin	13.48±0.37	10.43±0.47	30.05±0.39	15.83±0.54	15.60±1.68	9.68±1.15

[α]IC_{50} values are presented as the mean ± SD (standard error of the mean) from three separated experiments.

2.2.2 Investigation of Cell Cycle Distribution

The mechanism of the anti-proliferative effect of the **AA** derivatives was investigated by flow cytometric assay of the cell cycle distribution of T24 cells 48 h following treatment with 0, 2.5 or 5 μM of each analogue. As shown in Fig. 1, treatment of T24 cells with compound **3d** at each concentration enhanced cell cycle arrest at the G_1 phase, resulting in concomitant increase in the population in the G_1 phase (to 46.48% and 56.57%) compared with the control cells (38.51%). The S-phase population of T24 cells decreased by 48.27% and 36.97% compared with the control cells (57.65%). These results suggested that compound **3d** arrested the cell cycle of T24 cells in the G_1 stage.

The molecular events involved in cellular responses to the effective compound were investigated, and the expression of regulatory proteins implicated in G_1 arrest, including p21, cyclin A, p27, cyclin E1, and cyclin D1 were evaluated. The cell cycle regulatory proteins that control the G_1 to S-phase transition are cyclins and cyclin-dependent kinases (CDKs). The activity of cyclin D-dependent CDK4 is detected first in mid-G1 phase and then increases as cells approach the G1/S boundary. Cyclin E is expressed periodically with maximum levels occurring near the G1/S transition, binding to a different catalytic subunit, CDK2. The CDK inhibitors are tumour-suppressor proteins that interact with distinct cyclin-CDK complexes and thereby inhibit the activity of enzymes such as p21 and p27, which regulate the G1 to S-phase transition of the

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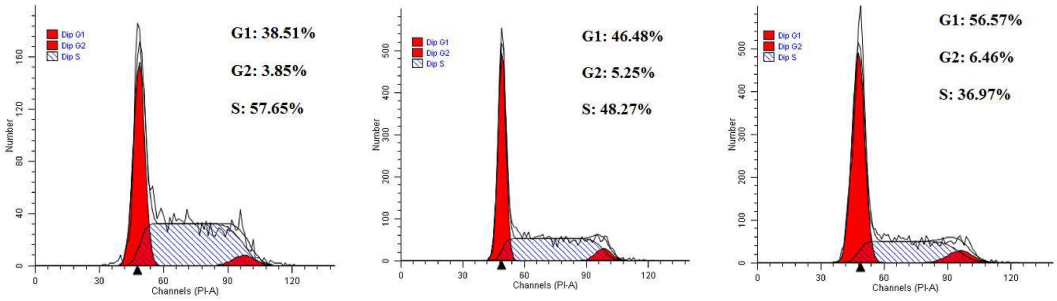
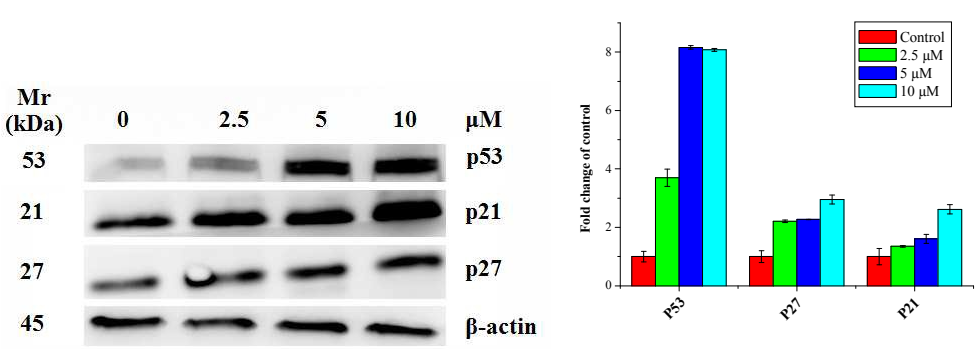
cell cycle [34–36]. Flow cytometry data clearly show that cell cycle arrest occurred at G_1 and possibly had an effect on cell cycle regulatory proteins. The levels of cyclins (cyclin A, cyclin D1 and cyclin E), CDKs (CDK2, and CDK4) and CDK inhibitors (p53, p16, p21 and p27) in T24 cells treated for 48 h with 2.5 μM, 5 μM and 10 μM **3d** were determined by western blot assay(Fig. 2). The T24 cell lysates revealed that **3d** reduced the levels of CDK2, CDK4, cyclin E and cyclin D1 compared with controls, and synchronously increased the levels of p21 and p27 in a concentration-dependent manner, indicating that the cells were effectively arrested at the G_1 phase of the cell cycle. However, **3d** treatment did not alter the levels of cyclin A in T24 cells.



(a) (b) (c)

Fig. 1. Cell cycle analysis of compound **3d** treated T24 cells. T24 cells were treated with different concentrations (0, 5 and 10 μM) of compound **3d** for 48 h to determine DNA fluorescence and cell cycle phase distribution.



(a)

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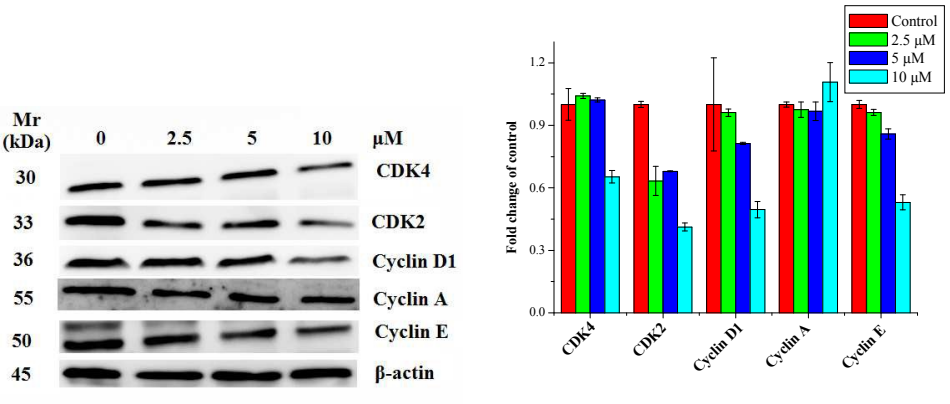
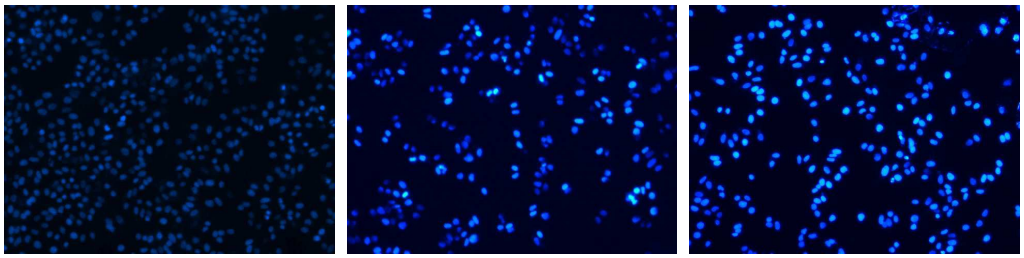


(b)

Fig. 2. Effect of asiatic acid derivatives on the expression of cyclins and associated proteins. T24 cells were treated with compound **3d** different concentrations for 48 h. Western blot analysis was carried out with antibodies against (cyclin D1, cyclin A and cyclin E); CDK2, CDK4 and *β*-actin was used as loading control.

2.2.3. Apoptosis Assay by Hoechst 33258 Staining

Apoptosis is a key pathway leading to cell death and is characterized by chromatin condensation and fragmented nuclei. It was considered of interest to investigate the apoptosis-inducing effect of compound **3d** by Hoechst 33258 staining of T24 bladder cancer cells. Cells treated with 10 μM and 20 μM **3d** for 24 h were stained with Hoechst 33258; cells not treated with **3d** were 24 h controls. The results (Fig. 3) showed that T24 control cells not treated with compound **3d** were normally stained blue (in the web version). Cells treated with **3d** for 12 and 24 h displayed strong blue fluorescence and had characteristic apoptotic morphologies. The observations revealed that compound **3d** induced apoptosis of T24 cells.



(a) (b) (c)

Fig. 3. Effects of compound **3d** on morphological changes of T24 cells after staining with Hoechst 33258 dye. (a) The cells not treated with **3d** were used as control, (b, c) compound **3d** treated T24 cells at concentrations of 10 and 20 μM, respectively.

2.2.4. Apoptosis Assay by Acridine Orange/Ethidium Bromide (AO/EB) Staining

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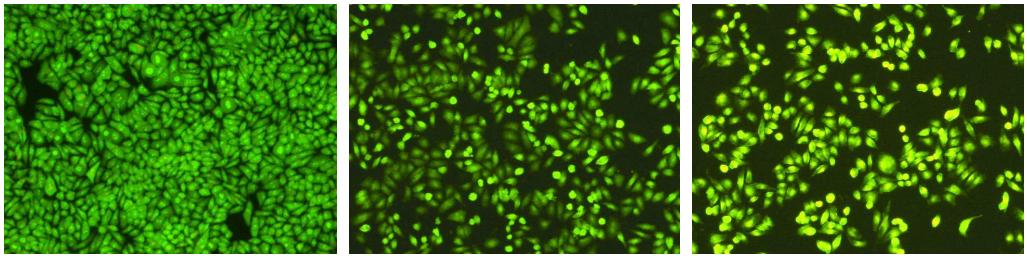
To further characterize the cell apoptosis induced by compound **3d**, AO/EB staining was carried out to evaluate the accompanying changes in morphology. The cytotoxicity of compound **3d** was evaluated in T24 cells following treatment with 10 μM and 20 μM for 24 h. T24 cells not treated with **3d** were 24 h controls. The results (Fig. 4) showed that at both concentrations, the morphology of **3d**-treated T24 cells had changed significantly. The cell nuclei were stained yellow green or orange, and the morphology showed pycnosis, membrane blebbing and cell budding characteristic of apoptosis. The nearly complete absence of red-stained cells showed that **3d** treatment was associated with very low cytotoxicity. The results thus demonstrated that compound **3d** induced apoptosis with low cytotoxicity, and were consistent with the results of Hoechst 33258 staining.



(a) (b) (c)

Fig. 4. Compound **3d** induced apoptotic in T24 cells were determined by AO/EB staining and were photographed via fluorescence microscopy. (a) Not dealt with compound **3d** was used as control at for 24 h, (b, c) dealt with compound **3d** for 24 h at concentrations of 10 and 20 μM, respectively.

2.2.5. Apoptosis Assay by Flow Cytometry

Following evaluation of the anticancer activity of **3d** and its effect on the cell cycle, we assayed its ability to trigger apoptosis in T24 cells by flow cytometry (Fig. 5). Untreated T24 cells (controls) or T24 cells treated with 10.0 μM or 20.0 μM compound **3d** for 24 h were double stained with fluorescein isothiocyanate (FITC)-annexin V and propidium iodide (PI) and analysed by flow cytometry. This assay allows permits the differentiation of live cells (annexin V^-/PI^-), early apoptotic cells (annexin V^+/PI^-), late apoptotic cells (annexin V^+/PI^+), and necrotic cells (annexin V^-/PI^+). Treatment of T24 cells with 10.0 μM compound **3d** (Fig. 5a, b) led to an increase in the number of apoptotic cells, from 7.39% in controls to 14.69% in treated cells (i.e., 10.2% early apoptotic cells and 4.49% late apoptotic cells). Treatment was also accompanied by a decrease in the percentage of live cells; 89.2% in controls and 81.5% in treated cells. Moreover, the effect on the apoptosis rate increased with concentration, and after treatment with 20 μM

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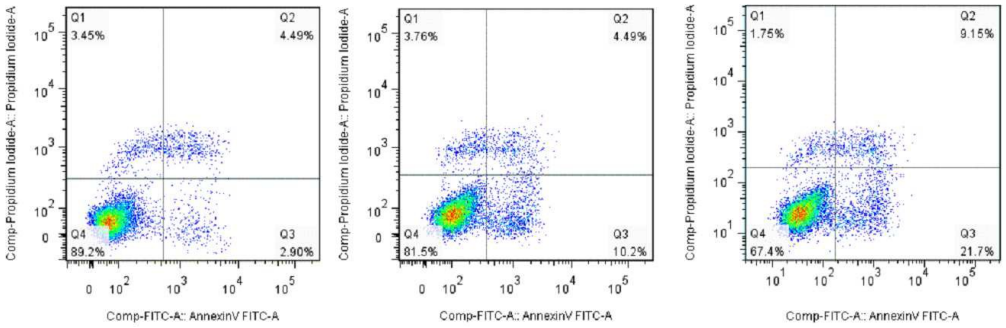
compound **3d,** 30.85% of the cells were apoptotic, a significantly higher percentage than observed in the controls. The results further demonstrated the apoptosis induced by compound **3d** in addition to its inhibition of cell proliferation.



(a) (b) (c)

Fig. 5. Flow cytometry analysis of cells stained with Annexin V-FITC and PI. (a) The T24 cells not treated with **3d** were used as control, (b, c) compound **3d** treated T24 cells for 24 h at concentration of 10 and 20 μM, respectively.

2.2.6. Increase of Intracellular ROS Level Induced in T24 Cells by Compound **3d**

ROS are a by-product of normal metabolism, and include free radicals such as the superoxide anion, hydroxyl and lipid radicals, as well as non-radical oxidizing species such as hydrogen peroxide, peroxynitrite and singlet oxygen. They often cause cellular damage and lead to cell death and tissue injury, especially at high concentrations. The generation of intracellular ROS may be responsible for the induction of apoptosis [37,38]. We studied the loss of mitochondrial transmembrane potential, and the resulting generation of ROS caused by compound **3d** *in vitro,* by fluorescence microscopy using the fluorescent probe 2,7-dichlorofluorescein diacetate. T24 cells treated with compound **3d** exhibited more intense fluorescence in the cytoplasm than untreated control cells. Under the same experimental procedures applied to **3d**-treated cells, the fluorescence detected in controls was weak and uniformly distributed within the cells. Figure 6 shows strong green fluorescence (in the web version) in T24 cells treated with compound **3d,** indicating that **3d** significantly induced apoptosis of T24 cells. An increase of ROS might thus be an early mediator in compound **3d**-induced apoptosis. These findings are consistent with an effect of compound **3d** on mitochondrial function and accumulation of ROS as cues for the induction of apoptosis.

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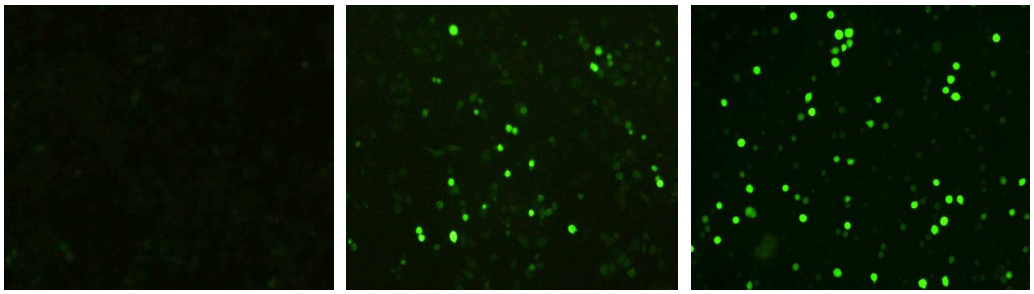
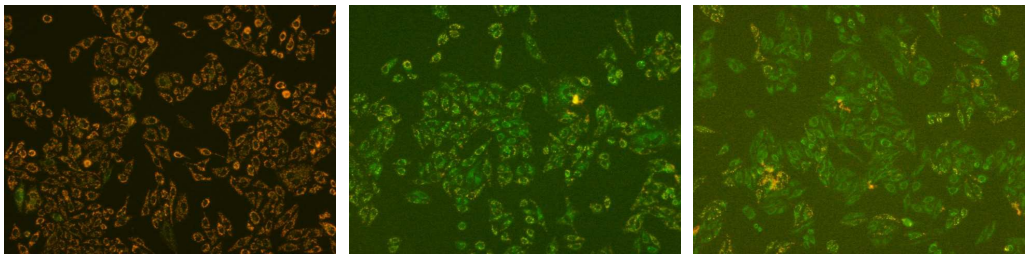


(a) (b) (c)

Fig. 6. Compound **3d** affected the levels of intracellular ROS in T24 cells. The T24 cells not treated with **3d** were used as control, (b, c) Compound **3d** treated T24 cells at concentrations of 10 and 20 μM, respectively.

2.2.7. Compound **3d**-induced Loss of Mitochondrial Membrane Potential (ΔΨm) in T24 Cells



(a) (b) (c)

Fig. 7. Effects of compound **3d** on morphological changes and mitochondrial membrane potential (Δψm) in T24 cells. (a) Not dealt with compound **3d** was used as control at for 24 h, (b, c) dealt with compound **3d** at concentration of 20 μM for 12 and 24 h , respectively.

Mitochondria play a central role in the life and death of cells and are known to be a major source and target of oxidative stress. Damage to mitochondrial integrity and the consequent loss of mitochondrial membrane potential are early events in the initiation and activation of apoptotic cascades [39]. To further investigate the apoptosis-inducing effect of compound **3d**, the fluorescent probe JC-1 was used to design and detect the changes of mitochondrial membrane potential. T24 cells treated with different concentrations of **3d** for 24 h were stained with JC-1; cells not treated with **3d** were used as 24 h controls. The results in Fig. 7 show that control cells not treated with compound **3d** were normally red (in the web version), while **3d**-treated cells showed strong green fluorescence and had typical apoptotic morphologies at both 12 and 24 h after treatment. These results indicate that apoptosis is the primary mode of cell death induced by compound **3d**.

2.2.8. Intracellular Ca^{2+} Release

Calcium is a ubiquitous second messenger involved in many cellular processes, including

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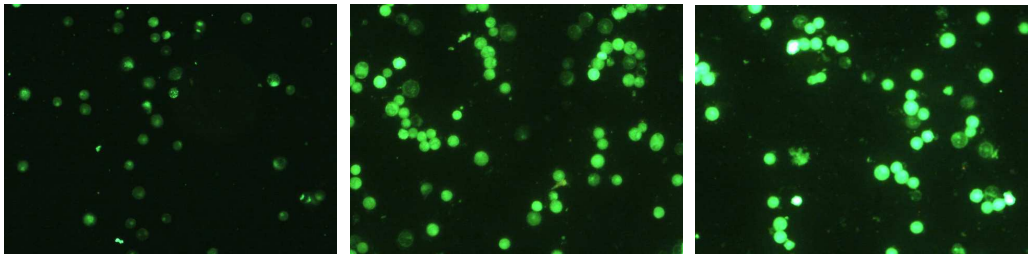
regulation of transcription, metabolism, proliferation and cell death. Because of its multiple effects, and because calcium overload can induce cell apoptosis, the intracellular Ca^{2+} concentration is tightly regulated. Mitochondria can be considered as a firewall that controls Ca^{2+} concentration in the cell and in cytoplasmic microdomains by tuning the frequency of oscillatory Ca^{2+} signals and by blunting the spread of cytosolic Ca^{2+} waves. We found that compound **3d** disrupted mitochondrial function and induced apoptosis. To determine the role of calcium signalling in **3d**-induced apoptosis, T24 cells were treated with **3d** for 24 h, and Ca^{2+} was detected by fluorescence microscopy using Fluo-3/AM, a calcium indicator dye. As shown in Fig. 8, **3d** treatment resulted in a dose-dependent elevation of intracellular Ca^{2+} concentration. The results suggest that **3d**-induced apoptosis was associated with an increase in Ca^{2+}concentration.



(a) (b) (c)

Fig. 8. Compound **3d** caused the levels of intracellular Ca^{+} elevation in T24 cells. The T24 cells not treated with **3d** were used as control, (b, c) Compound 3d treated T24 cells at concentrations of 10 and 20 μM, respectively.

2.2.9. Caspase-dependent Apoptosis in T24 Cells

To confirm the molecular mechanisms of apoptosis, we investigated the effects of compound **3d** on expression of proteins needed for mitochondria-mediated apoptosis. Cancer cells can limit or evade apoptosis in a number of ways, which often include perturbation of the Bcl-2 intrinsic apoptotic pathway. Members of the Bcl-2 family play important regulatory roles both as pro- and as anti-apoptotic proteins. Accordingly, the Bcl-2/Bax ratio is more important in the regulation of apoptosis than the individual levels of each Bcl-2 family protein. To further verify that **3d** promotes apoptosis in T24 cells, we assayed the expression of Bax, Bcl-2 and cytochrome c proteins following treatment with **3d**. As shown in Fig. 9a, treatment of T24 cells with **3d** led to an increase in Bax expression and decrease in Bcl-2 expression followed by the release of mitochondrial cytochrome c into the cytosol. These results indicate that **3d** induced apoptosis by regulating the levels of the Bcl-2 family proteins, Bax and Bcl-2.

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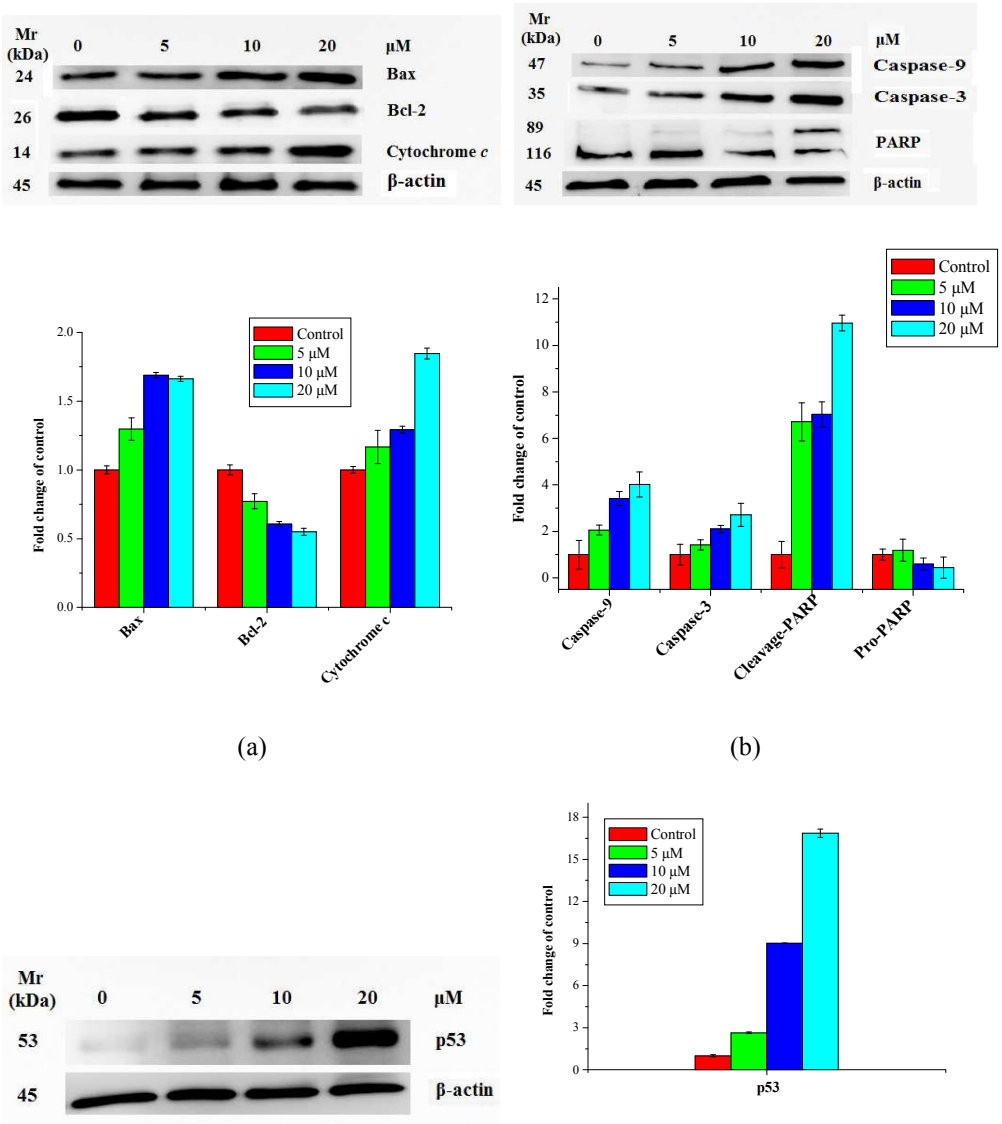

Fig. 9. Effects of compound **3d** on the level of cytochrome *c*, Bcl-2, Bax, Caspase-9, Caspase-3 and p53. T24 cells were treated with of compound **3d** for 24 h at concentrations of 0, 5, 10, 20 μM, respectively. Equal amount of protein was loaded on SDS-PAGE gel for western blot analysis as described in experimental section. *β*-actin was used as an internal control.

It is well-known that proteins of the Bcl-2 family play a pivotal role in apoptosis by interfering with caspases, which are the key effectors of programmed cell death. The caspase cascade is initiated by the proteolysis of inactive procaspases, and it is propagated by the cleavage of downstream caspases and substrates such as poly (ADP-ribose) polymerase (PARP) cleavage. To determine whether caspases are involved in **3d**-induced apoptosis, we assayed caspase-9, -3 and PARP expression in western blots. As shown in Fig. 9b, the expression of caspase-9, -3 and PARP in compound **3d**-treated T24 cells was significantly higher than that in control cells. These

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observations suggest that compound **3d** may exert pro-apoptotic effects through a mitochondria-mediated pathway and caspase cascade.

p53, a crucial tumour suppressor protein with a key role in many cellular processes, is one of the most relevant cellular components related to cancer growth. Depending on the cellular context, accumulation of p53 in response to various stresses including DNA damage induces cell cycle inhibition, senescence and apoptosis [40]. p53 protein is an attractive target of anticancer therapy because it can be functionally activated to eradicate tumours. Western blot analysis revealed a dose-dependent increase of p53 expression in T24 cells treated with **3d** compared with expression in controls (Fig. 9c). In line with our other observations, the effect of **3d** treatment on these cell cycle regulators was dose-dependent. These results indicate that enhanced p53 expression was likely involved in the mechanism of **3d**-induced G_1/S cell cycle arrest.

In summary, we successfully synthesized a series of **AA**–APA conjugates and evaluated their cytotoxicity in a panel of cancer cell lines. The target compounds exhibited remarkable anticancer activity with low cytotoxicity against normal HUVEC. The cell apoptosis-inducing activity of representative compound **3d** in T24 cells revealed that the anticancer activity of this compound depended on apoptosis of cancer cells via regulation of Bcl-2 family members, activation of caspase-9 and caspase-3 and subsequent cleavage of PARP. Cell cycle analysis confirmed that compound **3d** inhibited proliferation by G_1 phase arrest that depended on expression of p53 protein. In light of our results, the rational design of **AA** derivatives containing APA moieties represents a promising basis for the development of new anticancer agents.

3. Experimental Section

3.1. Chemistry

All chemicals were reagent grade and are commercially available. NMR spectra were measured on a BRUKER AVANCE AV500 spectrometer using tetramethyl silane, (TMS) as the internal standard. The mass spectra were obtained on a BRUKER ESQUIRE HCT spectrometer. Melting points were determined using an X-4 apparatus and were uncorrected. All chemical reagents including the aromatic aldehyde and solvents were analytical grade. GelRed nucleic acid stain was purchased from Biotium.

*3.1.1 General procedure for compound **1** (2α, 3β, 23-triacetoxyurs-12-ene-28-oic acid)*

Acetic anhydride (0.5 mL, 5.0 mmol) was added to a solution of **AA** (200 mg, 0.4 mmol) in pyridine (10 mL) and the mixture was stirred at 20^{o}C for 8 h. After dilution with ethyl acetate (25 mL), the mixture was washed with aqueous 1 M HCl (10 mL×5), saturated $CuSO_4$ (15 mL×2) and saturated NaCl solution (20 mL). The organic phase was dried over anhydrous sodium sulfate. Following filtration and evaporation of solvent at reduced pressure, the crude product was purified by column chromatography (petroleum ether : acetic ether = 3:1) to yield compound **1** as a white solid. Yield 85.5%. m.p. 151.2~154.6 ^{o}C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.19 (t, J = 3.2 Hz, 1H,

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H-12), 5.11 (td, *J* = 10.9, 4.6 Hz, 1H, H-2), 5.04 (d, *J* = 10.3 Hz, 1H, H-3), 3.80 (d, *J* = 11.8 Hz, 1H, H-23), 3.54 (d, *J* = 11.9 Hz, 1H, H-23), 2.14 (d, *J* = 11.3 Hz, 1H, H-9), 2.04 (s, 3H, CH_3CO), 1.98 (s, 3H, CH_3CO), 1.94 (s, 3H, CH_3CO), 1.89−1.11 (triterpene's H, 19H), 1.05 (s, 3H, CH_3-27), 1.03 (s, 3H, CH_3-24), 0.90 (s, 3H, CH_3-25), 0.83 (s, 3H, CH_3-26), 0.80 (d, *J* = 6.4 Hz, 3H, CH_3-29), 0.71 (d, *J* = 6.4 Hz, 3H, CH_3-30). ^{13}C NMR (126 MHz, $CDCl_3$) δ 184.04, 170.89, 170.54, 170.43, 138.03, 125.19, 74.77, 69.91, 65.21, 60.41, 52.39, 47.88, 47.53, 47.43, 43.66, 41.87, 39.45, 38.94, 38.75, 37.77, 36.61, 32.37, 30.54, 27.83, 23.90, 23.43, 23.29, 21.17, 21.09, 20.89, 20.79, 17.81, 17.01, 16.93, 14.19, 13.90; ESI-HRMS *m/z* Calc for $C_{36}H_{54}O_8$ $[M-H]^-$: 613.37459, founded: 613.37483.

3.1.2 General procedure for the preparation of compounds ***3***

Oxalyl chloride (1.2 mL) at 0°C was added dropwise to a solution of compound **2** (100.00 mg, 0.14 mmol) in CH_2Cl_2, and the mixture was stirred at room temperature for 8 h. The solvent was evaporated at reduced pressure. CH_2Cl_2 (10 ml × 3) was added to the mixture, with vacuum concentration. The mixture was the allowed to react with individual APAs (0.56 mmol) with stirring at room temperature for 4 h. After dilution with ethyl acetate (25 mL), the mixture was washed with water (20 mL × 3) and the organic phase was dried over anhydrous sodium sulfate. Following filtration and evaporation of solvent at reduced pressure, the residue was purified by column chromatography to yield a white solid.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-2-fluorophenyl)meth-yl}phosphonate ***(3a)***. Yield 65.5%. m.p. 135.7~139.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 (d, *J* = 7.2 Hz, 1H, Ar-H), 7.40–7.27 (m, 2H, Ar-H), 7.17−7.12 (m, 1H, Ar-H), 6.51–6.21 (m, 1H, NH), 5.82−5.67 (m, 1H, H-11), 5.30−5.20 (m, 1H, H-3), 5.16–5.10 (m, 1H, H-2), 5.04 (m, 1H, P-CH), 4.23–4.10 (m, 2H, -OCH_2), 3.40–3.75 (m, 2H, -OCH_2), 3.73–3.42 (m, 2H, H-23), 2.04 (s, 3H, $COCH_3$), 2.01(s, 3H, $COCH_3$), 1.97 (s, 3H, $COCH_3$), 1.96–1.10 (m, triterpene's H, 21H), 1.33 (t, *J* =5 Hz, 6H, CH_3×2), 1.10 (s, 3H, CH_3-27), 1.04 (s, 3H, CH_3-24), 1.00 (s, 3H, CH_3-26), 0.95 (d, *J* = 5.5 Hz, 3H, CH_3-30), 0.88 (s, 3H, CH_3-25), 0.82 (d, *J* = 3.3 Hz, 3H, CH_3-29). ^{13}C NMR (126 MHz, DMSO) δ 176.2, 170.7, 170.4, 170.3, 139.1, 138.2, 129.7, 129.2, 128.9, 126.9, 125.7, 125.2, 74.7, 69.8, 65.2, 63.1, 62.8, 54.1, 53.6, 47.9, 47.6, 43.7, 42.3, 42.1, 41.8, 39.6, 39.3, 38.9, 38.8, 32.5, 32.2, 30.7, 29.6, 27.6, 24.2, 23.2, 21.1, 21.0, 20.8, 20.7, 17.8, 17.6, 16.9, 16.8, 16.3, 15.9, 13.8. ESI-HRMS *m/z* Calc for $C_{47}H_{69}FNO_{10}P$ $[M+H]^+$: 858.47159 founded: 858.46844.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-3-fluorophenyl)met-hyl}phosphonate ***(3b)***. Yield 67.5%. m.p. 133.4~135.6 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.53 (d, *J* = 7.2 Hz, 1H, Ar-H), 7.38–7.26 (m, 2H, Ar-H), 7.15−7.10 (m, 1H, Ar-H), 6.49–6.19 (m, 1H, NH), 5.80−5.65 (m, 1H, H-11), 5.28−5.19 (m, 1H, H-3), 5.14–5.08 (m, 1H, H-2), 5.02 (m, 1H, P-CH), 4.21–4.09 (m, 2H, -OCH_2), 3.88–3.75 (m, 2H, -OCH_2), 3.71–3.42 (m, 2H, H-23), 2.02 (s, 3H, $COCH_3$), 1.99 (s, 3H, $COCH_3$), 1.95 (s, 3H, $COCH_3$), 1.94–1.08 (m, triterpene's H, 21H), 1.31 (t, *J* =5 Hz, 6H, CH_3×2), 1.07 (s, 3H, CH_3-27), 1.02 (s, 3H, CH_3-24), 0.98 (s, 3H, CH_3-26), 0.93 (d, *J* = 5.5 Hz, 3H,

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CH_3-30), 0.86 (s, 3H, CH_3-25), 0.80 (d, J = 3.3 Hz, 3H, CH_3-29). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.2, 170.7, 170.4, 170.3, 139.1, 138.2, 135.1, 133.0, 129.4, 127.5, 125.7, 125.2, 74.7, 69.8, 65.2, 63.1, 62.8, 54.2, 50.5, 47.9, 47.6, 43.7, 42.3, 42.1, 41.8, 39.6, 39.3, 38.9, 38.8, 32.5, 32.2, 30.7, 29.6, 27.7, 24.2, 23.2, 21.0, 21.0, 20.8, 20.7, 17.8, 17.6, 16.9, 16.8, 16.4, 15.9, 13.8. ESI-HRMS *m/z* Calc for $C_{47}H_{69}FNO_{10}P$ $[M+H]^+$: 858.47159 founded: 858.46891.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-4-fluorophenyl)met-hyl}phosphonate ***(3c)***. Yield 68.1%. m.p. 133.2~135.1 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39−7.32 (m, 2H, Ar-H), 7.02−6.98 (m, 2H, Ar-H), 6.67−6.43 (m, 1H, NH), 5.51−5.33 (m, 1H, H-11), 5.26 (dd, J = 11.3, 9.4 Hz, 1H, H-3), 5.15−5.08 (m, 1H, H-2), 5.04 (dd, J = 14.6, 10.3 Hz, 1H, P-CH), 4.15−4.02 (m, 2H, -OCH_2), 3.92−3.77 (m, 2H, -OCH_2), 3.73−3.50 (m, 2H, H-23), 2.10−1.10 (m, triterpene's H, 21H), 2.04 (s, 3H, $COCH_3$), 2.00 (s, 3H, $COCH_3$), 1.96 (s, 3H, $COCH_3$), (3×CH_3CO), 1.30 (td, J = 7.1 Hz, 6H, CH_3×2), 1.07 (s, 3H, CH_3-27), 1.04 (s, 3H, CH_3-24), 1.00 (s, 3H, CH_3-26), 0.94 (d, J = 4.6 Hz, 3H, CH_3-30), 0.86 (s, 3H, CH_3-25), 0.83 (d, J = 3.2 Hz, 3H, CH_3-29). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.50, 170.8, 170.4, 170.3, 139.2, 138.1, 130.9, 130.5, 129.7, 125.5, 125.2, 115.6, 74.8, 69.9, 65.3, 62.9, 62.7, 54.1, 50.4, 48.0, 47.6, 43.8, 42.3, 42.2, 41.8, 39.6, 39.5, 38.9, 38.8, 32.5, 32.4, 30.8, 29.6, 27.6, 24.3, 23.2, 21.1, 21.0, 20.8, 20.7, 17.8, 17.7, 17.0, 16.7, 16.3, 16.1. 13.9. ESI-HRMS *m/z* Calc for $C_{47}H_{69}FNO_{10}P$ $[M+H]^+$: 858.47159 founded: 858.46698.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-2-chlorophenyl)met-hyl}phosphonate ***(3d)***. Yield 64.9%. m.p. 141.3~144.6 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39−7.33 (m, 2H, Ar-H), 7.27–7.20 (m, 2H), 6.91−6.79 (m, 1H, NH), 5.82−5.72 (m, 1H, H-11), 5.20–5.13(m, 1H, H-3), 5.12–5.06 (m, 1H, H-2), 5.09−5.06 (m, 1H, P-CH), 4.19–4.09 (m, 2H, -OCH_2), 3.89−3.72 (m, 2H, -OCH_2), 3.73–3.47 (m, 2H, H-23), 2.03 (s, 2H, $COCH_3$), 1.99 (s, 3H, $COCH_3$), 1.96 (s, 3H, $COCH_3$), 1.95–1.09 (m, triterpene's H, 21H), 1.34−1.28 (m, 6H, CH_3×2), 1.06 (s, 3H, CH_3-27), 1.02 (s, 1H, CH_3-24), 0.99 (s, 3H, CH_3-26), 0.94 (d, J = 4.1 Hz, 3H, CH_3-30), 0.86 (s, 3H, CH_3-25), 0.81 (s, 3H, CH_3-29). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.8, 170.8, 170.5, 170.3, 139.1, 138.2, 133.2, 129.7, 129.2, 126.9, 125.7, 125.2, 74.8, 69.9, 65.2, 63.1, 62.8, 54.1, 47.9, 47.6, 47.4, 43.8, 42.3, 42.1, 41.9, 39.6, 39.3, 38.9, 32.5, 32.2, 30.7, 29.6, 27.7, 24.5, 23.4, 23.1, 21.1, 21.0, 20.8, 20.7, 17.8, 17.7, 16.9, 16.8, 16.4, 15.9, 13.9. ESI-HRMS *m/z* Calc for $C_{47}H_{69}ClNO_{10}P$ $[M+H]^+$: 874.44204 founded: 874.43933.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-3-chlorophenyl)met-hyl}phosphonate ***(3e)***. Yield 68.3%. m.p. 132.9~135.7 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35 (d, J = 15.0 Hz, 1H, Ar-H), 7.25 (m, 3H, Ar-H), 6.70–6.44 (m, 1H, NH), 5.49−5.33 (m, 1H, H-11), 5.28–5.22 (m, 1H, H-3), 5.15−5.10 (m, 1H, H-2), 5.06−5.01 (m, 1H, P-CH), 4.18–4.00 (m, 2H, -OCH_2), 3.95–3.77 (m, 2H, -OCH_2), 3.63–3.51 (m, 2H, H-23), 1.93−1.01 (m, triterpene's H, 21H), 2.03 (s, 3H, $COCH_3$), 1.99 (s, 3H, $COCH_3$), 1.96 (s, 3H, $COCH_3$), 1.30 (t, J = 7.1 Hz, 6H, CH_3×2), 1.07 (s, 3H, CH_3-27), 1.05 (s, 3H, CH_3-24), 0.94 (s, 3H, CH_3-26), 0.90 (d, J = 3.4 Hz, 3H, CH_3-30), 0.86 (s, 3H, CH_3-25), 0.83 (d, 3H, J = 4.3 Hz, CH_3-29). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.6, 170.8,

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170.4, 170.3, 139.0, 136.9, 134.5, 129.8, 128.3, 127.0, 126.1, 125.5, 74.8, 69.8, 65.2, 63.0, 62.8, 54.2, 50.8, 48.0, 47.4, 43.7, 42.3, 41.9, 41.8, 39.6, 39.4, 38.9, 32.5, 32.4, 30.7, 29.6, 27.6, 24.3, 23.4, 23.1, 21.1, 21.0, 20.8, 20.7, 17.8, 17.7, 17.0, 16.8, 16.4, 16.1, 13.9. ESI-HRMS *m/z* Calc for $C_{47}H_{69}ClNO_{10}P$ $[M+H]^+$: 874.44204 founded: 874.43604.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-4-chlorophenyl)met-hyl}phosphonate ***(3f)***. Yield 65.6%. m.p. 139.2~140.8 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.33 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.28 (d, *J* = 2.8 Hz, 2H, Ar-H), 6.69–6.42 (m, 1H, NH), 5.49–5.33 (m, 1H, H-11), 5.28−5.21 (m, 1H, H-3), 5.13−5.10 (m, 1H, H-2), 5.06−5.03 (m, 1H, P-CH), 4.13–4.06 (m, 2H, -OCH_2), 3.92–3.75 (m, 2H, -OCH_2), 3.57–3.51 (m, 1H, H-23), 2.03 (s, 3H, $COCH_3$), 2.00 (s, 3H, $COCH_3$), 1.96 (s, 3H, $COCH_3$), 1.95–1.00 (m, triterpene's H, 21H), 1.30 (t, *J* = 7.0 Hz, 6H, CH_3×2), 1.08–1.05 (m, 6H, CH_3-24/27), 0.94 (s, 3H, CH_3-26), 0.90 (d, *J* = 3.9 Hz, 3H, CH_3-30), 0.86 (s, 3H, CH_3-25), 0.83 (d , *J* = 4.1 Hz, 3H, CH_3-29). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.5, 170.8, 170.4, 170.3, 139.2, 138.2, 133.6, 130.2, 129.2, 128.8, 125.5, 125.3, 74.8, 69.8, 65.2, 63.0, 62.7, 54.1, 50.6, 48.0, 47.9, 43.7, 42.3, 41.9, 39.6, 39.4, 38.9, 38.8, 32.5, 32.4, 30.7, 29.6, 27.6, 23.4, 23.2, 21.1, 21.0, 20.8, 20.7, 17.8, 17.7, 17.0, 16.9, 16.7, 16.4, 16.3, 13.9. ESI-HRMS *m/z* Calc for $C_{47}H_{69}ClNO_{10}P$ $[M+H]^+$: 874.44204 founded: 874.43893.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-2-bromophenyl)met-hyl}phosphonate ***(3g)***. Yield 68.9%. m.p. 147.3~150.3 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.53 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.38–7.25 (m, 2H, Ar-H), 7.16−7.09 (m, 1H, NH), 5.79−5.67 (m, 1H, H-11), 5.19−5.13 (m, 1H, H-3), 5.12−5.06 (m, 1H, H-2), 5.06−5.01 (m, 1H, P-CH), 4.22−4.09 (m, 2H, -OCH_2), 3.89−3.76 (m, 2H, -OCH_2), 3.56−3.39 (m, 2H, H-23), 2.03 (s, 2H, $COCH_3$), 2.00 (s, 3H, $COCH_3$), 1.96 (s, 3H, $COCH_3$), 1.96−1.10 (m, triterpene's H, 21H), 1.35-1.30 (m, 6H, CH_3×2), 1.06 (s, 3H, CH_3-27), 1.03 (s, 3H, CH_3-24), 0.99 (s, 3H, CH_3-26), 0.94 (d, *J* = 4.9 Hz, 3H, CH_3-30), 0.86 (s, 3H, CH_3-25), 0.81 (d, *J* = 4.9 Hz, 3H, CH_3-29). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.3, 170.9, 170.6, 170.4, 138.2, 135.1, 133.1, 129.5, 129.2, 127.6, 125.8, 125.3, 74.8, 69.9, 65.3, 63.3, 62.9, 53.7, 50.6, 48.00, 47.7, 43.8, 42.3, 42.2, 41.9, 39.6, 39.0, 37.8, 32.5, 32.3, 30.8, 27.7, 24.6, 24.3, 23.5, 21.2, 21.1, 20.9, 20.8, 17.9, 17.7, 17.1, 16.9, 16.5, 16.0, 13.9. ESI-HRMS *m/z* Calc for $C_{47}H_{69}BrNO_{10}P$ $[M+H]^+$: 918.39152 founded: 918.38855.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-3-bromophenyl)met-hyl}phosphonate ***(3h)***. Yield 62.9%. m.p. 136.2~139.7 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.50 (d, *J* = 13.6 Hz, 1H, Ar-H), 7.41 (t, *J* = 10.0 Hz, 1H, Ar-H), 7.32 (dd, *J* = 15.6, 7.7 Hz, 1H, ArH), 7.18 (t, *J* = 7.8 Hz, 1H, Ar-H), 6.68 −6.44 (m, 1H, NH), 5.48–5.32 (m, 1H, H-11), 5.28–5.22 (m, 1H, H-3), 5.13 (td, *J* = 11.0, 4.5 Hz, 1H, H-2), 5.06−5.01 (m, 1H, P-CH), 4.16–4.02 (m, 2H, -OCH_2), 3.95−3.80 (m, 2H, -OCH_2), 3.67–3.52 (m, 2H, H-23), 2.04 (s, 3H, $COCH_3$), 2.00 (s, 3H, $COCH_3$), 1.97 (s, 3H, $COCH_3$), 1.95–1.09 (m, triterpene's H, 21H), 1.31 (t, *J* = 7.1 Hz, 6H, CH_3×2), 1.05 (s, 3H, CH_3-27), 1.01 (s, 3H, CH_3-24), 0.95 (s, 3H, CH_3-26), 0.91 (d, 3H, *J* = 3.5 Hz, CH_3-30), 0.86 (s, 3H, CH_3-25), 0.83 (d, *J* = 3.6 Hz, 3H, CH_3-29). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.6, 170.8,

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170.4, 170.3, 139.0, 138.1, 137.2, 131.6, 130.1, 127.5, 126.6, 125.5, 74.8, 69.9, 65.3, 63.0, 62.9, 54.2, 49.9, 48.1, 47.9, 43.8, 42.3, 41.9, 39.7, 39.5, 38.9, 37.8, 32.6, 32.4, 30.8, 27.6, 24.8, 23.4, 23.2, 21.1, 21.1, 20.9, 20.8, 17.9, 17.7, 17.0, 16.9, 16.5, 16.2, 16.1, 13.9. ESI-HRMS *m/z* Calc for $C_{47}H_{69}BrNO_{10}P$ $[M+H]^+$: 918.39152 founded: 918.38776.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-4-bromophenyl)met-hyl}phosphonate ***(3i)***. Yield 66.5%. m.p. 142.1~144.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 (dd, *J* = 8.2, 3.4 Hz, 2H, Ar-H), 7.28−7.22 (m, 2H, Ar-H), 6.71−6.42 (m, 1H, NH), 5.48−5.31 (m, 1H, H-11), 5.26−5.18 (m, 1H, H-3), 5.12 (td, *J* = 10.9, 4.5 Hz, 1H, H-2), 5.03 (dd, *J* = 13.0, 10.4 Hz, 1H, P-CH), 4.16−4.00 (m, 2H, -OCH_2), 3.92 − 3.76 (m, 2H, -OCH_2), 3.74 − 3.48 (m, 2H, H-23), 2.04 (s, 3H, CH_3CO), 1.99 (d, *J* = 2.7 Hz, 3H, CH_3CO), 1.95 (s, 3H, CH_3CO), (3×CH_3CO), 1.93−0.99 (m, triterpene's H, 21H), 1.29 (t, *J* = 7.1 Hz, 6H, CH_3×2), 1.07 (s, 3H, CH_3-27), 1.04 (s, 3H, CH_3-24), 0.93 (s, 3H, CH_3-26), 0.91 (d, *J* = 4.5 Hz, 3H, CH_3-30), 0.86 (s, 3H, CH_3-25), 0.84 (d, *J* = 3.4 Hz, 3H, CH_3-29). ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.5, 170.7, 170.4, 170.3, 139.1, 138.2, 134.1, 131.6, 130.5, 129.6, 125.5, 125.3, 74.8, 69.8, 65.2, 63.0, 62.7, 54.1, 50.8, 48.0, 47.9, 43.7, 42.2, 41.8, 39.6, 39.4, 38.9, 38.8, 32.5, 32.4, 30.7, 29.6, 27.6, 23.4, 23.1, 21.1, 21.0, 20.8, 20.7, 17.8, 17.6, 17.0, 16.9, 16.8, 16.3, 16.1, 13.9. ESI-HRMS *m/z* Calc for $C_{47}H_{69}BrNO_{10}P$ $[M+H]^+$: 918.39152 founded: 918.39420.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-2-methylphenyl)met-hyl}phosphonate ***(3j)***. Yield 61.9%. m.p. 130.9~134.1 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45−7.32 (m, 1H, Ar-H), 7.20–7.10 (m, 3H, Ar-H), 6.81− 6.47 (m, 1H, NH), 5.86–5.56 (m, 1H, H-11), 5.29 (d, *J* = 80.5 Hz, 1H, H-3), 5.21–5.00 (m, 2H, H-2, P-CH), 4.17−4.04 (m, 2H, -OCH_2), 3.88–3.76 (m, 2H, -OCH_2), 3.65–3.51 (m, 2H, H-23), 2.40 (s, 3H, Ar-CH_3), 2.03 (s, 3H, $COCH_3$), 1.99 (s, 3H, $COCH_3$), 1.96 (s, 3H, $COCH_3$), 1.96–1.06 (m, triterpene's H, 21H), 1.35–1.26 (m, 6H, CH_3×2), 1.05 (s, 3H, CH_3-27), 0.94 (s, 3H, CH_3-24), 0.86 (s, 3H, CH_3-26), 0.85 (d, *J* = 5.0 Hz, 3H, CH_3-30), 0.81 (s, 3H, CH_3-25), 0.80 (d, *J* = 3.3 Hz, 3H, CH_3-29). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.5, 170.8, 170.5, 170.4, 138.9, 138.3, 134.5, 133.5, 130.5, 128.0, 126.2, 125.5, 74.9, 69.9, 65.2, 62.8, 62.4, 54.3, 50.6, 47.9, 47.5, 43.8, 42.2, 41.9, 39.7, 39.3, 38.9, 37.6, 32.5, 32.4, 30.8, 27.7, 24.7, 24.3, 23.2, 21.1, 21.1, 20.8, 20.8, 19.6, 17.9, 17.7, 17.0, 16.8, 16.5, 16.0, 15.9, 13.9. ESI-HRMS *m/z* Calc for $C_{48}H_{72}NO_{10}P$ $[M+H]^+$: 854.49666 founded: 854.49109.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-3-methylphenyl)met-hyl}phosphonate ***(3k)***. Yield 68.5%. m.p. 134.8~136.9 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.25 − 7.20 (m, 2H, Ar-H), 7.10−7.07 (m, 2H, Ar-H), 6.66 − 6.42 (m, 1H, NH), 5.47−5.33 (m, 1H, H-11), 5.27 − 5.22 (m, 1H, H-3), 5.15 − 5.07 (m, 1H, H-2), 5.03 (dd, *J* = 17.7, 10.3 Hz, 1H, P-CH), 4.11−4.05 (m, 2H, -OCH_2), 3.91 − 3.75 (m, 2H, -OCH_2), 3.73−3.50 (m, 2H, H-23), 2.29 (s, 3H, Ar-CH_3), 2.02 (s, 3H, $COCH_3$), 1.99 (s, 3H, $COCH_3$), 1.95 (s, 3H, $COCH_3$), (3×CH_3CO), (3×CH_3CO), 2.08−1.10 (m, triterpene's H, 21H), 1.29 (td, *J* = 8.3, 5.8 Hz, 6H, CH_3×2), 1.04 (d, *J* = 3.7 Hz, 6H, CH_3-24/27), 0.98 (s, 3H, CH_3-26), 0.92 (d, , *J* = 3.9 Hz, 3H, CH_3-30), 0.85 (s, 3H, CH_3-25), 0.81 (d, *J* = 4.7 Hz,

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3H, CH_3-29). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.3, 170.8, 170.4, 170.3, 139.1, 138.2, 132.6, 131.7, 129.2, 128.6, 127.7, 125.4, 74.8, 69.8, 65.2, 62.7, 62.5, 54.1, 50.7, 48.6, 47.4, 43.7, 42.2, 41.8, 39.6, 39.3, 38.8, 37.7, 32.5, 32.3, 30.7, 27.6, 24.6, 24.3, 23.1, 21.1, 21.0, 20.8, 20.7, 19.9, 17.8, 17.6, 17.0, 16.9, 16.6, 16.2, 16.1, 13.8. ESI-HRMS *m/z* Calc for $C_{48}H_{72}NO_{10}P$ $[M+H]^+$: 854.49666 founded: 854.49299.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-4-methylphenyl)met-hyl}phosphonate ***(3l)***. Yield 64.3%. m.p. 138.1~141.6 °C. ^1H NMR (400 MHz, $CDCl_3$) δ 7.24 (dd, *J* = 8.5 Hz, 2H, Ar-H), 7.10 (d, *J* = 7.6 Hz, 2H, Ar-H), 6.71 − 6.42 (m, 1H, NH), 5.51 − 5.33 (m, 1H, H-11), 5.29 − 5.23 (m, 1H, H-3), 5.12 (m, 1H, H-2), 5.07 − 5.00 (m, 1H, P-CH), 4.15 − 4.02 (m, 2H, -OCH_2), 3.84−3.76 (m, 2H, -OCH_2), 3.57−3.51 (m, 2H, H-23), 2.30 (s, 3H, Ar-CH_3), 2.05 (s, 3H, $COCH_3$), 2.00 (s, 3H, $COCH_3$), 2.15−1.10 (m, triterpene's H, 21H), 1.96 (s, 3H, $COCH_3$), (3×CH_3CO), 1.33 − 1.26 (m, 6H, CH_3×2), 1.07 (s, 3H, CH_3-27), 1.02 (s, 3H, CH_3-24), 0.99 (s, 3H, CH_3-26), 0.94 (d, *J* = 4.5 Hz, 3H, CH_3-30), 0.86 (s, 3H, CH_3-25), 0.83 (d, *J* = 3.5 Hz, 3H, CH_3-29). ^{13}C NMR (126 MHz, $CDCl_3$) δ 175.2, 170.7, 170.4, 170.1, 138.0, 132.3, 131.4, 130.4, 129.5, 129.2, 128.2, 128.0, 74.9, 68.9, 65.2, 62.9, 62.7, 53.4, 50.1, 47.3, 47.1, 43.7, 42.4, 41.8, 39.1, 38.8, 38.5, 37.6, 32.5, 32.4, 30.4, 28.0, 24.4, 24.0, 23.2, 21.1, 21.0, 20.9, 20.7, 20.5, 18.2, 17.6, 17.3, 17.0, 16.5, 16.4, 16.1, 13.8. ESI-HRMS *m/z* Calc for $C_{48}H_{72}NO_{10}P$ $[M+H]^+$: 854.49666 founded: 854.48822.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-2-methoxyphenyl)-methyl}phosphonate ***(3m)***. Yield 69.0%. m.p. 132.3~135.2 °C. ^1H NMR (500 MHz, $CDCl_3$) δ 7.32−7.20 (t, 2H, Ar-H), 6.92−6.87 (m, 1H, Ar-H), 6.84 (m, 1H, NH), 5.78−5.67 (m, 1H, H-11), 5.16–5.11 (m, 1H, H-3), 5.10–5.08 (m, 1H, H-2), 5.03−5.00 (m, 1H, P-CH), 4.15–4.01 (m, 2H, -OCH_2), 3.90–3.75 (m, 5H, -OCH_2, Ar-OCH_3), 3.57−3.50 (m, 1H, H-23), 2.02 (s, 2H, $COCH_3$), 1.98 (s, 3H, $COCH_3$), 1.95 (s, 3H, $COCH_3$), 1.95–1.06 (m, triterpene's H, 21H), 1.28 (m, 6H, CH_3×2), 1.05–0.99 (m, 6H, CH_3-27/24), 0.98 (s, 3H, CH_3-26), 0.93 (d, *J* = 6.3 Hz, 3H, CH_3-30), 0.84 (s, 3H, CH_3-25), 0.80 (d, *J* = 4.4 Hz, 3H, CH_3-29). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.1, 170.8, 170.5, 170.3, 157.3, 138.8, 129.3, 125.4, 125.2, 123.3, 120.8, 111.0, 74.8, 69.9, 65.2, 62.6, 62.4, 55.7, 54.1, 47.9, 47.4, 43.7, 42.2, 41.8, 39.6, 39.3, 38.9, 37.6, 32.5, 32.2, 30.8, 27.6, 24.6, 24.3, 23.2, 23.0, 21.1, 21.0, 20.8, 20.7, 17.8, 17.7, 17.0, 16.8, 16.4, 16.1, 15.9, 13.9. ESI-HRMS *m/z* Calc for $C_{48}H_{72}NO_{11}P$ $[M+H]^+$: 870.49158 founded: 870.48547.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-3-methoxyphenyl)-methyl}phosphonate ***(3n)***. Yield 65.7%. m.p. 131.2~134.8 °C. ^1H NMR (500 MHz, $CDCl_3$) δ 7.20 (td, *J* = 7.9, 3.2 Hz, 1H, Ar-H), 6.97 − 6.88 (m, 2H, Ar-H), 6.79 (t, *J* = 8.2, 6.7 Hz, 1H), 6.68 − 6.44 (m, 1H, NH), 5.48 − 5.32 (m, 1H, H-11), 5.30 − 5.24 (m, 1H, H-3), 5.15 − 5.08 (m, 1H, H-2), 5.03 (dd, *J* = 16.2, 10.3 Hz, 1H, P-CH), 4.14 − 4.01 (m, 2H, -OCH_2), 3.94−3.79 (m, 2H, -OCH_2), 3.77 (d, *J* = 4.2 Hz, 3H, Ar-OCH_3), 3.73−3.53 (m, 2H, H-23), 2.04 (d, *J* = 12.5 Hz, 3H, $COCH_3$), 1.99 (d, *J* = 2.7 Hz, 3H, $COCH_3$), 1.96 (d, *J* = 2.6 Hz, 3H, $COCH_3$), 1.30 (t, *J* = 7.1 Hz, 6H, CH_3×2), 1.07 (s, 3H, CH_3-27), 1.04 (s, 3H, CH_3-24), 1.00 (s, 3H, CH_3-26), 0.94 (d, *J* = 5.3 Hz, 3H, CH_3-30), 0.85 (s, 3H,

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CH_3-25), 0.82 (d, J = 9.0 Hz, 3H, CH_3-29). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.4 (C-28), 170.7 ($COCH_3$), 170.4 ($COCH_3$), 170.3 ($COCH_3$), 159.7 (Ar-C), 139.0 (C-13), 129.6 (Ar-C), 125.5 (C-12), 121.0 (Ar-C), 120.2 (Ar-C), 114.5 (Ar-C), 113.5 (Ar-C), 74.8 (C-3), 69.8 (C-2), 65.2 (C-23), 62.7 (-OCH_2), 62.6 (-OCH_2), 55.2 (-OCH_3), 54.1 (P-CH), 50.3 (C-18), 48.0 (C-9), 47.8 (C-5), 43.7 (C-4), 42.3 (C-1), 41.9 (C-17), 41.8 (C-10), 39.60 (C-8), 39.3 (C-19), 38.9 (C-14), 38.8 (C-20), 32.5 (C-7), 32.4 (C-22), 30.7 (C-16), 29.6 (C-15), 27.6 (C-21), 23.4 (C-11), 23.1 (C-27), 21.1 (C-29), 21.0 ($COCH_3$), 20.8 ($COCH_3$), 20.7 ($COCH_3$), 17.8 (C-25), 17.7 (C-30), 17.0 (C-26), 16.8 (C-6), 16.7 (C-24), 16.2 (CH_3), 16.1 (CH_3). ESI-HRMS *m/z* Calc for $C_{48}H_{72}NO_{11}P$ $[M+H]^+$: 870.49158 founded: 870.48761.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-4-methoxyphenyl)-methyl}phosphonate ***(3o)***. Yield 61.9%. m.p. 133.4~135.8 °C. ^1H NMR (500 MHz, $CDCl_3$) δ 7.30–7.27 (m, 2H, Ar-H), 6.83 (dd, J = 8.7, 3.3 Hz, 2H, Ar-H), 6.63–6.50 (m, 1H, NH), 5.46−5.33 (m, 1H, H-11), 5.28−5.23 (m, 1H, H-3), 5.16−5.08 (m, 1H, H-2), 5.06−5.01 (m, 1H, P-CH), 4.15–4.00 (m, 2H, -OCH_2), 3.91–3.79 (m, 2H, -OCH_2), 3.77 (s, 3H, Ar-OCH_3), 3.70–3.51 (m, 2H, H-23), 2.03 (s, 3H, $COCH_3$), 2.00 (s, 3H, $COCH_3$), 1.96 (s, 3H, $COCH_3$), 1.95–1.08 (m, triterpene's H, 21H), 1.30 (t, J = 7.0 Hz, 6H, CH_3×2), 1.06–1.03 (m, 6H, CH_3-27/24), 1.00 (s, 3H, CH_3-26), 0.94 (d, J = 5.9 Hz, 3H, CH_3-30), 0.85 (s, 3H, CH_3-25), 0.83 (d, J = 7.4 Hz, 3H, CH_3-29). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.3, 170.8, 170.4, 170.3, 159.4, 139.2, 138.2, 130.0, 129.1, 126.8, 125.4, 114.0, 74.8, 69.9, 65.3, 62.7, 62.5, 55.3, 54.1, 47.9, 47.5, 43.8, 42.3, 41.9, 39.6, 39.4, 38.8, 37.7, 32.5, 32.4, 30.8, 27.6, 24.6, 24.3, 23.4, 23.1, 21.1, 21.0, 20.8, 20.7, 17.8, 17.7, 17.0, 16.9, 16.6, 16.3, 16.1, 13.9.ESI-HRMS *m/z* Calc for $C_{48}H_{72}NO_{11}P$ $[M+H]^+$: 870.49158 founded: 870.48566.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-phenyl)methyl}ph-osphonate ***(3p)***. Yield 66.2%. m.p. 129.8~132.1 °C. ^1H NMR (500 MHz, $CDCl_3$) δ 7.35 (td, J = 8.2, 3.9 Hz, 2H, Ar-H), 7.32−7.26 (m, 3H, Ar-H), 6.74−6.49 (m, 1H, NH), 5.47 − 5.31 (m, 1H, H-11), 5.31 − 5.21 (m, 1H, H-3), 5.14 − 5.07 (m, 1H, H-2), 5.05 − 4.99 (m, 1H, H-2, P-CH), 4.16 − 4.03 (m, 2H, -OCH_2), 3.89 − 3.75 (m, 2H, -OCH_2), 3.68 − 3.46 (m, 2H, H-23), 2.04 (d, J = 8.0 Hz, 3H, $COCH_3$), 1.99 (d, J = 2.7 Hz, 3H, $COCH_3$), 1.95 (d, J = 2.3 Hz, 3H, $COCH_3$), 1.32 − 1.26 (m, 6H, CH_3×2), 1.05 (s, 3H, CH_3-27), 1.00 (m, 3H, CH_3-24), 0.99 (s, 3H, CH_3-26), 0.93 (d, J = 6.1 Hz, 3H, CH_3-30), 0.84 (d, J = 1.9 Hz, 3H, CH_3-25), 0.81 (d, J = 4.8 Hz, 3H, CH_3-29). ESI-HRMS *m/z* Calc for $C_{47}H_{70}NO_{10}P$ $[M+H]^+$: 840.48101 founded: 840.47461.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-1-naphthyl)methyl}-phosphonate ***(3q)***. Yield 61.2%. m.p. 141.2~144.8 °C. ^1H NMR (500 MHz, $CDCl_3$) δ 7.88–7.83 (m, 4H, Ar-H), 7.56–7.48 (m, 3H, Ar-H), 6.82– 6.63 (m, 1H, NH), 5.74−5.53 (m, 1H, H-11), 5.39–5.31 (m, 1H, H-3), 5.18−5.12 (m, 1H, H-2), 5.09−4.98 (m, 1H, P-CH), 4.23–4.06 (m, 2H, -OCH_2), 3.96–3.72 (m, 2H, -OCH_2), 3.75–3.49 (m, 2H, H-23), 2.09 (s, 3H, $COCH_3$), 2.04(s, 3H, $COCH_3$), 2.01 (s, 3H, $COCH_3$), 1.99–1.12 (m, triterpene's H, 21H), 1.39−1.32 (m, 6H, CH_3×2), 1.08 (s, 3H, CH_3-27), 1.01 (s, 3H, CH_3-24), 0.99 (s, 3H, CH_3-26), 0.99 (d, J = 6.7 Hz, 3H, CH_3-30), 0.91 (s, 3H,

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CH_3-25), 0.87 (d, J = 8.2 Hz, 3H, CH_3-29). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.7, 170.8, 170.5, 170.3, 138.1, 132.3, 131.6, 128.9, 128.6, 126.7, 126.4, 125.9, 125.7, 125.1, 125.0, 123.5, 74.8, 69.8, 65.2, 62.9, 62.5, 54.1, 48.0, 47.3, 43.7, 42.2 41.8, 39.6, 39.4, 38.9, 37.7, 32.5, 32.2, 30.8, 27.6, 24.6, 24.3, 23.4, 23.0, 21.1, 21.0, 20.8, 20.7, 17.8, 17.5, 16.9, 16.5, 15.9, 15.7, 15.4, 13.8. ESI-HRMS *m/z* Calc for $C_{51}H_{72}NO_{10}P$ $[M+H]^+$: 890.49666 founded: 890.49374.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-12-ene-28-oyl]-2-naphthyl)methyl}-phosphonate ***(3r)***. Yield 69.0%. m.p. 137.1~140.2 °C. ^1H NMR (500 MHz, $CDCl_3$) δ 7.84–7.78 (m, 4H, Ar-H), 7.52–7.45 (m, 3H, Ar-H), 6.81–6.56 (m, 1H, NH), 5.71−5.44 (m, 1H, H-11), 5.35–5.28 (m, 1H, H-3), 5.14−5.03 (m, 1H, H-2), 5.03−4.94 (m, 1H, P-CH), 4.19– 4.06 (m, 2H, -OCH_2), 3.93–3.73 (m, 2H, -OCH_2), 3.67–3.46(m, 2H, H-23), 2.01 (s, 3H, $COCH_3$), 1.98 (s, 3H, $COCH_3$), 1.96 (s, 3H, $COCH_3$), 1.95–1.08 (m, 21H), 1.35−1.31 (m, 6H, CH_3×2), 1.00 (s, 3H, CH_3-27), 0.98 (s, 3H, CH_3-24), 0.95−0.97 (m, 6H, CH_3-26/30), 0.87 (s, 3H, CH_3-25), 0.84 (d, J = 8.2 Hz, 3H, CH_3-29). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.6, 170.8, 170.5, 170.3, 139.0, 138.2, 133.2, 133.0, 132.2, 128.4, 127.9, 127.6, 126.5, 126.4, 126.1, 125.4, 74.8, 69.9, 65.3, 62.9, 62.7, 54.2, 48.0, 47.6, 43.8, 42.2, 41.8, 39.7, 39.2, 38.9, 37.7, 32.4, 32.3, 30.8, 27.6, 24.7, 24.3, 23.4, 23.0, 21.1, 21.0, 20.8, 20.7, 17.7, 17.5, 17.0, 16.8, 16.5, 16.1, 16.0, 13.8. ESI-HRMS *m/z* Calc for $C_{51}H_{72}NO_{10}P$ $[M+H]^+$: 890.49666 founded: 890.49476.

3.1.3 General procedure for compound ***4*** *(2α, 3β, 23-Triacetoxyurs-11-oxo-12-ene-28-oic acid)*

A solution of **1** (100 mg, 0.16 mmol) and $K_2Cr_2O_7 \cdot 2H_2O$ (150 mg, 0.5 mmol) in 20 mL of acetic acid was refluxed for 5 h. The mixture was cooled to 20°C and neutralized with 10% $NaHCO_3$ to pH 7−8, diluted with ethyl acetate (20 mL), washed with water (10 mL × 5) and saturated NaCl solution (15 mL). The organic phase was dried over anhydrous sodium sulfate. Following filtration and evaporation of solvent at reduced pressure, the crude product was purified by silica gel chromatography with a gradient elution of CH_2Cl_2/MeOH (30:1, v:v) to yield a white solid (125 mg). Yield 85.5%. m.p. 191.2~194.8 °C. ^1H NMR (400 MHz, $CDCl_3$) δ 5.72 (s, 1H, H-12), 5.27−5.21 (m, 1H, H-2), 5.00 (d, J = 10.2 Hz, 1H, H-3), 3.78 (d, J = 11.8 Hz, 1H, H-23), 3.55 (d, J = 11.9 Hz, 1H, H-23), 3.16 (dd, J = 12.8, 4.5 Hz, 1H, H-19), 2.39 (s, 1H, H-9), 2.21−1.06 (triterpene's H, 17H), 2.05 (s, 3H, CH_3CO), 1.98 (s, 3H, CH_3CO), 1.93 (s, 3H, CH_3CO) (3×CH_3CO), 1.31 (s, 3H, CH_3-27), 1.22 (s, 3H, CH_3-24), 0.97 (d, J = 6.1 Hz, 3H, CH_3-29), 0.89 (d, J = 6.4 Hz, 3H, CH_3-30), 0.85 (s, 6H, CH_3-25/26). ^{13}C NMR (101 MHz, $CDCl_3$) δ 199.05, 182.78, 170.84, 170.54, 170.25, 163.15, 130.58, 74.89, 69.03, 65.25, 61.12, 52.52, 47.50, 47.38, 44.64, 44.18, 43.76, 41.96, 38.60, 38.53, 37.71, 36.01, 32.48, 30.25, 28.33, 23.56, 21.04, 20.99, 20.93, 20.70, 20.80, 19.07, 17.80, 17.04, 16.97, 13.90; ESI-HRMS *m/z* Calc for $C_{42}H_{56}FNO_8$ $[M+H]^+$: 629.36841, founded: 629.36667.

3.1.4 General procedure for the preparation of compounds ***6***

Oxalyl chloride (1.2 mL) at 0°C was added dropwise to a solution of compound **4** (100.00 mg,

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0.14 mmol) in CH_2Cl_2, and the mixture was stirred at room temperature for 8 h. The solvent was evaporated at reduced pressure, and CH_2Cl_2 (10 ml × 3) was added to the mixture, with vacuum concentration. The mixture was allowed to react with individual APAs (0.56 mmol) at room temperature for 4 h with stirring. After dilution with ethyl acetate (25 mL), the mixture was washed with water (20 mL × 3) and the organic phase was dried over anhydrous sodium sulfate. Following filtration and evaporation of solvent at reduced pressure, the residue was purified by column chromatography to yield a white solid.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-11-oxo-12-ene-28-oyl]-4-bromoph-enyl)methyl}phosph onate ***(6i)***. Yield 62.0%. m.p. 157.1~160.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42 (dd, *J* = 11.2, 8.5 Hz, 2H, Ar-H), 7.27– 7.19 (m, 2H, Ar-H), 6.69 (d, *J* = 47.9 Hz, 1H), 5.62 (s, 1H, H-12), 5.41−5.28 (m, 1H, H-3), 5.25 (m, 1H, H-2), 5.00 (dd, *J* = 10.2, 7.0 Hz, 1H, P-CH), 4.15–4.02 (m, 2H, -OCH_2), 3.92–3.78 (m, 2H, -OCH_2), 3.69–3.52 (m, 2H, H-23), 2.05 (s, 3H, CH_3CO), 1.99 (s, 3H, CH_3CO), 1.94 (s, 3H, CH_3CO), 1.93−1.30 (m, 19H), 1.30 (t, *J* = 12.9, 6.5 Hz, 6H, CH_3×2), 1.14 (s, 3H, CH_3-27), 1.08 (s, 3H, CH_3-24), 1.06 (s, 3H, CH_3-26), 0.96 (d, *J* = 5.0 Hz, 3H, CH_3-30), 0.88 (s, 3H, CH_3-25), 0.86 (d, *J* = 5.2 Hz, 3H, CH_3-29). ^{13}C NMR (126 MHz, $CDCl_3$) δ 198.1, 175.7, 170.7, 170.4, 170.0, 162.6, 134.8, 133.9, 131.9, 131.7, 130.4, 130.3, 130.0, 129.8, 74.9, 68.9, 65.2, 63.1, 62.9, 61.0, 52.9, 50.5, 47.4, 44.1, 43.7, 41.9, 39.1, 38.8, 38.6, 37.6, 32.5, 30.4, 27.9, 24.4, 24.0, 20.9, 20.9, 20.8, 20.7, 18.5, 18.3, 17.6, 17.0, 16.9, 16.5, 16.4, 16.1, 13.8. ESI-HRMS *m/z* Calc for $C_{47}H_{67}BrNO_{11}P$ $[M+H]^+$: 932.37079 founded: 932.36475.

O,O'Diethyl{N-[2α,3β,23-Triacetoxyurs-11-oxo-12-ene-28-oyl]-4-methylphe-nyl)methyl}phosp honate ***(6n).*** Yield 65.1%. m.p. 161.2~164.8 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.21 (dd, *J* = 28.2, 6.7 Hz, 2H, Ar-H), 7.08 (dd, *J* = 11.3, 8.0 Hz, 2H, Ar-H), 6.71−6.60 (m, 1H, NH), 5.61 (d, *J* = 7.0 Hz, 1H, H-12), 5.51−5.36 (m, 1H, H-3), 5.27−5.22 (m, 1H, H-2), 4.99 (dd, *J* = 10.2, 8.1 Hz, 1H, P-CH), 4.12−4.02 (m, 2H, -OCH_2), 3.88−3.76 (m, 2H, -OCH_2), 3.64−3.51 (m, 2H, H-23), 2.3 (s, 3H, Ar-CH_3), 2.04 (s, 3H, CH_3CO), 1.98 (s, 3H, CH_3CO), 1.93 (s, 3H, CH_3CO), 1.92−1.06 (m, 19H), 1.31−1.28 (m, 6H, CH_3×2), 1.21 (s, 3H, CH_3-27), 1.03 (s, 3H, CH_3-24), 1.01 (s, 3H, CH_3-26), 0.95 (d, *J* = 6.3 Hz, 3H, CH_3-30), 0.85 (s, 3H, C-25), 0.84 (d, *J* = 3.7 Hz, 3H, CH_3-29). ^{13}C NMR (126 MHz, $CDCl_3$) δ 198.0, 175.1, 170.7, 170.4, 170.1, 162.7, 132.3, 131.3, 130.3, 129.4, 129.2, 128.2, 128.0, 74.8, 68.9, 65.1, 62.8, 62.7, 61.0, 53.4, 50.0, 47.2, 44.2, 43.7, 41.8, 39.1, 38.5, 37.6, 36.6, 32.4, 32.3, 30.4, 27.7, 24.3, 23.9, 21.1, 21.0, 20.8, 20.7, 18.4, 18.1, 17.6, 17.3, 17.0, 16.5, 16.4, 16.1, 13.8. ESI-HRMS *m/z* Calc for $C_{48}H_{70}NO_{11}P$ $[M+H]^+$: 868.47593 founded: 868.47266.

3.2. Biological Assays

3.2.1. Cytotoxicity of **AA** Derivatives

A549 human non-small cell lung carcinoma cells, T24 human bladder carcinoma cells, Hct-116 human colorectal carcinoma cells, SK-OV-3 human ovarian carcinoma cells, HUVEC and Spca-2 human lung adenocarcinoma cells were all obtained from the Institute of Biochemistry and Cell

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Biology, China Academy of Sciences. They were cultured in a humidified, 5% CO_2 atmosphere at 37°C and maintained in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin and 100 mg/mL penicillin. Chemosensitivity was assessed with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, exponentially growing cells were seeded into 96-well plates and treated with the indicated concentrations of **AA** derivatives for 48 h, and then 10 mL of MTT (10 mg/mL) was added. After incubation for 4 h at 37°C, the purple formazan crystals (a reduced form of MTT) generated in viable cells were dissolved by adding 100 μL DMSO to each well. The plates were swirled gently for 10 min to dissolve the precipitate, and quantified by measuring the optical density of the plates at 490 nm using a plate reader (TECAN infinite M1000). Each concentration was repeated in three wells and the same experimental conditions were maintained for all testing procedures. The MTT assays were repeated three times for each cell line.

3.2.2. Hoechst 333258 Staining

Cells grown on a sterile cover slips in six-well tissue culture plates were treated with test compounds for the indicated time. The culture medium containing the compounds was removed, and the cells were fixed in 4% paraformaldehyde for 10 min. After washing twice with phosphate buffered saline (PBS), the cells were stained with 0.5 mL of Hoechst 33258 (Beyotime) for 5 min and again washed twice with PBS. Nuclear staining was observed with a Nikon ECLIPSETE2000-S fluorescence microscope at 350 nm excitation and 460 nm emission wavelengths.

3.2.3. AO/EB Staining

Cells were seeded at a concentration of 5×10^4 cell/mL in a volume of 2 mL on sterile cover slips in six-well tissue culture plates. Following incubation, the medium was removed and replaced with fresh medium plus 10% FBS and supplemented with compound **3d**. After treatment, cover slips with cell monolayers were inverted on a glass slide with 20 μL of AO/EB stain (100 mg/mL). Fluorescence was read on a Nikon ECLIPSETE2000-S fluorescence microscope (OLYMPUS Co., Japan).

3.2.4. Mitochondrial Membrane Potential Staining

Mitochondrial depolarization was assayed in T24 cells using a JC-1 probe. Briefly, cells cultured in six-well plates after the indicated treatment were incubated with an equal volume of JC-1 staining solution (5 μg/mL) at 37°C for 20 min and rinsed twice with PBS. Mitochondrial membrane potentials were monitored by determining the relative amounts of dual emissions from mitochondrial JC-1 monomers or aggregates using a Nikon ECLIPSETE2000-S fluorescent microscope. Mitochondrial depolarization was indicated by an increase in the green/red fluorescence intensity ratio.

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3.2.5. Apoptosis Analysis

Apoptosis was assayed by annexin V-FITC and PI. Cells were seeded at 2×10^6/well in 10% FBS–DMEM into six-well plates and treated with test compounds for 24 h. The cells were then washed twice with cold PBS and resuspended in 1×binding buffer (0.1 M pH 7.4 Hepes/NaOH, 1.4 M NaCl, 25 mM $CaCl_2$) at a concentration of 1×10^6 cells/mL. A 100 μL volume of the solution (1×10^5 cells) was transferred to a 5 mL culture tube; 5 μL of FITC Annexin V (BD, Pharmingen) and 5 μL PI were added to each tube. The cell suspension was gently vortexed and incubated for 30 minutes at room temperature (25°C) in the dark, and then 200 μl PBS was added to each tube. The apoptosis assay was carried out by flow cytometry (FACSVerse, BD, USA) at 488 nm excitation. The lower left quadrant included viable cells (annexin V^-PI^-); lower right quadrant included early apoptotic cells (annexin V^+/PI^-); upper right quadrant included late apoptotic cells (annexin V^+/PI^+); and the upper left quadrant included necrotic cells (annexin V^-/PI^+). The percentage of PI^+ and/or Annexin V-$FITC^+$ cells inside the quadrants was reported.

3.2.6. Cell Cycle Analysis

Cell cultures were treated with the indicated concentrations of compound **3d** and after 48 h incubation, the cells were washed twice with ice-cold PBS, fixed and permeabilized with ice-cold 70% ethanol at −20°C overnight. The cells were treated with 100 μg/mL RNase A at 37°C for 30 min after washing with ice-cold PBS, and finally stained with 1 mg/mL PI in the dark at 4°C for 30 min. Cell cycle analysis was performed by flow cytometry (FACSVerse, BD, USA) at an excitation of 488 nm and an emission of 620 nm.

3.2.7. ROS Assay

T24 cells were seeded into six-well plates, and following treatment, were incubated with 10 mM DCFH-DA (Beyotime, Haimen, China) dissolved in cell-free medium for 30 min at 37°C and in the dark. They were then washed three times with PBS. Cellular fluorescence was measured with a Nikon ECLIPSETE2000-S fluorescence microscope at 485 nm excitation and 538 nm emission.

3.2.8. Calcium Analysis

To monitor the effect of **AA** APA derivatives on calcium release, T24 cells were seeded into six-well plates, and loaded with 5 mM of the membrane-permeable calcium indicator Fluo-3 acetoxymethyl ester (Beyotime, Haimen, China) in PBS buffer for 40 min at 37°C. After loading with the Fluo-3 dye, cells were washed with PBS and suspended in Ca-free PBS containing 5 mM EGTA. Fluo-3 was excited by argon laser light at 488 nm; fluorescence was measured at 515 nm, and quantified with a Nikon ECLIPSETE2000-S fluorescence microscope.

3.2.9. Western Blot Assay

The western blot procedure was performed as described previously [3]. T24 cells were collected after treatment with compound **3d** (20 μM) for 0, 6, 12 or 24 h and then lysed in ice-cold lysis

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buffer (1% sodium dodecyl sulfate in 25 mM pH 7.5 Tris–HCl, 4 mM EDTA, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin and 10 mg/mL soybean trypsin inhibitor). Whole-cell lysates were centrifuged at 12,000×*g* for 5 min. Thereafter, the protein concentration was determined with a bicinchoninic acid protein assay kit (Beyotime Co, China). An aliquot of cell lysate (40–50 μg) was fractionated by SDS-PAGE on 12% polyacrylamide gels for 2 h and transferred to polyvinylidene difluoride membranes. After blocking with 5% non-fat dry milk in PBS-t for 1 h at room temperature, the membranes were incubated with β-actin, cytochrome c, caspase-9, caspase-3, Bax or Bcl-2 antibodies (Bioworld Technology Inc, USA) overnight at 4^{o}C, washed with tris-buffered saline and Tween 20, and then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Proteins were detected by electrochemiluminescence, Thermo Fisher Scientific, USA) and analysed by Image J software.

3. 2. 10 Statistical Analysis

Data are expressed as mean ± SD for three different determinations. Statistical significance was analyzed by one-way ANOVA. Mean separations were performed using the least significant difference method. $P<0.05$was defined as statistically significant.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

All authors of this paper have directly participated in the planning or drafting of this manuscript and have read and approved the final version submitted.

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