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Synthesis and biological evaluation of novel asiatic acid derivatives with anticancer activity

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Bruno M. F. Gonçalves,[a] Jorge A. R. Salvador,[a,b,*] Silvia Marín,[c,d] and Marta Cascante[c,d,*]

Asiatic acid (**AA**) is a pentacyclic triterpenoid with recognized anticancer properties. Structural modification of **AA** may afford derivatives with improved anticancer potency. Hence, in this paper, a series of new lactol and A-nor **AA** derivatives were prepared, and their antiproliferative activities against several human cancer cell lines and a non-tumoral fibroblast cell line (BJ) were tested. Among all the derivatives tested, compound **24** proved to be the most active compound, with IC_{50} values ranging from 0.11 μM to 0.65 μM for cancer cell lines. The molecular mechanisms underlying its antiproliferative activity were further investigated using the HeLa cell line. Our results showed that treatment of HeLa cells with compound **24** led to cell-cycle arrest at the G0/G1 phase, which was associated with an upregulation of $p21^{cip1/waf1}$ and $p27^{kip1}$ and a downregulation of cyclin D_3. Moreover, compound **24** induced apoptotic HeLa cell death via the activation of caspase-9, caspase-8, and caspase-3. The downregulation of the Bcl-2 and Bid proteins and the upregulation of the Bax protein suggest that the mitochondrial pathway is activated during the apoptotic process.

1. Introduction

Despite recent advances in cancer therapy, this complex disease remains one of the leading causes of death worldwide[1,2]. To solve this public health problem, research efforts toward the development of new therapeutic strategies and more effective anticancer drugs have been made.

Pentacyclic triterpenoids (PTs) are a large class of natural products that are widely distributed in nature and have a vast range of unique biological activities[3–5]. In the last decades, the anticancer and anti-inflammatory activities of PTs and their semi-synthetic derivatives have been extensively studied, and some papers support the contention that these compounds are ideal candidates for the development of new anticancer therapies[4,6–11]. Asiatic acid (**AA**) **(1)** (Fig. 1) is a PT that is mainly extracted from *Centella asiatica* that, in addition to other important pharmacological activities, shows promising anticancer effects. The mechanisms underlying the anticancer effect of **AA (1)** include cell-cycle arrest[12], strong antiangiogenic activity[13] , inhibition of cancer cell proliferation, and induction of apoptosis in several cancer cell lines[13–16].

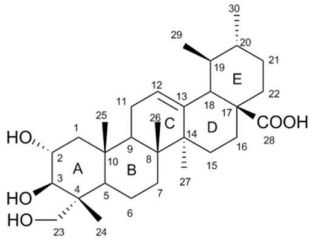

Fig.1 Chemical structure of asiatic acid **1**

Apoptotic cell death induced by **AA (1)** has been reported to be mediated by alterations in calcium homeostasis in the HepG2, PCC-1, and U87-MG cancer cell lines[14,16,17]; activation of the mitochondrial pathway in SW480 human colon cancer cells[18]; and generation of reactive oxygen species (ROS), upregulation of BAX, and activation of caspase-3 in SK-MEL-2 human melanoma cells[15].

Because of its promising anticancer activity, low toxicity, and commercial availability, **AA (1)** has been receiving increased attention from scientists who aim for the development of new anticancer drugs. Several groups performed structural modifications of the **AA (1)** backbone; some of the semi-synthetic derivatives obtained displayed improved antiproliferative activity in several cancer cell lines compared with **AA (1)**[19–22]. However, further investigation is needed to develop and synthesize new **AA (1)** derivatives as anticancer agents.

Bhagirath Sing and coworkers reported the conversion of the hexameric ring A of **AA (1)** into a pentameric ring containing an α,β-unsaturated carbonyl group[23]. It has been well established in several studies that the presence of an

[a] *Laboratory of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Coimbra, 3000-548, Coimbra, Portugal. Fax: +351 239 488 503; Tel: +351 239 488 400.*
[b] *Center for Neuroscience and Cell Biology, Coimbra, Portugal.*
[c] *Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, Diagonal 643, 08028 Barcelona, Spain; Tel +34-934021593.*
[d] *Institute of Biomedicine of University of Barcelona (IBUB) and Associated Unit to CSIC,Barcelona, Spain.*
*Corresponding authors: Jorge A. R. Salvador, E-mail: salvador@ci.uc.pt and Marta Cascante, E-mail: martacascante@ub.edu†
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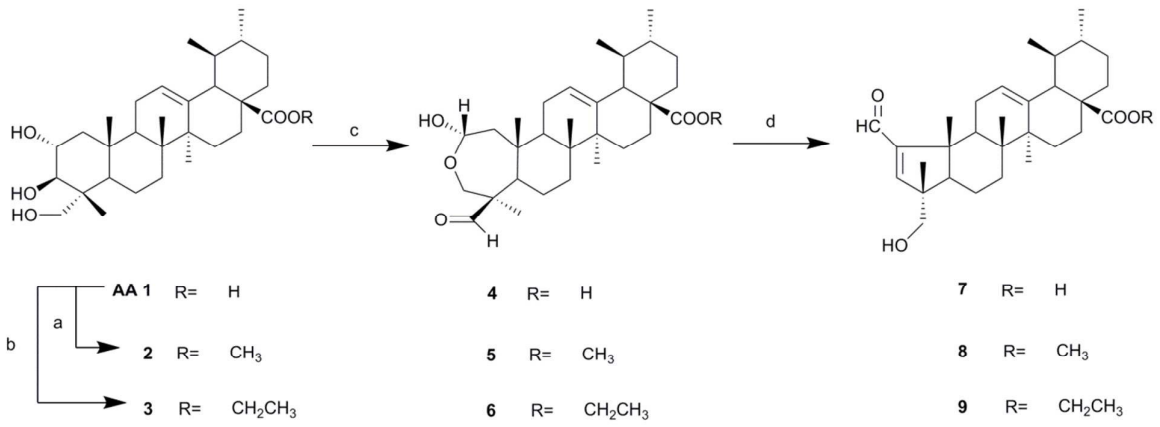

Sheme 1 Synthesis of Asiatic acid derivatives **1-9.** Reagents and conditions: a) CH_3I, K_2CO_3, DMF, rt.; b) CH_3CH_2I, K_2CO_3, DMF, rt.; c) $NaIO_4$, MeOH / H_2O, r.t.; d) i- Acetic acid, piperidine, dry benzene, reflux 60°C, N_2; ii -Anhydrous $MgSO_4$, reflux 60 °C, N_2.

α,β-unsaturated carbonyl group in the A-ring of PTs significantly enhances their biological activities[4,24–26]. However, the transformations performed in the A ring of **AA (1)** were not thoroughly explored, which encouraged us to develop and synthesize a series of new **AA (1)** derivatives containing a 5-carbon ring A with an α,β-unsaturated carbonyl moiety, combined with additional modifications at C-23, C-11, and C-28, to obtain **AA (1)** derivatives with improved anticancer activity.

In addition, the nitrile group has been gaining great importance in the design and development of new drugs[27,28], because its presence in organic molecules plays several biologically important roles[27–32]. This functional group could work as a hydroxyl and carboxyl surrogate, as nitrile is a strong hydrogen acceptor[27]. Taking this into account, we also designed and prepared a series of pentameric A-ring **AA (1)** derivatives containing a nitrile group.

The *in vitro* antiproliferative activities of the newly synthesized derivatives against the MCF-7, HT-29, and HeLa cell lines were tested, and a structure–activity relationship (SAR) was established. Most of the new derivatives showed improved cytotoxic activities compared with **AA (1)**. Compound **24** exhibited the best antiproliferative profile among all new derivatives and was selected for further experiments aimed at exploring its mechanism of action in HeLa cells.

2. Results and discussion

2.1 Chemistry

A series of new **AA (1)** derivatives containing a pentameric ring A were synthesized and their structures were fully elucidated. The synthesis started by the treatment of **AA (1)** with anhydrous potassium carbonate (K_2CO_3) and methyl iodide or ethyl iodide in DMF, to give the methyl derivative **2** and the ethyl derivative **3**, respectively, in almost quantitative yields (Scheme 1). The lactol derivatives **4**, **5**, and **6** were obtained in good yield via the reaction of compounds **1**, **2**, and **3,** respectively, with sodium periodate ($NaIO_4$) in methanol/water[23]. Further treatment of compounds **4**, **5**, and **6** with catalytic amounts of piperidine and acetic acid in benzene at 80 °C afforded the pentameric A-ring derivatives **7**, **8**, and **9**, respectively[23].

The proton of the aldehyde group of the lactol derivatives **4–6** appeared as a singlet at δ = 9.94ppm on ^{1}H NMR spectra, and the ^{13}C NMR signal for the CHO carbon was observed around δ = 206 ppm. In the ^{1}H NMR spectra of compounds **7–9**, the signals of the aldehyde and olefinic protons in the A ring were consistently observed as two singlet signals at δ = 9.72 ppm and δ = 6.66 ppm, respectively. The ^{13}C NMR signal for the CHO carbon was observed at δ= 190.81–190.85 ppm, whereas the signals for the carbons of the A ring double bond appeared at δ= 159.24–159.29 ppm and δ= 158.90–159.95 ppm. The characteristic IR bands for the C=O and C=C stretching vibrations of the α,β-unsaturated aldehydes were observed around 1689 cm^{-1} and 1581 cm^{-1}, respectively.

To study the impact of the introduction of a carbonyl group at C-11 on the anticancer activity, we decided to synthesize several **AA (1)** derivatives with an α,β-unsaturated ketone in the C ring. As shown in Scheme 2, the reaction of compound **2** with acetic anhydride in the presence of DMAP in THF at room temperature gave compound **10**, which was further oxidized

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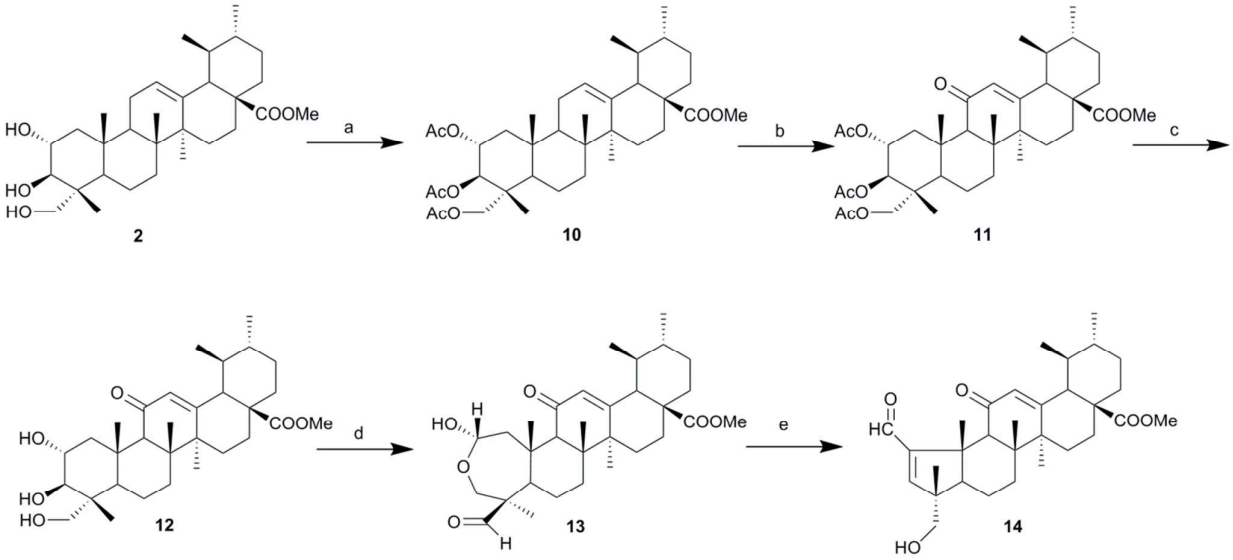

Sheme 2 Synthesis of Asiatic acid derivatives **10–14**. Reagents and conditions: a) Acetic anhydride, DMAP, THF, r.t.; b) $KMnO_4$, $Fe_2(SO_4)_3.nH_2O$, t-BuOH, H_2O, CH_2Cl_2, r.t.; c) KOH, MeOH, reflux, 30 min.; d) $NaIO_4$, MeOH / H_2O, r.t.; e) i- Acetic acid, piperidine, dry benzene, reflux 60 °C, N_2; ii -Anhydrous $MgSO_4$, reflux 60 °C, N_2.

with a mixture of potassium permanganate and iron sulfate, to afford the 11-oxo-12-en derivative **11** in 95% yield[33,34]. Compound **11** was then deacetylated with potassium hydroxide (KOH) in methanol, to afford intermediate **12**. The lactol derivative **13** was prepared by reaction of compound **12** with sodium periodate ($NaIO_4$) in methanol/water at room temperature. The α,β-unsaturated aldehyde **14** was obtained from **13** using the procedure that was described previously for the preparation of compounds **7–9**.

Taking into consideration that the nitrile group can act as a carbonyl bioisostere[27], and considering its pharmaceutical importance, we prepared a panel of **AA (1)** nitrile derivatives. The treatment of compounds **7**, **8**, and **14** with iodine and 25% aqueous ammonium solution in THF at room temperature[35] afforded the derivatives **17**, **16**, and **18**, respectively, which have an α,β-unsaturated nitrile group in the pentameric A ring (Scheme 3). The direct conversion of aldehyde into nitrile was confirmed by the specific IR absorption band for CN stretching vibration observed at 2215.8–2217.7 cm^{-1} and by the ^{13}C NMR signal for the CN carbon observed around δ = 117 ppm. Moreover, no signal corresponding to the proton of the CHO group was observed on the ^{1}H NMR spectra. These structural data were consistent for all derivatives that had a pentameric A ring with an α,β-unsaturated nitrile **(16-18** and **25-31)**.

In addition, we decided to investigate the influence of C-23 hydroxyl substitution on the anticancer activity of compounds **14** and **16**. Thus, starting from these two compounds, we prepared a panel of C-23-substituted ester, carbamate, and mesylate derivatives (Schemes 4 and 5). The ester derivatives **19**, **20**, and **25–28** were prepared in moderate-to-good yields via the reaction of compound **14** or compound **16** with the corresponding anhydrides in the presence of DMAP at room temperature. The treatment of **14** or **16** with cynamoyl chloride and DMAP in dry benzene at 60 °C afforded the 23-cinnamic ester derivatives **21** and **29**, respectively. As shown in Scheme 4, compound **14** was treated with CDI or CBMI in THF at reflux, to give the carbamate derivatives **22** and **23** in 12% and 33% yield, respectively, after FCC. Derivative **30** was prepared in 59% isolated yield via the reaction of **16** with CBMI in THF at 80 °C (Scheme 5). The successful preparation of the carbamate derivatives **22**, **23**, and **30** was confirmed by the IR band observed at 1747–1764 cm^{-1}, corresponding to the C=O stretching vibration, and by the ^{13}C NMR signal for the carbamate carbonyl carbon observed at 148.56–149.47 ppm. In addition, in the ^{1}H NMR spectra of compounds **23** and **30**, the two specific hydrogen atoms of the methyl imidazole moiety appeared as two peaks at 7.27–7.28 ppm and 6.84–6.87 ppm. In the case of the derivative **22**, the imidazole protons appeared as three singlets at 8.09, 7.30, and 7.06 ppm[36,37] on the ^{1}H NMR spectra.

Finally, the 23-methanesulfonyloxy derivatives **15**, **24**, and **31** were obtained in moderate yields by treatment of

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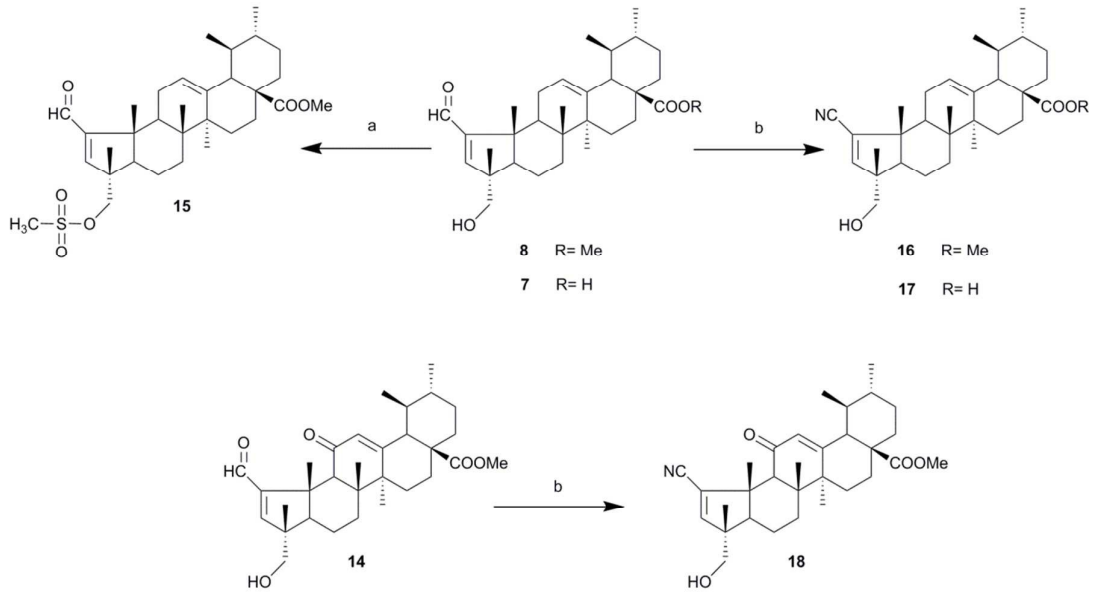
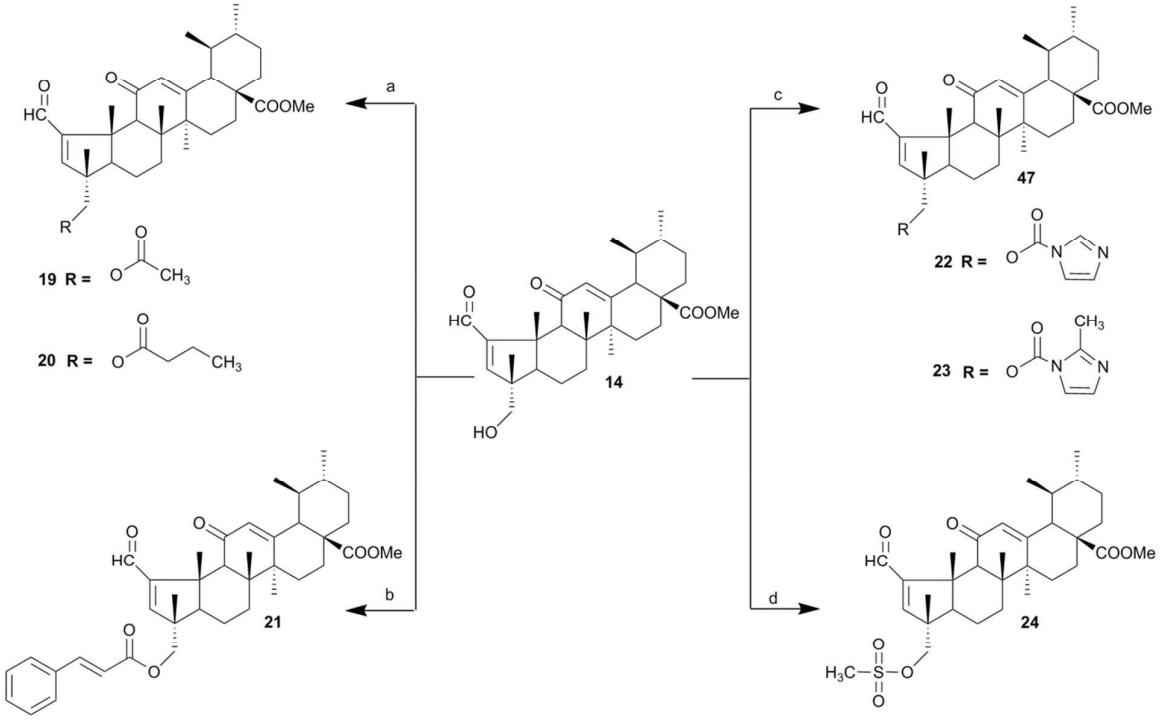
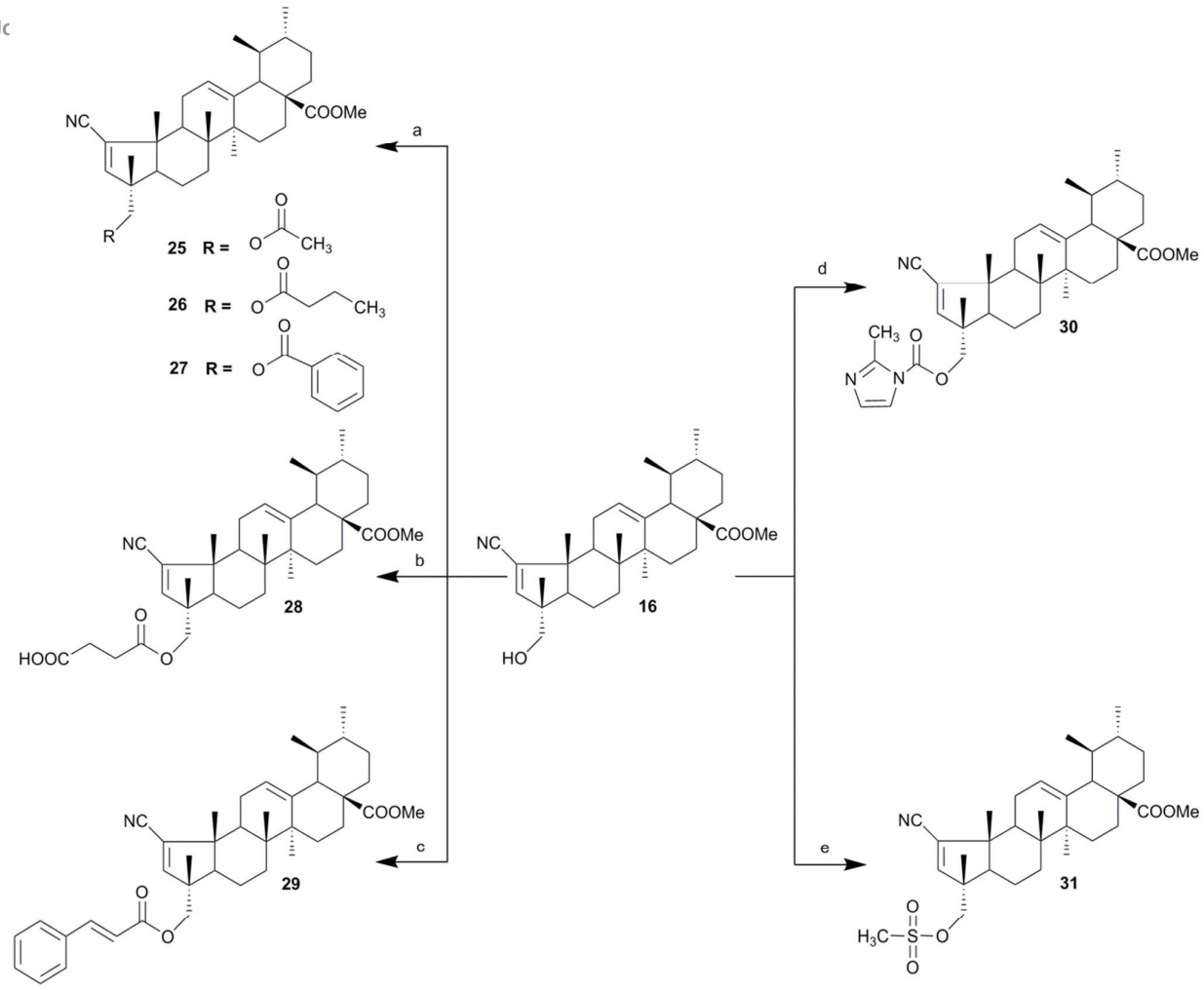


Sheme 3 Synthesis of Asiatic acid derivatives **15–18.** Reagents and conditions: a) Methanesulfonyl chloride, triethylamine, dry CH_2Cl_2, r.t. b) I_2, aq. NH_3, THF, r.t.



Sheme 4 Synthesis of Asiatic acid derivatives **19–24.** Reagents and conditions: a) Acetic anhydride or butyric anhydride, DMAP, THF, r.t.; b) Cinnamoyl chloride, dry benzene, DMAP, 60 °C, N_2; c) CDI or CBMI, THF, 70 °C, N_2; d) Methanesulfonyl chloride, triethylamine, dry CH_2Cl_2, r.t.



Sheme 5 Synthesis of Asiatic acid derivatives **25–31**. Reagents and conditions: a) Acetic or butyric or benzoic anhydride, DMAP, THF, r.t.; b) Succinic anhydride, DMAP, CH_2Cl_2, r.t.; c) Cinnamoyl chloride, dry benzene, DMAP, 60 °C, N_2; d) CBMI, THF, 70 °C, N_2; e) Methanesulfonyl chloride, triethylamine, dry CH_2Cl_2, r.t.

compounds **8**, **14**, and **16**, respectively, with methanesulfonyl chloride and triethylamine in dry dichloromethane at room temperature (Schemes 3, 4, and 5). The successful preparation of compounds **15**, **24**, and **31** was confirmed by the characteristic IR absorptions for the asymmetric and symmetric S=O vibrations observed around 1355–1359 cm^{-1} and 1174–1176 cm^{-1}. In the ^1H NMR spectra, the protons from the mesylate methyl group were observed as a singlet at 3.01–3.03 ppm.

2.2 Biological activities

2.2.1 Evaluation of antiproliferative activity The antiproliferative activity of **AA (1)** and all the newly synthesized derivatives against MCF-7 (human breast adenocarcinoma), HT-29 (human colon adenocarcinoma), and HeLa (human cervix adenocarcinoma) cell lines was evaluated by MTT assay. The intermediates (**2**, **4**, **5**, **7**, **8**, **10**, **11**, **12**) and the control drug cisplatin were tested against the HeLa cell line. The cell lines were treated with increasing concentrations of each compound, and the IC_{50} values (concentration that inhibited 50% of cell growth) were determined after 72 h of incubation.

The analysis of IC_{50} values (Table 1) revealed that the great majority of the new derivatives showed better antiproliferative activities than **AA (1)** against the tested cell lines. These new derivatives were particularly active against the HeLa cell line, with the exception of compound **29**; therefore, their antiproliferative activities against HeLa cells were used to establish a SAR (Fig. 2).

According to previous studies, the small carbon chain ester derivatives at C-28 of ursane-type triterpenoids present increased cytotoxic activity[38,39]. Similar results were obtained in our study, the methyl ester derivatives **2**, **5**, and **8** and the ethyl ester derivatives **3**, **6**, and **9** exhibited increased cytotoxic activity compared with the parent compounds that had a free carboxyl group (**AA (1)**, **4**, and **7**, respectively). The antiproliferative activity of C-28 ester derivatives was significantly increased with the increase in the length of the carbon chain.

The direct comparison of the antiproliferative activities (against the HeLa cell line) of compounds **15** and **24**, as well as compounds **8** and **14**, indicated that the introduction of a keto group at position C-11 improves antiproliferative activity. However, an opposite effect was observed when we compared

Table 1 Cytotoxic activities (expressed as IC_{50}) of AA, its derivatives and cisplatin against breast (MCF-7), colon (HT-29) and cervix (HeLa) cancer cell lines.

Compound	Cell Line [a] / IC_{50} (μM)		
	MCF-7	HT-29	HeLa
Asiatic acid (1)	68.50 ± 2.12	64.33 ± 3.21	52.47 ± 0.06
2	N.D.	N.D.	27.50 ± 2.50
3	N.D.	N.D.	20.67 ± 1.53
4	N.D.	N.D.	21.67 ± 1.04
5	N.D.	N.D.	4.70 ± 0.40
6	4.00 ± 0.02	5.70 ± 0.46	4.75 ± 0.21
7	N.D.	N.D.	5.30 ± 0.2
8	N.D.	N.D.	0.60 ± 0.07
9	0.70 ± 0.05	0.64 ± 0.05	0.51 ± 0.03
10	N.D.	N.D.	0.60 ± 0.04
11	N.D.	N.D.	3.13 ± 0.32
12	N.D.	N.D.	37.17 ± 2.57
13	12.03 ± 0.40	12.20 ± 0.26	8.48 ± 1.31
14	0.74 ± 0.05	0.59 ± 0.04	0.30 ± 0.02
15	0.47 ± 0.02	0.45 ± 0.05	0.30 ± 0.02
16	14.33 ± 1.53	10.27 ± 1.55	8.90.± 0.53
17	>60	>60	43.17 ± 3.55
18	16.10 ± 1.40	17.00 ± 1.32	14.60 ± 1.65
19	1.02 ± 0.11	0.97 ± 0.09	0.60 ± 0.05
20	0.88 ± 0.07	0.77 ± 0.03	0.49 ± 0.00
21	1.03 ± 0.04	0.84 ± 0.05	0.53 ± 0.01
22	1.20 ± 0.05	0.73 ± 0.02	0.53 ± 0.03
23	0.98 ± 0.02	0.66 ± 0.05	0.54 ± 0.02
24	0.65 ± 0.04	0.59 ± 0.02	0.24 ± 0.02
25	11.50 ± 1.32	12.20 ± 1.04	10.75 ± 0.35
26	>30	>60	12.00 ± 1.41
27	>60	>60	15.50 ± 0.71
28	30.00 ± 1.41	>30	26.80 ± 2.34
29	>60	>60	>60
30	7.00 ± 0.00	6.13 ± 0.32	6.33 ± 0.15
31	19.50 ± 2.12	9.77 ± 0.23	7.27 ± 0.91
Cisplatin	19.10 ± 4.50	6.11[40]	2.28 ± 0.26

[a]The cell lines were treated with increasing concentrations of each compound for 72h. IC_{50} Values were determined by MTT assay and are expressed as means ± SD (standard deviation) of three independent experiments. N.D. Not Determined. IC_{50} is the concentration of compound that inhibits 50% of cell growth.

the activity of the following pairs of compounds: **2** and **12**, **10** and **11**, and **5** and **13**. These results suggest that there is no direct relationship between the introduction of a keto group at C-11 and the antiproliferative activity of the compound, which is in accordance with the results of a previous study[41].

The conversion of hexameric ring A into the corresponding heptameric lactol ring substantially improved the growth inhibitory activity in all tested cell lines. The lactol derivatives (**4**, **5**, **6**, and **13**) showed IC_{50} values ranging from 4.70 μM (**5**) to 8.48 μM (**13**), which was 6–11 times lower than the IC_{50} of **AA (1)** (52.47 μM) against the HeLa cell line.

The group of derivatives that had a pentameric ring A with an α,β-unsaturated carbonyl moiety proved to be the most active among all tested derivatives in all tested cancer cell lines. These compounds presented IC_{50} values ranging from 0.24 μM (compound **24**) to 5.30 μM (compound **7**) against the HeLa cell line. Moreover, with the exception of compound **7**, all the derivatives of this group were more active than cisplatin, which indicated that the α,β-unsaturated carbonyl moiety in ring A is important for the antiproliferative activity of these compounds against cancer cell lines.

Compounds **17**, **16**, and **18**, with a pentameric ring A with an α,β-unsaturated nitrile, were 1.2-, 5.9-, and 3.6-fold more potent than was **AA (1)**, respectively, against the HeLa cell line.

The effect of different substituents at the C-23 position of compounds **14** and **16** on their antiproliferative activities was also investigated. We found that, with the exception of compound **30**, only the introduction of the methanesulfonyloxy group at C-23 (compounds **15**, **24**, and **31**) resulted in a marked increase in antiproliferative activity. In fact, compounds **15** and **24** were the most active compounds among all the synthesized derivatives, as they were approximately 175- and 218-fold more active than **AA (1)**, respectively, against the HeLa cell line.

The most active compounds, **9**, **14**, **15**, and **24**, were selected and their antiproliferative activity was further evaluated against a panel of other four cancer cell lines (Jurkat, PC-3, MIA PaCa-2, and A-375) and against a nontumoral fibroblast cell line (BJ), to evaluate selectivity. As depicted in Table 2, the selected compounds markedly inhibited the proliferation of all tested cancer cell lines. Compounds **14**, **15**, and **24** exhibited a decreased toxicity for the normal fibroblast cell line BJ compared with cancer cell lines. Compound **24** presented the best antiproliferative profile, and was especially active against the HeLa and Jurkat cell lines, with IC_{50} values of 0.24 μM and 0.11 μM, respectively. Thus, this compound was selected for further studies aimed at exploring the mechanism underlying its antiproliferative effect against the HeLa cell line.

2.2.2 Effects of compound 24 on cell-cycle distribution First, we investigated the effect of compound **24** on the cell-cycle distribution of HeLa cells. Cells were treated with increasing concentrations of **24** (0 μM, 0.24 μM, 0.48 μM, 0.96 μM, and 1.44 μM) for 24 h and the distribution of the cell cycle was assessed by FACS analysis after staining the cells with propidium iodide (PI). As observed in Figs. 3A and 3B, treatment of HeLa cells with 0.96 μM of compound **24** increased the percentage of cells in the G0/G1phase, from 50.53% in control cells to 68.65% in treated cells. Concomitantly, the percentage of cells in the S phase decreased from 36.24% in control cells to 12.89% in treated cells. These results suggest that compound **24** inhibits the proliferation of HeLa cells via cell-cycle arrest at the G0/G1 phase.

We also observed an increase of around 17% in the percentage of cells in the sub-G0/G1 phase (cells with hypodiploid DNA) after treatment of HeLa cells with 1.44 μM of compound **24**, which suggests that this compound has the ability to induce cell death in a dose-dependent manner.

2.2.3 Effects of compound 24 on the levels of cell-cycle-related proteins As the experiment described above indicated that compound **24** arrested the cell cycle at the G0/G1 phase, we explored the effects of this compound on the levels of some cell-cycle-regulatory proteins using western blot analysis. As shown in Fig. 3C, treatment of HeLa cells with compound **24** at 0.96 μM significantly increased the levels of $p27^{kip1}$, which was in agreement with the observation of cell-cycle arrest at the G0/G1 phase.

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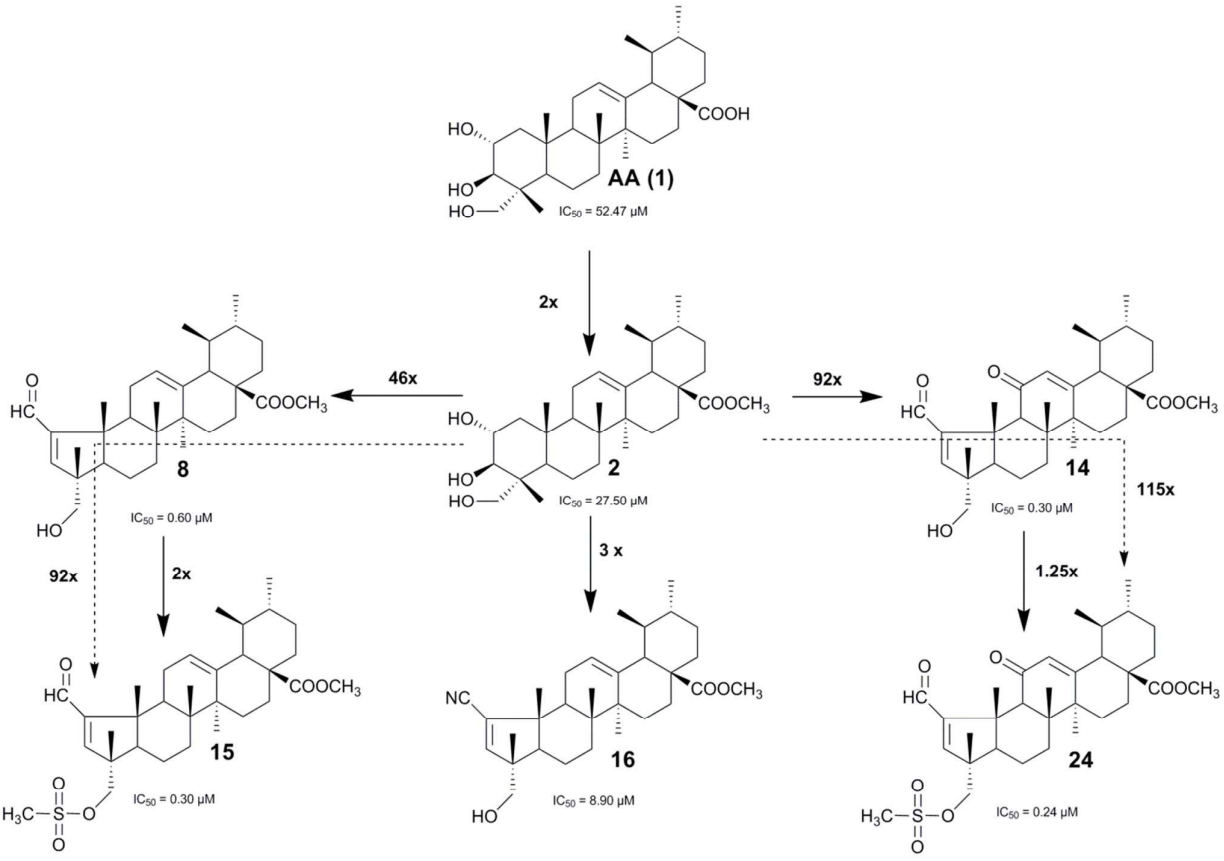

Fig. 2- Schematic representation of the SAR for the antiproliferative activity against HeLa cell line. The SAR was established based on IC_{50} values. The derivatives with pentameric ring A containing an α,β – unsaturated carbonyl moiety showed the higher antiproliferative activities, while the compounds containing an hexameric ring A showed the lower antiproliferative activities. The introduction of the methanesulfonyloxy group at C-23 significantly increases the antiproliferative activity. Derivative **2**, is 2-fold more potent than Asiatic acid **1**. Derivatives **8** and **14** are 46-fold and 92-fold more potent than derivative **2**, respectively. The most active compound **24** is 115-fold more potent than derivative **2**.

Table 2- Cytotoxic activities of asiatic acid, derivatives **9**, **14**, **15**, **24** and cisplatin against leukemia (Jurkat), prostate (PC-3), pancreas (MIA PaCa-2), melanoma (A375) cancer cell lines and non-tumoral fibroblast cell line (BJ).

Compound	Cell lines [a] / IC_{50} (μM)				
	Jurkat	PC-3	MiaPaca-2	A-375	BJ
Asiatic acid 1	37.18 ± 3.75	67.25 ± 0.35	50.67 ± 1.15	50.33 ± 2.57	88.70 ± 0.58
9	0.45 ± 0.04	0.57 ± 0.04	0.83 ± 0.04	0.63 ± 0.05	N.D.
14	0.18 ± 0.02	0.60 ± 0.05	0.60 ± 0.06	0.38 ± 0.01	3.33 ± 0.25
15	0.27 ± 0.01	0.41 ± 0.02	0.60 ± 0.06	0.36 ± 0.03	1.94 ± 0.08
24	0.11 ± 0.01	0.42 ± 0.02	0.46 ± 0.04	0.25 ± 0.01	2.43 ± 0.11
Cisplatin	1.94[42]	4.60[43]	5.00 ± 1.00[44]	3.11 ± 0.98[45]	10.10 ± 2.00

[a] The cell lines were treated with increasing concentrations of each compound for 72h. IC_{50} Values were determined by MTT assay in PC-3, MIA PaCa-2, A375 and BJ cell lines and by XTT assay in Jurkat cell line. The results shown are expressed as means ± SD (standard deviation) of three independent experiments. N.D. Not Determined. IC_{50} is the concentration of compound that inhibits 50% of cell growth.

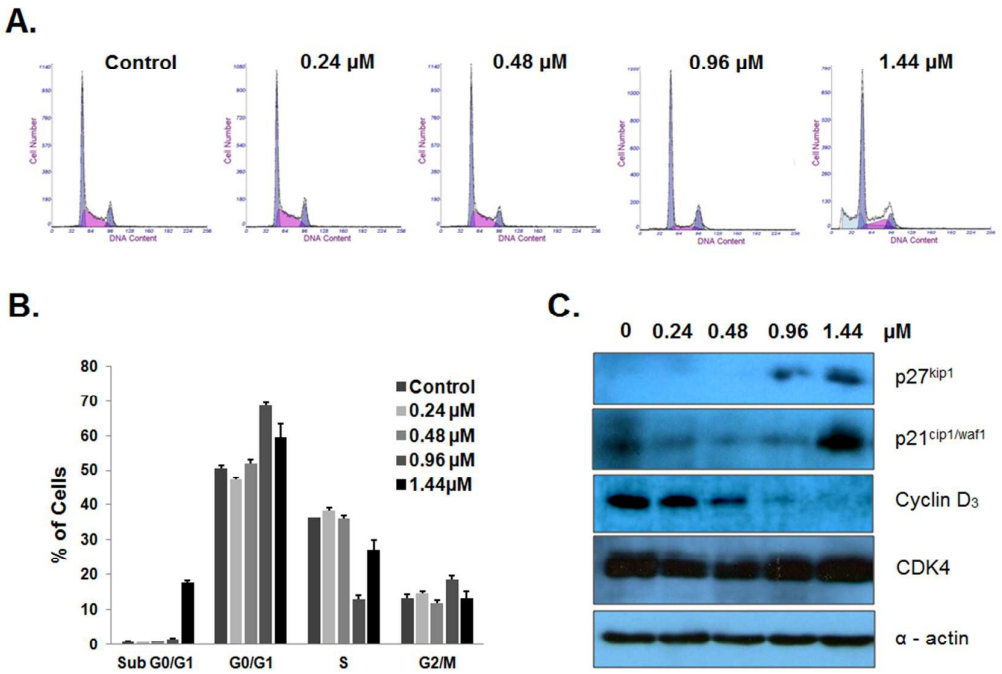

Fig. 3 Effects of compound **24** on cell cycle distribution. **A**. Representative histograms of cell cycle analysis of HeLa cells untreated (control) or treated with the indicated concentrations of compound **24** during 24h. After treatment, cells were stained with PI and analysed by flow cytometry. **B**. Cell cycle analysis of HeLa cells after treatment with compound **24** at specified concentrations for 24h. The results are presented as means ± SD of a minimum three independent experiments **C**- Effect of compound **24** on the levels of cell-cycle-related proteins. HeLa cells were treated with compound **24** at the indicated concentrations during 24h. Protein levels were analysed by western blot. α – actin was used as loading control.

Upregulation of $p21^{waf1/cip1}$ was also observed after treatment with 1.44 μM compound **24**. These data suggest that **24** preferably targets $p27^{kip1}$. Compound **24** also decreased the levels of cyclin D_3 in a concentration-dependent manner, but did not affect the levels of CDK4.

Considering that activated cyclin D/CDK4 complexes promote the progression of the G1 phase of the cell cycle and that the CDKIs $p21^{waf1/cip1}$ and $p27^{kip1}$ inhibit the kinase activity of such complexes, our results suggest that the upregulation of $p27^{kip1}$ and $p21^{waf1/cip1}$ and the downregulation of cyclin D3 induced by compound **24** lead to cell-cycle arrest at the G0/G1 phase, with the consequent inhibition of cell proliferation.

2.2.4 Annexin V-FITC/PI flow cytometric assay We also explored the possibility that compound **24** induces apoptosis in HeLa cells. In the early stages of the apoptotic process, the membrane loses its symmetry and externalization of phosphatidylserine (PS) occurs. Annexin V-FITC specifically binds to externalized PS, thus allowing the quantitative assessment of apoptosis[46]. In the late stages of apoptosis, the membrane loses its integrity and PI can enter the cell[46]. Untreated (control) or treated HeLa cells with compound **24** at concentrations of 0.24 μM, 0.48 μM, 0.96 μM and 1.44 μM for 24 h, were double stained with Annexin V-FITC/PI and analyzed by flow cytometry. This assay permits the differentiation between live cells (Annexin V –/PI –), early apoptotic cells (Annexin V +/PI –), late apoptotic cells (Annexin V +/PI +), and necrotic cells (Annexin V –/PI +).

We observed that treatment of HeLa cells with 1.44 μM of compound **24** led to an increase in the number of apoptotic cells, from 2.9% in control cells to 19.17 % in treated cells (i.e., 7.53% of early apoptotic cells and 11.64% of late apoptotic cells) (Fig. 4A). Concomitantly, the percentage of live cells decreased from 96.53% in the control to 78.61% in treated cells. Treatment with 0.24 μM or 0.48 μM of compound **24** did not change the apoptotic rates significantly. These results suggest that compound **24** at 1.44 μM induces apoptosis in HeLa cells.

2.2.5 Morphological analysis of HeLa cells treated with compound 24 using phase-contrast microscopy and fluorescence microscopy after Hoechst 33258 staining Taking into consideration that apoptosis is characterized by typical morphological features[47], we analyzed the morphology of HeLa cells untreated or treated with compound **24** at 0.96 μM and 1.44 μM for 24 h using microscopic observation, to evaluate further the proapoptotic effect of compound **24.**

As shown in the phase-contrast microscopic pictures (Fig. 4B, upper panel), treatment of HeLa cells with compound **24** reduced the cell density and induced remarkable morphological changes. Compared with control cells, treated cells became smaller and nonadherent and acquired a rounded morphology.

To assess the nuclear morphological changes in greater detail, HeLa cells were stained with Hoechst 33258 after treatment with compound **24** and were analyzed by

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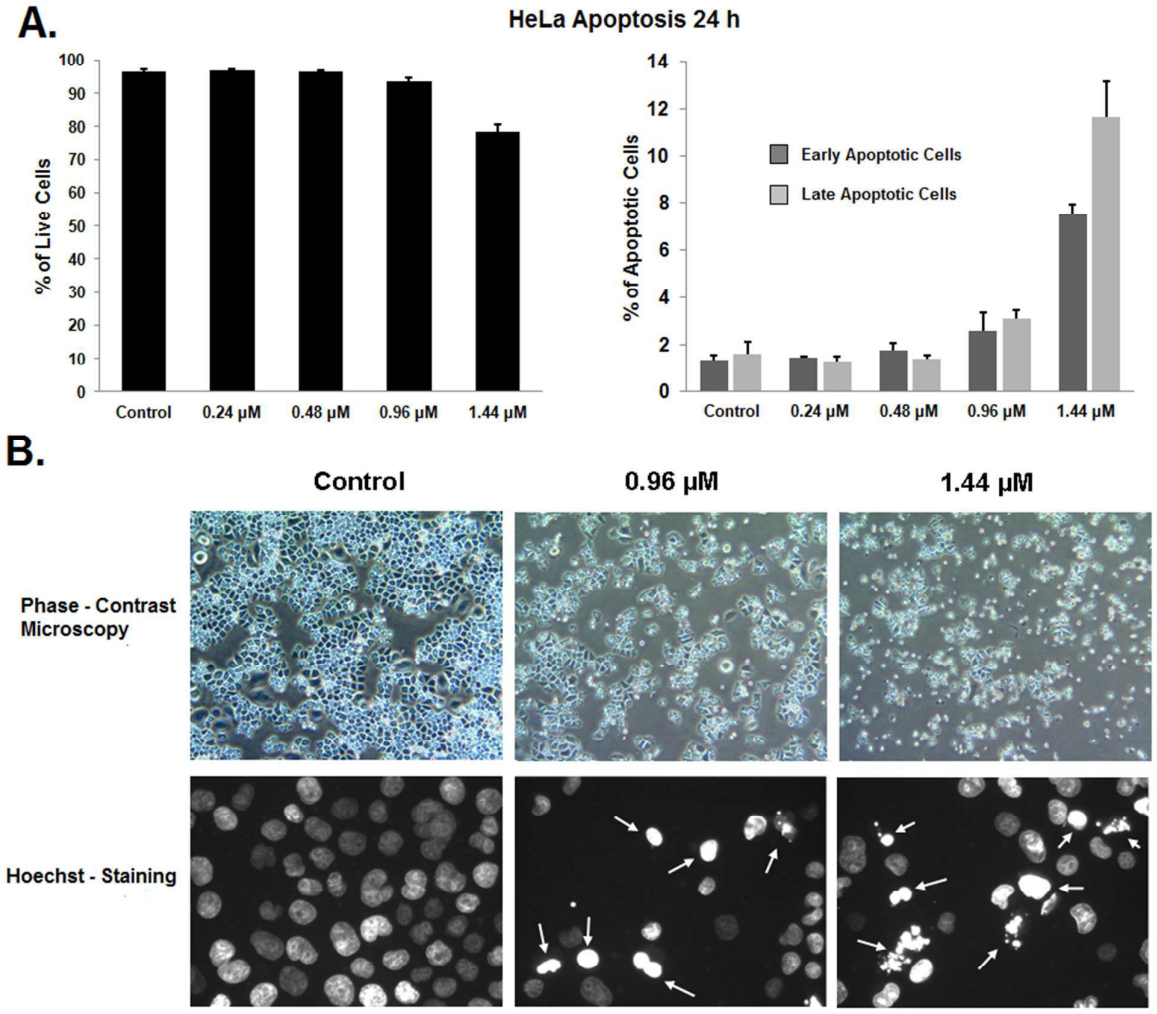

Fig. 4 A. Flow cytometric quantification of apoptosis in HeLa cells untreated (control) or treated with compound 24 at specified concentrations for 24h. After treatment, HeLa cells were stained with Annexin V-FICT/PI and analysed by Flow Cytometry. Black bars represent the percentage of live cells. Dark grey bars represent the percentage of late apoptotic cells and light grey bars represent the percentage of early apopotic cells. The results shown are means ± SD of a minimum three independent experiments **B.** Morphological analysis. Upper panel: Representative phase-contrast images of HeLa cells untreated (control) or treated with compound **24** at specified concentrations for 24h. Lower panel: Representative fluorescence microscopic images of HeLa cells untreated (control) or treated with compound **24** at specified concentrations for 24h. Hela cells were stained with Hoechst 33258 before analysis by fluorescence microscopy. White arrows represent apoptotic cells.

fluorescence microscopy. As shown in Fig. 4B, lower panel, control cells were uniformly stained and presented a normal morphology. Conversely, HeLa cells treated with 0.96 µM of compound **24** exhibited typical apoptotic morphological changes, such as chromatin condensation and cell shrinkage. Nuclear fragmentation and membrane blebbing were evident after treatment with 1.44 µM of compound **24**. A decrease in the number of cells with increasing drug concentrations was also observed. The morphological changes induced by compound **24** were consistent with an apoptotic cell death process.

2.2.6 Effects of compound 24 on the levels of apoptosis-related proteins To gain deeper insight into the mechanism via which compound **24** induces apoptosis in HeLa cells, we investigated the effects of this compound (0–1.44 µM over 24 h) on the levels of some apoptosis-related proteins using western blot analysis (Fig. 5). The activation of caspases is one of the most important mechanisms underlying the execution of apoptosis[48]. Therefore, we

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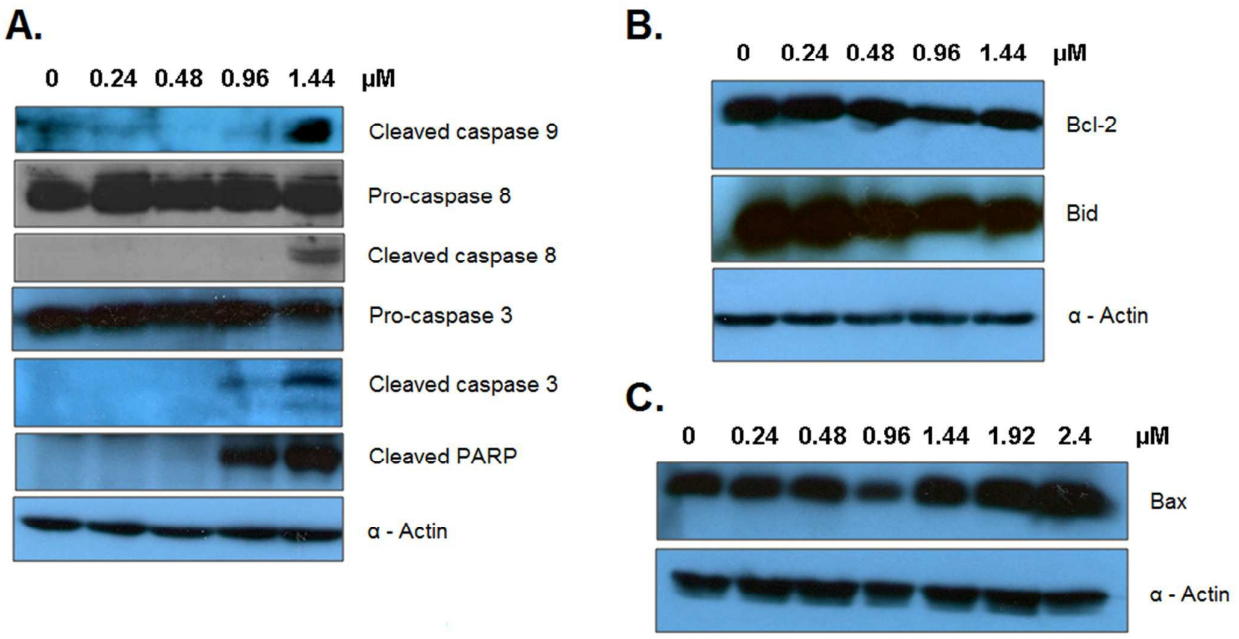

Fig. 5 Effect of compound 24 on levels of apoptosis-related proteins. HeLa cells were treated with the indicated concentrations of compound 24 for 24 hours. The levels of apoptosis-related proteins were assessed by western blot analysis. A. Compound 24 induced the caspase-9, caspase-8 and caspase-3 activation and the cleavage of PARP B. Compound 24 downregulated the levels of Bcl-2 and Bid. C. Compound 24 upregulated the levels of Bax.

first investigated the effect of compound **24** on the levels of caspase-9, caspase-8, caspase-3, and cleaved PARP. Cleaved PARP is an 89 kDa fragment that is generated during apoptosis from the full length PARP via the proteolytic action of active caspase-3 and is considered a biomarker of apoptosis. As shown in Fig. 5A, treatment of HeLa cells with 1.44 μM of compound **24** led to the cleavage of procaspase-8 and procaspase-3 into their active forms, and to increased levels of cleaved caspase-9 (active form of caspase-9). Compound **24** also induced a significant increase in the levels of cleaved PARP. The activation of caspases 8 and 9 suggest that compound **24** induces apoptosis in HeLa cells via a caspase-driven mechanism with activation of both extrinsic and intrinsic pathways.

Subsequently, we explored the effect of compound **24** on the levels of Bcl-2 protein family members, such as Bcl-2 (antiapoptotic), Bax (proapoptotic), and Bid (proapoptotic). As depicted in Figs. 5B and 5C, the treatment of HeLa cells with compound **24** caused a downregulation of Bcl-2 in a concentration-dependent manner. These data support that the mitochondrial pathway is involved in compound **24**-induced apoptosis. Compound **24** also downregulated the levels of Bid, suggesting the activation of Bid into t-Bid. However, bands corresponding to t-Bid were not detected on western blots, which can be justified by the short half-life and small size of t-Bid. Further studies are needed to confirm and understand better the role of the intrinsic and extrinsic pathways in this apoptotic mechanism.

3. Conclusions

In summary, we successfully synthesized a series of new pentameric A-ring **AA** derivatives. Our results showed that the conversion of the hexameric ring A of **AA** into a 5-carbon ring with α,β-unsaturated carbonyl or α,β-unsaturated nitrile moieties significantly improved the antiproliferative activity of compounds. Compound **24** displayed the best antiproliferative profile, with IC_{50} values ranging from 0.11 μM to 0.65 μM. This compound arrested the cell cycle at the G0/G1 phase and induced apoptosis in HeLa cells via the activation of caspases-9, -8, and -3, cleavage of PARP, and modulation of the ratio of Bax/Bcl-2. In light of our results, this compound might represent a promising lead for the development of new anticancer agents.

4. Experimental

4.1 Chemistry

4.1.1 General

Asiatic acid and all reagents were purchased from Sigma-Aldrich Co. The solvents used in the reactions were obtained from Merck Co and were purified and dried according to usual procedures. The solvents used in the workups were purchased from VWR Portugal. Thin layer chromatography (TLC) analysis was carried out in Kieselgel 60HF254/Kieselgel 60G. Separation and purification of compounds by flash column chromatography (FCC) was performed using Kieselgel 60 (230-400 mesh, Merck). Melting points were measured using a BUCHI melting point B-540 apparatus and are uncorrected. IR spectra were obtained on a Fourier transform spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on Brucker Digital NMR—Avance 400 apparatus spectrometer, using $CDCl_3$ as internal standard. The chemical shifts (δ) were reported in parts per million (ppm), and coupling constants (*J*) in hertz (Hz). The mass spectrometry was carried out on Quadrupole/Ion Trap Mass Spectrometer (QIT-MS) (LCQ Advantage MAX, THERMO FINNINGAN). Elemental analysis was performed in an Analyser Elemental Carlo Erba 1108 by chromatographic combustion.

4.1.2 Methyl 2α,3β,23-trihydroxyurs-12-en-28-oate (2)

To a stirred solution of **AA (1)** (1000 mg, 2.05 mmol) and anhydrous potassium carbonate (707.20 mg, 5.12 mmol, 2.5 eq.) in dry DMF (20 mL), methyl iodide (254.7 µL; 4.04 mmol, 2 eq.) was added. The reaction mixture was stirred at room temperature in anhydrous conditions. After 2 hours the reaction mixture was evaporated under reduced pressure to remove the organic phase. The obtained crude was dispersed by water (100 mL) and extracted with ethyl acetate (3 × 100 mL). The resulting organic phase was washed with 5% aqueous HCl (2 × 100 mL), 10% aqueous $NaHCO_3$ (2 × 100 mL), 10% aqueous Na_2SO_3 (100 mL), water (100 mL) and brine (100 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to afford **2** as a white powder (1040 mg, quantitative). Mp: 211.6–214.1 °C. ν_{max}/cm^{-1} (KBr): 3419.17, 2946.70, 2925.48, 2873.42, 1725.98, 1454.06, 1049.09. ^{1}H NMR (400MHz, $CDCl_3$): δ = 5.23 (t, *J* = 3.09 Hz, 1H, H-12), 3.79-3.71 (m, 1H, H-2), 3.59 (m, 4H), 3.40 (d. *J* = 9.97 Hz, 1H, H-23), 3.38 (d, *J* = 10.32 Hz, 1H, H-23), 1.07 (s, 3H), 1.01 (s, 3H), 0.94 (d, *J* = 5.93 Hz, 3H), 0.85 (d, *J* = 6.48 Hz, 3H), 0.81 (s, 3H), 0.72 (s, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 178.06 (C28), 138.25 (C13), 125.22 (C12), 79.83, 69.51, 68.73, 52.80, 51.45, 48.64, 48.04, 47.41, 46.3, 42.58, 42.05, 39.48, 39.02, 38.83, 38.10, 36.59, 32.53, 30.61, 27.95, 24.18, 23.69, 23.33, 21.16, 18.24, 17.15, 17.03, 16.91, 12.89 ppm; DI-ESI-MS m/z: 502.98 ($[M+H]^+$); Calcd. For $C_{31}H_{50}O_5.0.25H_2O$: C, 73.41; H, 10.03. Found: C, 73.29; H, 10.46%.

4.1.3 Ethyl 2α,3β,23-trihydroxyurs-12-en-28-oate (3)

Accordingly to the method described for **2,** using **AA (1)** (500 mg, 1.02 mmol), anhydrous potassium carbonate (353.50 mg, 2.56 mmol, 2.5 eq.), dry DMF (10 mL) and ethyl iodide (246.77 µL; 3.07 mmol, 3 eq.) for 3 hours at room temperature, to afford **3** as a white powder (507.3 mg, 96%). Mp: 177.5–179.1 °C. ν_{max}/cm^{-1} (KBr): 3417.24, 2973.70, 2925.48, 2871.49, 1724.05, 1454.06, 1037.52. ^{1}H NMR (400MHz, $CDCl_3$): δ = 5.24 (t, *J* = 3.10 Hz, 1H, H-12), 4.05 (q, *J* = 7.20 Hz, 2H, $COO\underline{CH_2}CH_3$), 3.79-3.72 (m, 1H, H-2), 3.66 (d, *J* = 10.36 Hz, 1H, H-3), 3.44 (d, *J* = 9.05 Hz, 1H, H-23), 3.41 (d, *J* = 9.40 Hz, 1H, H-23), 1.21 (t, *J* = 7.00 Hz, 3H, $COOCH_2\underline{CH_3}$), 1.08 (s, 3H), 1.03 (s, 3H), 0.94 (d, *J* = 6.05 Hz, 3H), 0.87 (s, 3H), 0.85 (d, *J* = 6.20 Hz, 3H), 0.76 (s, 3H) ppm.^{13}C NMR (100MHz, $CDCl_3$): δ = 177.53 (C28), 138.25 (C13), 125.18 (C12), 68.75, 60.02, 52.81, 47.83, 47.47, 46.24, 42.13, 39.57, 39.07, 38.85, 38.15, 38.62, 32.70, 30.58, 27.91, 24.13, 23.58, 23.34, 21.18, 18.32, 17.16, 17.11, 17.01, 14.21, 12.76 ppm; DI-ESI-MS m/z: 516.97 ($[M+H]^+$); Calcd. For $C_{32}H_{52}O_5.0.5H_2O$: C, 73.10; H, 10.16. Found: C, 73.31; H, 10.32%.

4.1.4 2α,23-Lactol-urs-12-ene-28-oic acid (4)

To a stirred solution of **AA (1)** (200 mg, 0.41 mmol) in methanol / water (5 mL/0.25 mL (20:1)), $NaIO_4$ (131.30 mg; 0.61 mmol, 1.5 eq.) was added. The reaction mixture was stirred at room temperature. After 2 hours the reaction mixture was evaporated under reduced pressure to remove the organic phase. The obtained crude was dispersed by water (40 mL) and extracted with ethyl acetate (3 × 40 mL). The resulting organic phase was washed with water (4 × 40 mL) and brine (40 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to afford **4** as a white powder (quantitative). Mp: 198.5–201.4 °C. ν_{max}/cm^{-1} (KBr): 3421.1, 2948.63, 2927.41, 2871.49, 2732.64, 2630.43, 1716.34, 1695.12, 1456.99, 1378.85, 1037.52. ^{1}H NMR (400MHz, $CDCl_3$): δ = 9.94 (s, 1H, CHO), 5.29 (t, *J* = 3.25 Hz, 1H, H-12), 5.14-5.11 (m, 1H, H-2), 3.94 (d, *J* = 13.41 Hz, 1H), 3.75 (d, *J* = 13.17 Hz, 1H), 1.08 (s, 3H), 1.06 (s, 3H), 0.99 (s, 3H), 0.95 (d, *J* =5.96 Hz, 3H), 0.86 (s, 3H), 0.85 (d, *J* =5.42 Hz, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 206.09 (CHO), 182.94 (C28), 138.05 (C13), 125.98 (C12), 93.68, 65.37, 61.16, 53.41, 62.71, 48.07, 45.15, 43.65, 42.57, 40.05, 39.98, 38.94, 38.77, 38.58, 33.57, 30.59, 27.83, 24.63, 24.06, 23.17, 21.10, 20.58, 20.37, 17.88, 16.93, 14.57 ppm; DI-ESI-MS m/z: 487.15 ($[M+H]^+$); Calcd. For $C_{30}H_{46}O_5.H_2O$: C, 71.39; H, 9.59. Found: C, 71.49; H, 9.85%.

4.1.5 Methyl 2α,23-lactol-urs-12-ene-28-oate (5)

Accordingly to the method described for **4,** using compound **2** (1000 mg, 1.99 mmol), methanol / water (25 mL/1.25 mL [20:1]) and $NaIO_4$ (645.60 mg; 3.02 mmol, 1.52 eq.) for 3 hours to afford **5** as a white powder (978.70 mg, 98%). Mp: 144.7–147.1 °C. ν_{max}/cm^{-1} (KBr): 3444.24, 2948.63, 2927.41, 2871.49, 2730.71, 2626.57, 1722.12, 1454.06, 1378.85, 1037.52. ^{1}H NMR (400MHz, $CDCl_3$): δ = 9.94 (s, 1H, CHO), 5.30 (t, *J* = 3.26 Hz, H-12), 5.13-5.09 (m, 1H, H-2), 3.93 (d, *J* = 13.31 Hz, 1H), 3.74 (d, *J* = 13.31 Hz, 1H), 3.60 (s, 3H, $COO\underline{CH_3}$), 1.08 (s, 3H), 1.06 (s, 3H), 0.99 (s, 3H), 0.94 (d, *J* = 6.03 Hz), 0.85-0.83 (m, 6H) ppm.^{13}C NMR (100MHz, $CDCl_3$): δ = 205.85 (CHO), 177.99 (C28); 138.30 (C13); 125.75 (C12); 93.67; 65.41, 61.12, 53.45, 53.03, 51.49, 48.23, 45.17, 43.67, 42.59, 40.03, 39.97, 38.94, 38.81, 36.52, 33.62, 30.64, 27.86, 24.63, 24.17, 23.17, 21.10, 20.60, 20.38, 17.86, 16.97, 14.52 ppm; DI-ESI-MS m/z: 500.99 ($[M+H]^+$); Calcd. For $C_{31}H_{48}O_5.0.25H_2O$: C, 73.70; H, 9.68. Found: C, 73.75; H, 9.97%.

4.1.6 Ethyl 2α,23-lactol-urs-12-ene-28-oate (6)

Accordingly to the method described for **4,** using compound **3** (480 mg, 0.93 mmol), methanol/water (12.90 mL/ 0.65 mL (20:1)) and $NaIO_4$ (299.45 mg; 1.40mmol, 1.5eq.) for 1h 30min to afford **6** as a white powder (477.90 mg, quantitative). Mp: 138.1–142.0 °C. ν_{max}/cm^{-1} (KBr): 3444.24, 2950.55, 2927.41, 2971.49, 2732.64, 1720.19, 1454.06, 1378.85, 1230.36, 1141.65, 1037.52. ^{1}H NMR (400MHz, $CDCl_3$): δ = 9.94 (s, 1H, CHO), 5.29 (t, *J* = 3.10 Hz, 1H, H-12), 5.12-5.09 (m, 1H, H-2), 4.06 (q, *J* = 7.15 Hz, 2H, $COO\underline{CH_2}CH_3$), 3.93 (d, J = 13.29 Hz, 1H), 3.75 (d, *J* = 13.29 Hz, 1H), 1.21 (t, *J* = 7.14 Hz, 3H, $COOCH_2\underline{CH_3}$), 1.08 (s, 3H), 1.06 (s, 3H), 0.99 (s, 3H), 0.94 (d, *J* = 6.10 Hz, 3H), 0.84 (m, 6H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 205.85 (CHO), 177.45 (C28), 138.31 (C13), 125.70 (C12), 93.65, 65.39, 61.12, 60.04, 53.43, 53.01, 47.99, 45.21, 43.68, 42.65, 40.11, 39.94, 38.97, 38.81, 36.53, 33.71, 30.68, 27.80, 24.63, 24.12, 23.07, 21.10, 20.60, 20.37, 18.05, 16.95, 14.53, 14.21 ppm. DI-ESI-MS m/z: 514.92 ([M+H]$^+$); Calcd. For $C_{32}H_{50}O_5.0.25H_2O$: C, 74.02; H, 9.80. Found: C, 73.84; H, 10.05%.

4.1.7 2-Formyl-23-hydroxy-A(1)-norurs-2,12-diene-28-oic acid (7)

To a stirred solution of compound **4** (500 mg, 1.03 mmol) in dry benzene (50 mL), piperidine (3 mL) and acetic acid (3 mL) were added. The resultant solution was heat-refluxed at 60 °C. After 1 hour, anhydrous magnesium sulfate (500 mg) was added and the reaction mixture was heat-refluxed under nitrogen atmosphere for 4h 20 min. The reaction mixture was evaporated under reduced pressure to remove the organic phase. The obtained crude was dispersed by water (50 mL) and extracted with ethyl acetate (3 × 50mL). The resulting organic phase was washed with water (4 × 50mL) and brine (50 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to afford a yellow powder. The crude solid was purified by flash column chromatography (petroleum ether / ethyl acetate 3:1→1:1) to afford **7** as a white solid (353.77 mg, 73%). Mp: 183.5–186.1 °C. ν_{max}/cm^{-1} (KBr): 3428.81, 2946.7, 2925.48, 2869.56, 2726.85, 2632.36, 1689.34, 1581.34, 1454.06, 1380.78, 1041.37. ^{1}H NMR (400MHz, $CDCl_3$): δ = 9.72 (s, 1H, CHO), 6.66 (s, 1H, H-3), 5.28 (t, *J* = 3.05 Hz, 1H, H-12), 3.62 (d, *J* = 10.67 Hz, 1H, H-23), 3.45 (d, *J* = 10.70 Hz, 1H, H-23), 1.25 (s, 3H), 1.10 (s, 3H), 1.01 (s, 3H), 0.93 (d, *J* = 6.24 Hz, 3H), 0.88 (s, 3H), 0.84 (d, *J* = 6.32 Hz, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ =190.85 (CHO), 183.27 (C28), 159.29 (C3), 158.90 (C2), 137.49 (C13), 126.60 (C12), 69.36, 56.26, 52.57, 50.88, 49.41, 47.87, 44.10, 42.41, 41.36, 38.78 (2C), 36.63, 33.53, 30.55, 28.19, 27.09, 23.99 (2C), 21.16, 19.03, 18.74, 17.35, 16.98, 15.92 ppm; DI-ESI-MS m/z: 469.03 ([M+H]$^+$); Calcd. For $C_{30}H_{44}O_4.H_2O$: C, 74.04; H, 9.53. Found: C, 73.68; H, 9.75%.

4.1.8 Methyl 2-formyl-23-hydroxy-A(1)-norurs-2,12-diene-28-oate (8)

Accordingly to the method described for **7**, using compound **5** (500 mg, 0.99 mmol), dry benzene (50 mL), piperidine (2.5 mL) acetic acid (2.5 mL) and anhydrous magnesium sulfate (327 mg), to afford compound **8** as a yellow powder (488.5 mg, quantitative). Mp: 119.7–122.4 °C. ν_{max}/cm^{-1} (KBr): 3448.10, 2948.63, 2925.48, 2869.56, 2724.92, 2634.29, 1724.05, 1687.41, 1581.34, 1455.99, 1382.71, 1232.29, 1145.51, 1047.16. ^{1}H NMR (400MHz, $CDCl_3$): δ = 9.72 (s, 1H, CHO), 6.66 (s, 1H, H-3), 5.28 (t, *J* = 3.20 Hz, 1H, H-12), 3.62-3.60 (m, 4H), 3.46 (d, *J* = 10.72 Hz, 1H, H-23), 1.25 (s, 3H), 1.09 (s, 3H), 1.02 (s, 3H), 0.93 (d, *J* = 6.15 Hz, 3H), 0.83 (m, 6H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ =190.81 (CHO), 178.09 (C28), 159.24 (C3), 158.93 (C2), 137.74 (C13), 126.34 (C12), 69.39, 56.27, 52.81, 51.45, 50.90, 49.42, 48.03, 44.12, 42.43, 41.34, 38.83, 38.81, 36.57, 33.55, 30.59, 28.25, 27.09, 24.16, 23.99, 21.17, 19.00, 18.67, 17.37, 17.04, 15.99 ppm; DI-ESI-MS m/z: 482.98 ([M+H]$^+$); Calcd. For $C_{31}H_{46}O_4.0.25H_2O$: C, 76.42; H, 9.62. Found: C, 76.58; H, 9.78%.

4.1.9 Ethyl 2-formyl-23-hydroxy-A(1)-norurs-2,12-diene-28-oate (9)

Accordingly to the method described for **7**, using compound **6** (300 mg, 0.583 mmol), dry benzene (30 mL), piperidine (1.5 mL), acetic acid (1.5 mL) and anhydrous magnesium sulfate (300 mg) to afford a yellow crude. The crude solid was purified by flash column chromatography (petroleum ether / ethyl acetate, 3:1→2:1) to afford **9** as a white solid (193.2 mg, 67%). Mp: 207.5–209.7 °C. ν_{max}/cm^{-1} (KBr): 3550.31, 2977.55, 2960.20, 2946.70, 2925.48, 2867.63, 2809.78, 2726.85, 1708.62, 1689.34, 1581.34, 1452.11, 1238.08, 1143.58, 1045.23. ^{1}H NMR (400MHz, $CDCl_3$): δ = 9.72 (s, 1H, CHO), 6.66 (s, 1H, H-3), 5.28 (t, *J* = 3.00 Hz, 1H, H-12), 4.09 – 4.02 (m, 2H, $COO\underline{CH_2}CH_3$), 3.62 (d, *J* = 8.50 Hz, 1H, H-23), 3.46 (d, *J* = 8.78 Hz, 1H, H-23), 1.25 (s, 3H), 1.22 (t, *J* = 7.20 Hz, 3H, $COOCH_2\underline{CH_3}$), 1.09 (s, 3H), 1.02 (s, 3H), 0.93 (d, *J* = 6.07 Hz, 3H), 0.86 (s, 3H), 0.83 (d, *J* = 6.54 Hz, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 190.84 (CHO), 177.57 (C28), 159.29 (C3), 158.95 (C2), 137.79 (C13), 126.28 (C12), 69.39 (C23), 60.02, 56.25, 52.81, 50.91, 49.43, 47.79, 44.15, 42.49, 41.44, 38.84 (2C), 36.57, 33.62, 30.64, 28.19, 27.10, 24.11, 23.90, 21.17, 19.01, 18.81, 17.37, 17.02, 15.93, 14.18 ppm. DI-ESI-MS m/z: 497.07 ([M+H]$^+$). Calcd. For $C_{32}H_{48}O_4$: C, 77.38; H, 9.74. Found: C, 76.94; H, 10.12%.

4.1.10 Methyl 2α,3β,23-triacetoxyurs-12-ene-28-oate (10)

To a stirred solution of compound **2** (953 mg, 1.89 mmol) in dry THF (28 mL), acetic anhydride (1163.30 uL; 12.33 mmol, 6,5 eq.) and a catalytic amount of DMAP (95.30 mg) were added. The reaction mixture was stirred at room temperature in anhydrous conditions. After 5 hours the reaction mixture was evaporated under reduced pressure to remove the organic phase. The obtained crude was dispersed by water (80 mL) and extracted with ethyl acetate (3 × 80 mL). The resulting organic phase was washed with 5% aqueous HCl (2 × 80 mL), 10% aqueous $NaHCO_3$ (2 × 80 mL), 10% aqueous Na_2SO_3 (80 mL), water (80 mL) and brine (80mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to afford **10** as a white powder (1182 mg, 99% quantitative). Mp: 118.8–121.1 °C. ν_{max}/cm^{-1} (KBr): 2948.63, 2927.41, 2871.49, 1745.26, 1455.99, 1369.21, 1234.22, 1043.30. ^{1}H NMR (400MHz, $CDCl_3$): δ = 5.24 (t, *J* = 3.00 Hz, 1H, H-12), 5.19-5.13 (m, 1H, H-2), 5.08 (d, *J* = 10.29 Hz, 1H, H-3), 3.85 (d, J = 11.80 Hz, 1H, H-23), 3.57 (d, *J* = 11.80 Hz, 1H, H-23), 3.60 (s, 3H, $COO\underline{CH_3}$), 2.08 (s, 3H, CH_3CO), 2.01 (s, 3H, CH_3CO), 1.97 (s, 3H, CH_3CO), 1.09 (s, 3H), 1.06 (s, 3H), 0.94 (d, *J* = 6.06 Hz, 3H), 0.88 (s, 3H), 0.84 (d, *J* = 6.23 Hz, 3H), 0.74 (s, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 177.95 (C28), 170.85 (OCO), 170.44 (OCO), 170.37 (OCO),

138.30 (C13), 125.01 (C12), 74.85, 69.91, 65.28, 52.81, 51.46, 48.04, 47.59, 47.49, 43.75, 41.98, 41.89, 39.50, 39.00, 38.81, 37.77, 36.54, 32.44, 30.60, 27.90, 24.12, 23.42, 23.34, 21.14, 21.06, 20.86, 20.76, 17.88, 17.00, 16.95, 16.85, 13.90 ppm; DI-ESI-MS m/z: 629.36 ($[M+H]^+$); Calcd. For $C_{37}H_{56}O_8$: C, 70.67; H, 8.98. Found: C, 70.55; H, 8.98%.

4.1.11 Methyl 2α,3β,23-triacetoxy-11-oxours-12-ene-28-oate (11)

To a stirred solution of compound **10** (100 mg, 0.16 mmol) in dry dichloromethane (2 mL), a mixture of $KMnO_4$ (440 mg) and $Fe_2(SO_4)_3.nH_2O$ (220 mg), previous reduced to fine powder, water (22 µL) and *t*-butanol (0.1 mL) were added. The reaction mixture was stirred at room temperature. After 4h 45 min the reaction mixture was diluted with diethyl ether (20 mL) and filtered thought a Celite pad, with further washing with diethyl ether (100 mL). The resulting organic phase was washed with 10% aqueous $NaHCO_3$ (2 × 50 mL) and water (2× 50 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to afford **11** as a white powder (97.5mg, 95%). Mp: 139.2–141.5 °C. ν_{max}/cm^{-1} (KBr): 2977.55, 2950.55, 2875.34, 1747.19, 1662.34, 1457.92, 1369.21, 1234.22, 1043.30. ^1H NMR (400MHz, $CDCl_3$): δ = 5.62 (s, 1H, H-12), 5.31-5.25 (m. 1H, H-2), 5.04 (d, *J* = 10.30 Hz, 1H, H-3), 3.83 (d, J = 11.76 Hz, 1H, H-23), 3.57 (d, *J* = 11.76 Hz, 1H, H-23), 3.59 (s, 3H, $COO\underline{CH_3}$), 2.07 (s, 3H, CH_3CO), 2.00 (s, 3H, CH_3CO), 1.94 (s, 3H, CH_3CO), 1.28 (s, 6H), 0.96 (d, *J* = 6.31 Hz, 3H), 0.90 (s, 6H), 0.85 (d, *J* = 6.49 Hz, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 198.82 (C11), 177.09 (C28), 170.79 (OCO), 170.46 (OCO), 170.10 (OCO), 163.26 (C13), 130.43 (C12), 74.88, 68.96, 65.21, 61.11, 52.71, 51.87 (2C), 47.61, 47.32, 44.55, 44.17, 43.72, 41.88, 38.58, 37.65, 35.88, 32.45, 30.26, 28.29, 23.84, 20.98, 20.95 (2C), 20.88, 20.75, 18.81, 17.70, 17.05, 16.99, 13.85 ppm; DI-ESI-MS m/z: 643.12 ($[M+H]^+$); Calcd. For $C_{37}H_{54}O_9.0.25H_2O$: C, 68.65; H, 8.49. Found: C, 68.46; H, 8.38%.

4.1.12 Methyl 2α,3β,23-trihydroxy-11-oxours-12-ene-28-oate (12)

To a stirred solution of compound **11** (353 mg, 0.55 mmol) in methanol (23.5 mL), KOH (2350 mg) was added and the reaction mixture was heated under reflux. After 30 min the reaction mixture was evaporated under reduced pressure to remove the methanol, and acidified (pH 5-6) with 6M aqueous HCl solution. Water (20 mL) was added and the aqueous layer was extracted with ethyl acetate (3 × 80 mL). The resulting organic phase was washed with 10% aqueous $NaHCO_3$ (3 × 50 mL), water (50 mL) and brine (50 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to afford **12** as a white powder (280.2 mg, quantitative). Mp: 174.9–177.3 °C. ν_{max}/cm^{-1} (KBr): 3424.96, 2948.63, 2929.34, 2873.42, 1727.91, 1660.41, 1455.99, 1388.50, 1201.43, 1047.16. ^1H NMR (400MHz, $CDCl_3$): δ = 5.60 (s, 1H, H-12), 3.87-3.81 (m, 1H, H-2), 3.64 (d, *J* = 11.09 Hz, 1H, H-23), 3.60 (s, 3H, $COO\underline{CH_3}$), 3.42 (d, *J* = 9.51 Hz, 1H, H3) 3.36 (d, *J* = 11.09 Hz, 2H, H-23), 1.30 (s, 3H), 1.21 (s, 3H), 0.96 (d, *J* = 6.10 Hz, 3H), 0.89 (s, 3H), 0.86 (d, *J* = 6.31 Hz, 3H), 0.82 (s, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 199.70 (C11), 177.19 (C28), 163.51 (C13), 130.43 (C12), 68.50, 61.04, 52.73, 51.85 (2C), 47.64, 46.89, 44.66, 43.86, 42.78, 38.63, 38.56, 38.01, 35.94, 32.54, 30.28, 28.35, 23.90, 21.19, 20.96 (2C), 18.91, 17.88, 17.21, 17.09, 12.99 ppm; DI-ESI-MS m/z: 516.93 ($[M+H]^+$); Calcd. For $C_{31}H_{48}O_6.H_2O$: C, 69.63; H, 9.42. Found: C, 69.19; H, 8.92%.

4.1.13 Methyl 2α,23-lactol-11-oxours-12-ene-28-oate (13)

Accordingly to the method described for **4**, using compound **12** (1950 mg, 3.78 mmol), methanol / water (48.70 mL/ 2.40 mL (20:1)) and $NaIO_4$ (1227.40 mg; 5.74 mmol, 1.52 eq.) for 1 hour at room temperature. The crude solid was purified by flash column chromatography (petroleum ether/ethyl acetate, 4:1→1:1) to afford **13** as a white solid (1280.30 mg, 66%). Mp: 154.0–157.0 °C. ν_{max}/cm^{-1} (KBr): 3436.53, 2948.63, 2938.98, 2873.42, 2734.57, 1725.98, 1658.48, 1455.99, 1205.29, 1141.65, 1037.52. ^1H NMR (400MHz, $CDCl_3$): δ = 9.97 (s, 1H, CHO), 5.64 (s, 1H, H-12), 5.36-5.33 (m, 1H, H-2), 3.98 (d, *J* = 13.23 Hz, 1H), 3.71 (d, *J* = 13.59 Hz, 1H), 3.60 (s, 3H, $COO\underline{CH_3}$), 1.34 (s, 3H), 1.32 (s, 3H), 0.97 (m, 6H), 0.93 (s, 3H), 0.86 (d, *J* = 6.37 Hz, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 205.65 (CHO), 198.89 (C11), 177.12 (C28), 162.74 (C13), 130.85 (C12), 93.49, 65.25, 61.38, 57.40, 53.20, 52.75, 51.89, 47.71, 45.70, 44.65, 44.15, 39.21, 38.60, 38.57, 35.92, 33.16, 30.30, 28.35, 23.88, 20.93, 20.70, 20.47, 19.60, 19.28, 17.03, 14.38 ppm; DI-ESI-MS m/z: 515.42 ($[M+H]^+$); Calcd. For $C_{31}H_{46}O_6.0.75H_2O$: C, 70.49; H, 9.06. Found: C, 70.31; H, 9.41%.

4.1.14 Methyl 2-formyl-11-oxo-23-hydroxy-A(1)-norurs-2,12-diene-28-oate (14)

Accordingly to the method described for **7**, using compound **13** (300 mg, 0.58 mmol), dry benzene (30 mL), piperidine (1.45 mL), acetic acid (1.45 mL) and anhydrous magnesium sulfate (300 mg). The crude solid was purified by flash column chromatography (petroleum ether/ethyl acetate, 2:1→1:1) to afford **14** as a white solid (142.90 mg, 49%). Mp: 144.8–146.6 °C. ν_{max}/cm^{-1} (KBr): 3442.31, 2977.55, 2948.63, 2929.34, 2871.49, 1727.91, 1662.34, 1614.13, 1581.34, 1455.99, 1226.50. ^1H NMR (400MHz, $CDCl_3$): δ = 10.15 (s, 1H, CHO), 6.35 (s, 1H, H-3), 5.67 (s, 1H, H-12), 3.62 (s, 3H, $COO\underline{CH_3}$, H28), 3.56 (d, J = 11.08 Hz, 1H), 3.39 (d, *J* = 11.08 Hz, 1H),1.45 (s, 3H), 1.33 (s, 3H), 1.00 (s, 3H), 0.97 (m, 6H), 0.85 (d, *J* = 6.35 Hz, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 199.38 (C11), 195.49 (CHO), 177.17 (C28), 164.67 (C13), 157.47 (C2); 144.75 (C3), 129.47 (C12), 69.24, 58.26, 54.83, 52.99, 51.88, 48.70, 48.23, 47.57, 45.83, 44.44, 38.61, 38.36, 35.87, 33.44, 30.21, 28.77, 23.87, 21.39, 21.06, 20.96, 20.54, 17.11, 16.90, 16.10 ppm. DI-ESI-MS m/z: 497.47 ($[M+H]^+$). Anal. Calcd. For $C_{31}H_{44}O_5.0.5H_2O$: C, 73.63; H, 8.97. Found: C, 73.27; H, 8.50%.

4.1.15 Methyl 2-formyl-23-methanesulfonyloxy-A(1)-norurs-2,12-diene-28-oate (15)

To a stirred solution of compound **8** (250 mg, 0.52 mmol) in dry dicloromethane (10 mL), triethylamine (144.60 µL; 1.04 mmol, 2 eq.) and methanesulfonyl chloride (80.20 µL, 1.04 mmol, 2 eq.) were added. The reaction mixture was stirred at room temperature. After 4 hours, the reaction mixture was evaporated under reduced pressure to remove the organic phase. The obtained crude was dispersed by water (50 mL) and extracted with ethyl acetate (3 × 50 mL). The resulting organic phase was washed with water (4 × 50 mL) and brine (50 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to afford a yellow crude. The crude solid was purified by flash

column chromatography (petroleum ether/ethyl acetate, 4:1→2:1) to afford **15** as a white solid (206.9 mg, 71 %). Mp: 105.6–107.2 °C. ν_{max}/cm^{-1} (KBr): 2948.63, 2925.48, 2871.49, 2728.78, 1722.12, 1689.34, 1585.2, 1455.99, 1357.64, 1176.36, 958.45. ^{1}H NMR (400MHz, $CDCl_3$): δ = 9.73 (s, 1H, CHO), 6.60 (s, 1H, H-3), 5.26 (t, *J* = 3.01 Hz, 1H, H-12), 4.13 (d, *J* = 9.59 Hz, 1H, H-23), 4.06 (d, *J* = 9.59 Hz, 1H, H-23), 3.61 (s, 3H, COO$\underline{CH_3}$), 3.03 (s, 3H, (S-$\underline{CH_3}$), 1.25 (s, 3H), 1.10 (s, 3H), 1.08 (s, 3H), 0.93 (d, *J* = 5.87 Hz, 3H), 0.84 – 0.83 (m, 6H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 190.56 (CHO), 178.03 (C28), 158.95 (C2), 155.79 (C3), 137.85 (C13), 126.10 (C12), 74.70, 56.93, 52.79, 51.45, 50.85, 48.01, 47.69, 44.29, 42.43, 41.30, 38.82, 38.78, 37.49, 36.53, 33.43, 30.56, 28.17, 27.03, 24.11, 23.86, 21.16, 18.78, 18.66, 17.30, 17.05, 15.88 ppm. DI-ESI-MS m/z: 560.91 ([M+H]+); Calcd. For $C_{32}H_{48}O_6S.0.5H_2O$: C, 67.45; H, 8.67; S, 5.63. Found: C, 67.56; H, 8.73; S, 5.27%.

4.1.16 Methyl 2-cyano-23-hydroxy-A(1)-norurs-2,12-diene-28-oate (16)

To a stirred solution of compound **8** (295 mg, 0.61 mmol) in THF (4.40 mL), aqueous ammonium solution 25% (13.70 mL) and iodine (340.75 mg, 1.34 mmol, 2.20 eq.) were added. The mixture was stirred at room temperature for 8 hours (the dark solution became colorless). The reaction mixture was charged with 5% aqueous $Na_2S_2O_2$ (50 mL) and extracted with ethyl acetate (3 × 60 mL). The resulting organic phase was washed with water (50 mL) and brine (50 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to afford a yellow powder. The crude solid was purified by flash column chromatography (petroleum ether/ethyl acetate, 3:1→2:1) to afford **16** as a white solid (146.20 mg, 50%). Mp: 115.1–118.1 °C. ν_{max}/cm^{-1} (KBr): 3504.02 (OH), 2948.63, 2925.48, 2871.49, 2215.81, 1724.05, 1652.70, 1587.13, 1455.99, 1362.71, 1234.22, 1197.58, 1145.51, 1049.09. ^{1}H NMR (400MHz, $CDCl_3$): δ = 6.48 (s, 1H, H-3), 5.26 (t, *J* = 3 Hz, 1H, H-12), 3.61 (s, 3H, COO$\underline{CH_3}$), 3.55 (d, *J* = 10.07 Hz, 1H, H-23), 3.39 (d, *J* = 9.92 Hz, 1H, H-23), 1.27 (s, 3H), 1.13 (s, 3H), 1.02 (s, 3H), 0.94 (d, *J* = 5.36 Hz, 3H), 0.87 (d, *J* = 6.13 Hz, 3H), 0.83 (s, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 178.02 (C28), 153.59 (C3), 138.68 (C13), 127.16 (C2), 125.01 (C12), 117.64 (CN), 69.26, 55.37, 52.90 (2C), 51.48, 50.83, 48.03, 43.55, 42.39, 40.92, 38.83 (2C), 36.54, 33.41, 30.57, 28.20, 24.55, 24.09, 24.01, 21.16, 19.27, 18.44, 17.65, 17.08, 16.01 ppm. DI-ESI-MS m/z: 480.08 ([M+H]$^+$); Calcd. For $C_{31}H_{45}NO_3.0.5H_2O$: C, 76.19; H, 9.49; N, 2.87. Found: C, 76.41; H, 9.62; N, 2.97%.

4.1.17 2-cyano-23-hydroxy-A(1)-norurs-2,12-diene-28-oic acid (17)

Accordingly to the method described for **16**, using compound **7** (240 mg, 0.51 mmol), THF (2.40 mL), aqueous ammonium solution 25% (9.60 mL) and iodine (142.96 mg, 0.56 mmol, 1.10 eq.) at room temperature for 2 hours. The crude solid was purified by flash column chromatography (petroleum ether/ethyl acetate, 4:1→2:1) to afford **17** as a white solid (134.4 mg, 56%). Mp: 147.1–150.1 °C. ν_{max}/cm^{-1} (KBr): 3423.03, 2948.63, 2925.48, 2871.49, 2217.74, 1697.05, 1455.99, 1382.71, 1043.30. ^{1}H NMR (400MHz, $CDCl_3$): δ = 6.48 (s, 1H, H-3), 5.25 (t, *J* = 2.99 Hz, 1H, H-12), 3.54 (d, *J* = 10.58 Hz, 1H, H-23), 3.38 (d, *J* = 10.58 Hz, 1H, H-23), 1.27 (s, 3H), 1.13 (s, 3H), 1.01 (s, 3H), 0.95 (d, *J* = 6.04 Hz, 3H), 0.87 (m, 6H) ppm. $_{13}$C NMR (100MHz, $CDCl_3$): δ = 183.40 (C28), 153.72 (C3), 138.50 (C13), 127.02 (C2), 125.13 (C12), 117.62 (CN), 69.17 (C23), 55.34, 52.85, 52.65, 50.81, 47.89, 43.53, 42.37, 40.92, 38.79 (2C), 36.63, 33.38, 30.54, 28.13, 24.54, 24.00, 23.93, 21.15, 19.29, 18.46, 17.62, 17.03, 16.00 ppm. DI-ESI-MS m/z: 466.03 ([M+H]$^+$); Calcd. For $C_{30}H_{43}NO_3.0.75H_2O$: C, 75.20; H, 9.36; N, 2.92. Found: C, 75.10; H, 9.83; N, 2.79%.

4.1.18 Methyl 2-cyano-11-oxo-23-hydroxy-A(1)-norurs-2,12-diene-28-oate (18)

Accordingly to the method described for **16**, using compound **14** (300 mg, 0.60 mmol), THF (3 mL), aqueous ammonium solution 25% (12 mL) and iodine (168.60 mg, 0.66 mmol, 1.10 eq.) at room temperature for 3hours. The crude solid was purified by flash column chromatography (petroleum ether/ethyl acetate, 3:1→1:1) to afford **18** as a white solid (144.6 mg, 48%). Mp: 139.1–142.0 °C. ν_{max}/cm^{-1} (KBr): 3444.24, 2948.63, 2929.34, 2871.49, 2217.74, 1725.98, 1670.05, 1612.2, 1581.34, 1457.92, 1226.50. ^{1}H NMR (400MHz, $CDCl_3$): δ = 6.57 (s, 1H, H-3), 5.71 (s, 1H, H-12), 3.60 (s, 3H, COO$\underline{CH_3}$), 3.55 (d, J = 10.93 Hz, 1H, H-23), 3.38 (d, *J* = 10.92 Hz, 1H, H-23), 1.40 (s, 3H), 1.33 (s, 3H), 1.01 (s, 3H), 0.97 (d, *J* = 6.26 Hz, 3H), 0.91 (s, 3H), 0.87 (d, *J* = 6.43 Hz, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 197.16 (C11), 177.19 (C28), 163.92 (C13), 155.23 (C3), 129.34 (C12), 128.72 (C2), 117.24 (CN), 68.76, 57.20, 54.68, 52.93, 51.88, 50.86, 49.96, 47.56, 45.43, 44.25, 38.61, 38.45, 35.91, 33.14, 30.20, 28.74, 23.81, 21.51, 20.96, 20.76, 20.44, 17.23, 16.86, 15.86 ppm. DI-ESI-MS m/z: 494.37 ([M+H]$^+$); Calcd. For $C_{31}H_{43}NO_4.0.5H_2O$: C, 74.07; H, 8.82; N, 2.79. Found: C, 74.10; H, 8.83; N, 2.94%.

4.1.19 Methyl 2-formyl-11-oxo-23-acetoxy-A(1)-norurs-2,12-diene-28-oate (19)

To a stirred solution of compound **14** (95 mg, 0.19 mmol) in dry THF (2.85 mL), acetic anhydride (27 µL; 0.29 mmol, 1.50 eq.) and a catalytic amount of DMAP (9.5 mg) were added. The mixture was stirred at room temperature in anhydrous conditions. After 1h 30 min, the reaction mixture was evaporated under reduced pressure to remove the organic phase. The obtained crude was dispersed by water (30 mL) and extracted with ethyl acetate (3 × 30 mL). The resulting organic phase was washed with 5% aqueous HCl (2 × 30 mL), 10% aqueous $NaHCO_3$ (2 × 30 mL), 10% aqueous Na_2SO_3 (30 mL), water (30 mL) and brine (30 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to afford **19** as a white powder (73.2 mg, 71%). Mp: 104.2–107.3 °C. ν_{max}/cm^{-1} (KBr): 2948.63, 2933.20, 2873.42, 1735.62, 1662.34, 1614.13, 1585.2, 1455.99, 1384.84, 1232.29, 1039.44. ^{1}H NMR (400MHz, $CDCl_3$): δ = 10.15 (s, 1H, CHO), 6.39 (s, 1H, H-3), 5.67 (s, 1H, H-12), 3.97 (d, J = 10.78 Hz, 1H, H-23), 3.89 (d, *J* = 10.76 Hz, 1H, H-23), 3.61 (s, 3H, COO$\underline{CH_3}$), 2.05 (s, 3H, CH_3CO), 1.45 (s, 3H), 1.31 (s, 3H), 1.05 (s, 3H), 0.96 (m, 6H), 0.86 (d, *J* = 6.51 Hz, 3H) ppm. $_{13}$C NMR (100MHz, $CDCl_3$): δ = 199.12 (C11), 195.25 (CHO), 177.13 (C28), 171.05 (OCO), 164.61 (C13), 156.37 (C2), 143.96 (C3), 129.46 (C12), 70.75, 58.41, 56.75, 52.99, 51.88, 48.01, 47.56, 46.64, 45.77, 44.37, 38.61, 38.36, 35.84, 33.49, 30.19, 28.71, 23.85, 21.21, 21.02, 20.95, 20.85, 20.25, 17.12, 17.00, 16.28

ppm. DI-ESI-MS m/z: 539.88 ([M+H]$^+$); Calcd. For $C_{33}H_{46}O_6$.0.75H_2O: C, 71.77; H, 8.67. Found: C, 71.80; H, 8.65%.

4.1.20 Methyl 2-formyl-11-oxo-23-butyroxy-A(1)-norurs-2,12-diene-28-oate (20)

Accordingly to the method described for **19**, using compound **14** (300 mg, 0.60 mmol), dry THF (9 mL), butyric anhydride (98.80 µL; 0.60 mmol, 1 eq) and a catalytic amount of DMAP (30 mg). at room temperature for 1 hour. The crude solid was purified by flash column chromatography (petroleum ether/ethyl acetate, 7:1→5:1) to afford **20** as a white solid (59.1 mg, 17%). Mp: 77.8–80.3 °C. ν_{max}/cm^{-1} (KBr): 2950.55, 2933.20, 2873.42, 1731.76, 1662.34, 1614.13, 1585.20, 1457.92, 1224.58, 1197.58, 1174.44. ^{1}H NMR (400MHz, $CDCl_3$): δ = 10.15 (s, 1H, CHO), 6.39 (s, 1H, H-3), 5.68 (s, 1H, H-12), 3.98 (d, J = 10.78 Hz, 1H, H-23), 3.90 (d, *J* = 10.63 Hz, 1H, H-23), 3.62 (s, 3H, $COOCH_3$), 1.46 (s, 3H), 1.31 (s, 3H), 1.05 (s, 3H), 0.96 – 0.92 (m, 6H), 0.86 (d, *J* = 6.23 Hz, 3H) ppm. $_{13}$C NMR (100MHz, $CDCl_3$): δ = 199.13 (C11), 195.22 (CHO), 177.14 (C28), 173.61 (OCO), 164.59 (C13), 156.36 (C2), 144.10 (C3), 129.49 (C12), 70.33, 58.41, 56.64, 53.00, 51.89, 47.98, 47.57, 46.73, 45.79, 44.37, 38.62, 38.38, 36.16, 35.86, 33.51, 30.20, 28.72, 23.86, 21.21, 21.01, 20.97, 20.30, 18.47, 17.14, 16.97, 16.31, 13.73 ppm. DI-ESI-MS m/z: 567.40 ([M+H]$^+$). Anal. Calcd. For $C_{35}H_{50}O_6$: C, 74.17; H, 8.89. Found: C, 73.79; H, 9.21%.

4.1.21 Methyl 2-formyl-11-oxo-23-cinnamoxy-A(1)-norurs-2,12-diene-28-oate (21)

To a stirred solution of compound **14** (300 mg, 0.60 mmol) in dry benzene (18 mL), cinnamoyl chloride (402.50 mg, 2.41 mmol, 4 eq.) and DMAP (295.20 mg, 2.41mmol, 4 eq) were added. The mixture was stirred at 60 °C under nitrogen atmosphere. After 3 hours, the reaction mixture was evaporated under reduced pressure to remove the organic phase. The obtained crude was dispersed by water (50 mL) and extracted with ethyl acetate (3 × 50 mL). The resulting organic phase was washed with water (4 × 50 mL) and brine (50 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to afford a light yellow crude. The crude solid was purified by flash column chromatography (petroleum ether/ethyl acetate, 6:1→4:1) to afford **21** as a white solid (268.7 mg, 71%). Mp: 97.0–100.2 °C. ν_{max}/cm^{-1} (KBr): 3083.62, 3060.48, 3025.76, 2948.63, 2931.27, 2873.42, 1718.26, 1664.27, 1637.27, 1452.14, 1382.71, 1309.43, 1272.79, 1228.43, 1201.43, 1164.79. ^{1}H NMR (400MHz, $CDCl_3$): δ= 10.17 (s, 1H, CHO), 7.69 (d, *J* = 15.98 Hz, 1H, H-3'), 7.54 - 7.52 (m, 2H, H-2'' and H-6''), 7.40 – 7.38 (m, 3H, H-3'',H-4'', H-5''), 6.47 (s, 1H, H-3), 6.43 (d, *J* = 16.02 Hz, 1H, H-2'), 5.68 (s, 1H, H-12), 4.11 (d, J = 11.01 Hz, 1H, H-23), 4.07 (d, *J* = 11.05 Hz, 1H, H-23), 3.62 (s, 3H, $COO\underline{CH_3}$), 1.48 (s, 3H), 1.29 (s, 3H), 1.11 (s, 3H), 0.96 – 0.95 (m, 6H), 0.84 (d, *J* = 6.36 Hz, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 199.18 (C11), 195.29 (CHO), 177.14 (C28), 166.94 (OCO), 164.66 (C13), 156.39 (C2), 145.30, 144.13 (C3), 134.22, 130.43, 129.43 (C12), 128.89 (2C), 128.19 (2C), 117.61, 70.67, 58.42, 56.73, 52.98, 51.88, 48.03, 47.55, 46.92, 45.78, 44.37, 38.59, 38.32, 35.84, 33.51, 30.18, 28.69, 23.83, 21.19, 21.02, 20.94, 20.29, 17.10, 17.03, 16.37 ppm. DI-ESI-MS^2 m/z : 627.36 ([M+H]$^+$,42%), 609.48 (62), 566.37 (72), 479.38 (100), 419.39 (29); Calcd. For $C_{40}H_{50}O_6$.H_2O: C, 74.50; H, 8.13. Found: C, 74.10; H, 7.76%.

4.1.22 Methyl 2-formyl-11-oxo-23-(1H-imidazole-1-carbonyloxy)-A(1)-norurs-2,12-diene-28-oate (22)

To a stirred solution of compound **14** (280 mg, 0.56 mmol) in dry THF (11 mL), CDI (184.10 mg, 1.14 mmol, 2 eq.) was added. The reaction mixture was stirred at reflux temperature under nitrogen atmosphere. After 2 hours, the reaction mixture was evaporated under reduced pressure to remove the organic phase. The obtained crude was dispersed by water (50 mL) and extracted with ethyl acetate (3 × 50 mL). The combined organic phase was washed with water (4 × 50 mL) and brine (50 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to afford a yellowish powder. The crude solid was purified by flash column chromatography (petroleum ether/ethyl acetate, 1:1) to afford **22** as a white solid (41.4 mg, 12%). Mp: 111.6–114.1 °C. ν_{max}/cm^{-1} (KBr): 3129.90, 2948.63, 2873.42, 1764.55, 1725.98, 1662.34, 1614.13, 1587.13, 1457.92, 1400.07, 1288.22, 1240.00, 1004.73. ^{1}H NMR (400MHz, $CDCl_3$): δ = 10.14 (s, 1H, CHO), 8.09 (s, 1H, H-2''), 7.30 (s, 1H, H-5''), 7.06 (s, 1H, H-4''), 6.37 (s, 1H, H-3), 5.67 (s, 1H, H-12), 4.29 (d, J = 10.50, 1H, H-23), 4.24 (d, *J* = 10.33 Hz, 1H, H-23), 3.60 (s, 3H, $COO\underline{CH_3}$), 1.24 (s, 3H), 1.13 (s, 3H), 0.95 (s, 6H), 0.84 (d, *J* = 5.41 Hz, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 198.78 (C11), 194.76 (CHO), 177.06 (C28), 164.76 (C13), 157.09 (C2), 148.56 (OCO), 142.24 (C3), 136.89, 130.90, 129.34 (C12), 116.90, 73.85 , 58.35, 56.69, 52.95, 51.85, 48.10, 47.50, 46.95, 45.68, 44.29, 38.56, 38.29, 35.77, 33.46, 30.12, 28.66, 23.77, 21.01, 20.97, 20.90, 20.20, 17.11, 17.05, 16.20 ppm. DI-ESI-MS m/z: 591.52 ([M+H]$^+$); Calcd. For $C_{35}H_{46}N_2O_6$.0.25H_2O: C, 70.62; H, 7.87; N, 4.71. Found: C, 70.30; H, 7.71; N, 4.35%.

4.1.23 Methyl 2-formyl-11-oxo-23-(2'-Methyl-1H-imidazole-carbonyloxy)-A(1)-norurs-2,12-diene-28-oate (23)

Accordingly to the method described for **22**, using compound **14** (300 mg, 0.60 mmol), dry THF (12.5 mL), and CBMI (287.20 mg, 1.51 mmol, 2.5 eq.) at reflux temperature under nitrogen atmosphere for 6 hours. The crude solid was purified by flash column chromatography (petroleum ether/ethyl acetate, 2:1→1:2) to afford **23** as a white solid (121.4 mg, 33%). Mp: 128.7–131.0 °C. ν_{max}/cm^{-1} (KBr): 3122.19, 2948.63, 2931.27, 2873.42, 1758.76, 1725.98, 1660.41, 1614.13, 1587.13, 1552.42, 1511.92, 1457.92, 1398.14, 1295.93, 1143.58. ^{1}H NMR (400MHz, $CDCl_3$): δ = 10.15 (s, 1H, CHO), 7.28 (d, *J* = 1.47 Hz, 1H, H-5''), 6.84 (d, *J* = 1.48 Hz, 1H, H-4''), 6.39 (s, 1H, H-3), 5.67 (s, 1H, H-12), 4.27 (d, *J* = 10.81 Hz, 1H, H-23), 4.20 (d, *J* = 10.81 Hz, 1H, H-23), 3.61 (s, 3H, $COO\underline{CH_3}$), 2.62 (s, 3H, CH_3 Imidazole), 1.47 (s, 3H), 1.23 (s, 3H), 1.13 (s, 3H), 0.97 - 0.95 (m, 6H), 0.85 (d, *J* = 6.30 Hz, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 198.80 (C11), 194.81 (CHO), 177.08 (C28), 164.75 (C13), 157.03 (C2), 149.47 (OCO), 147.91, 142.61 (C3), 129.39 (C12), 128.20, 117.78, 73.30, 58.38, 56.42, 52.98, 51.88, 48.07, 47.52, 46.93, 45.72, 44.32, 38.59, 38.33, 35.81, 33.48, 30.15, 28.70, 23.80, 21.06, 20.98, 20.92, 20.26, 17.13, 16.94, 16.85, 16.26 ppm. DI-ESI-MS m/z: 605.40 ([M+H]$^+$); Calcd. For $C_{36}H_{48}N_2O_6$: C, 71.50; H, 8.00; N, 4.63. Found: C, 71.16; H, 8.46; N, 4.36%.

4.1.24 Methyl 2-formyl-11-oxo-23-methanesulfonyloxy-A(1)-norurs-2,12-diene-28-oate (24)

Accordingly to the method described for **15**, using compound **14** (300 mg, 0.60 mmol), dry CH_2Cl_2 (12.50 mL), triethylamine (168.50 µL; 1.21 mmol, 2 eq.) and methanesulfonyl chloride (93.50 µL, 1.21 mmol, 2 eq.) at room temperature in anhydrous conditions for 1 h 10 min. The crude solid was purified by flash column chromatography (petroleum ether/ethyl acetate, 5:1→1.5:1) to afford **24** as a white solid (183.1 mg, 53%); Mp: 114.0–116.5 °C. ν_{max}/cm^{-1} (KBr): 2948.63, 2933.20, 2873.42, 1725.98, 1662.34, 1614.13, 1587.13, 1457.92, 1355.71, 1174.44, 958.45 cm^{-1}. ^{1}H NMR (400MHz, $CDCl_3$): δ = 10.15 (s, 1H, CHO), 6.30 (s, 1H, H-3), 5.68 (s, 1H, H-12), 4.07 (d, *J* = 10.05 Hz, 1H, H-23), 4.04 (d, *J* = 10.05 Hz, 1H, H-23), 3.62 (s, 3H, COO$\underline{CH_3}$), 3.01 (s, 3H, S-$\underline{CH_3}$), 1.46 (s, 3H), 1.33 (s, 3H), 1.09 (s, 3H), 0.97 – 0.96 (m, 6H), 0.86 (d, *J* = 6.21 Hz, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 199.04 (C11), 194.93 (CHO), 177.14 (C28), 164.93 (C13), 157.18 (C2), 141.96 (C3), 129.35 (C12), 75.09, 58.28, 56.08, 53.01, 51.89, 48.15, 47.56, 46.91, 45.75, 44.44, 38.61, 38.35, 37.55, 35.84, 33.28, 30.18, 29.69, 23.83, 21.25, 21.05, 20.95, 20.27, 17.12, 16.94, 16.06 ppm. DI-ESI-MS m/z: 575.42 ([M+H]$^+$), 597.30 ([M+Na]$^+$). Anal. Calcd. For $C_{32}H_{46}O_7S$: C, 66.87; H, 8.07; S, 5.58. Found: C, 66.48; H, 8.45; S, 5.20%.

4.1.25 Methyl 2-cyano-23-acetoxy-A(1)-norurs-2,12-diene-28-oate (25)

Accordingly to the method described for **19**, using compound **16** (200 mg, 0.42 mmol), dry THF (6 mL), acetic anhydride (43.30 µL; 0.46 mmol, 1.10 eq.) and a catalytic amount of DMAP (20 mg) at room temperature for 1 hour. The crude solid was purified by flash column chromatography (petroleum ether/ethyl acetate, 6:1→4:1) to afford **25** as a white solid (126.4 mg, 58%). Mp: 86.7–88.6 °C. ν_{max}/cm^{-1} (KBr): 2948.63, 2925.48, 2871.49, 2215.81, 1745.26, 1724.05, 1650.77, 1589.06, 1455.99, 1382.71, 1236.15, 1039.44. ^{1}H NMR (400MHz, $CDCl_3$): δ = 6.44 (s, 1H, H-3), 5.26 (t, *J* = 2.95 Hz, 1H, H-12), 3.97 (d, *J* = 11.05 Hz, 1H, H-23), 3.90 (d, *J* = 10.95 Hz, 1H, H-23), 3.60 (s, 3H, COO$\underline{CH_3}$), 2.06 (s, 3H, CO$\underline{CH_3}$), 1.26 (s, 3H), 1.11 (s, 3H), 1.05 (s, 3H), 0.94 (d, *J* = 5.92 Hz, 3H), 0.87 (d, *J* = 6.32 Hz, 3H), 0.83 (s, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 177.95 (C28), 170.88 (OCO), 152.65 (C3), 138.72 (C13), 127.03 (C2), 124.93 (C12), 117.46 (CN), 69.73, 56.16, 52.89, 52.61, 51.47, 48.99, 48.01, 43.79, 42.33, 40.88, 38.81, 38.81, 36.51, 33.42, 30.54, 28.12, 24.54, 24.06, 23.81, 21.15, 20.80, 19.02, 18.42, 17.49, 17.11, 16.19 ppm. DI-ESI-MS m/z: 522.31 ([M+H]$^+$); Calcd. For $C_{33}H_{47}NO_4$: C, 75.97; H, 9.08; N, 2.68. Found: C, 76.22; H, 9.25; N, 2.85%.

4.1.26 Methyl 2-cyano-23-butyroxy-A(1)-norurs-2,12-diene-28-oate (26)

Accordingly to the method described for **19**, using compound **16** (290 mg, 0.61 mmol), dry THF (11.6 mL), butyric anhydride (108.8 µL, 0.67 mmol, 1.10 eq.) and a catalytic amount of DMAP (30 mg,) at room temperature for 1 h 15 min. The crude solid was purified by flash column chromatography (petroleum ether/ethyl acetate, 7:1→6:1) to afford **26** as a white solid (101.1 mg, 30%); Mp: 65.5–68.0 °C. ν_{max}/cm^{-1} (KBr): 2948.63, 2927.41, 2873.42, 2215.81, 1735.55, 1652.70, 1589.06, 1455.99, 1172.51. ^{1}H NMR (400MHz, $CDCl_3$): δ = 6.44 (s, 1H, H-3), 5.27 (t, *J* = 3.0 Hz, 1H, H-12), 3.98 (d, *J* = 10.94 Hz, 1H, H-23), 3.90 (d, *J* = 10.94 Hz, 2H, H-23), 3.61 (s, 3H, COO$\underline{CH_3}$), 1.27 (s, 3H), 1.10 (s, 3H), 1.04 (s, 3H), 0.97 – 0.93 (m, 6H), 0.87 (d, *J* = 6.09 Hz, 3H), 0.83 (s, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 177.96 (C28), 173.45 (OCO), 152.81 (C3), 138.70 (C13), 126.99 (C2), 124.95 (C12), 117.47 (CN), 69.27, 55.99, 52.88, 52.60, 51.48, 49.09, 48.01, 43.76, 42.33, 40.89, 38.82 (2C), 36.52, 36.16, 33.43, 30.55, 28.13, 24.54, 24.06, 23.79, 21.15, 19.06, 18.48, 18.40, 17.44, 17.12, 16.23, 13.71 ppm. DI-ESI-MS m/z: 550.33 ([M+H]$^+$); Calcd. For $C_{35}H_{51}NO_4$: C, 76.46; H, 9.35; N, 2.55. Found: C, 76.75; H, 9.62; N, 2.58%.

4.1.27 Methyl 2-cyano-23-benzoxy-A(1)-norurs-2,12-diene-28-oate (27)

Accordingly to the method described for **19**, using compound **16** 250 mg, 0.52 mmol), dry THF(10 mL), benzoic anhydride (141.50 mg, 0.63 mmol, 1.20 eq.) and a catalytic amount of DMAP (25 mg) at room temperature for 1 h 30 min. The crude solid was purified by flash column chromatography (petroleum ether/ethyl acetate, 10:1→7:1) to afford **27** a white solid (158.2 mg, 52%). Mp: 100.2–103.1 °C. ν_{max}/cm^{-1} (KBr): 3089.40, 3060.48, 2948.63, 2925.48, 2871.49, 2215.81, 1722.12, 1602.56, 1585.20, 1452.14, 1270.86, 1112.73. ^{1}H NMR (400MHz, $CDCl_3$): δ = 8.00 (d, *J* = 7.23 Hz, 2H, H2'' and H6''), 7.58 (t, *J* = 7.34 Hz, 1H, H4''), 7.45 (t, *J* = 7.75 Hz, 2H, H3'' and H5''), 6.54 (s, 1H, H-3), 5.26 (t, *J* = 2.80 Hz, 1H, H-12), 4.24 (d, J = 11.00 Hz, 1H, H-23), 4.14 (d, *J* = 11.00 Hz, 1H, H-23), 3.61 (s, 3H, COO$\underline{CH_3}$), 1.30 (s, 3H), 1.15 (s, 3H), 1.03 (s, 3H), 0.94 (d, *J* = 5.79 Hz, 3H), 0.86 (d, *J* = 6.64 Hz, 3H). 0.84 (s, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 177.95 (C28), 166.27 (OCO), 152.76 (C3), 138.73 (C13), 133.26, 129.75, 129.54, 128.55, 127.18 (C2), 124.92 (C12), 117.45 (CN), 69.85, 56.08, 52.87, 52.67, 51.47, 49.39, 48.00, 43.88, 42.31, 40.90, 38.83, 38.78, 36.52, 33.49, 30.54, 28.09, 24.57, 24.04, 23.63, 21.11, 19.00, 18.41, 17.49, 17.12, 16.36 ppm. DI-ESI-MS m/z: 584.29 ([M+H]$^+$); Calcd. For $C_{38}H_{49}NO_4$: C, 78.18; H, 8.46; N, 2.40. Found: C, 77.80; H, 8.58; 2.52%.

4.1.28 Methyl 2-cyano-23-succinoxy-A(1)-norurs-2,12-diene-28-oate (28)

To a stirred solution of compound **16** (250 mg, 0.52 mmol) in dry CH_2Cl_2 (15 mL), succinic anhydride (130.38 mg; 1.30 mmol, 2.5 eq.) and DMAP (95.50 mg, 0.78 mmol, 1.50 eq.) were added. The reaction mixture was stirred at room temperature in anhydrous conditions. After 3 hours, the reaction mixture was evaporated under reduced pressure to remove the organic phase. The obtained crude was dispersed by water (60 mL) and extracted with ethyl acetate (3 × 60 mL). The resulting organic phase was washed with 5% aqueous HCl (2 × 60 mL), 10% aqueous Na_2SO_3 (60 mL), water (60 mL) and brine (60 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to afford a yellow powder. The crude solid was purified by flash column chromatography (petroleum ether/ethyl acetate, 3:1→1:1) to afford **28** as a white solid (213.9 mg, 71%). Mp: 105.8–107.7 °C. ν_{max}/cm^{-1} (KBr): 2948.63, 2927.41, 2871.49, 2657.43, 2559.08, 2215.81, 1743.33, 1727.91, 1712.48, 1589.06, 1455.99, 1201.43, 1162.87. ^{1}H NMR (400MHz, $CDCl_3$):

δ = 6.43 (s, 1H, H-3), 5.26 (t, *J* = 2.85 Hz, 1H, H-12), 4.01(d, *J* = 11.01 Hz, 1H, H-23), 3.92 (d, *J* = 11.01 Hz, 1H, H-23), 3.60 (s, 3H, COO$\underline{CH_3}$), 2.68-2.64 (m, 4H, H-2' and H-3'), 1.26 (s, 3H), 1.11 (s, 3H), 1.05 (s, 3H), 0.94 (d, *J* = 5.96 Hz, 3H), 0.87 (d, *J* = 6.33 Hz, 3H), 0.83 (s, 3H). ^{13}C NMR (100MHz, $CDCl_3$): δ = 178.00 (C28), 177.25, 171.89, 152.49 (C3), 138.71 (C13), 127.10 (C2), 124.91 (C12), 117.41 (CN), 70.06, 56.21, 52.88, 52.62, 51.49, 49.05, 48.02, 43.74, 42.33, 40.88, 38.80 (2C), 36.51, 33.40, 30.54, 28.76, 28.70, 28.10, 24.53, 24.06, 23.82, 21.15, 19.02, 18.41, 17.49, 17.10, 16.17 ppm. DI-ESI-MS m/z: 580.35 ($[M+H]^+$); Calcd. For $C_{35}H_{49}NO_6.H_2O$: C, 70.32; H, 8.60; N, 2.34. Found: C, 70.22; H, 8.50; N, 2.38%.

4.1.29 Methyl 2-cyano-23-cinnamoxy-A(1)-norurs-2,12-diene-28-oate (29)

Accordingly to the method described for **21**, using compound **16 (**300 mg, 0.63 mmol), dry benzene (18 mL), cynamoil chloride (416.75 mg, 2.50 mmol, 4.0 eq.) and DMAP (305.60 mg, 2.50 mmol, 4 eq) at 60 °C under nitrogen atmosphere for 1h 40 min. The crude solid was purified by flash column chromatography (petroleum ether/ethyl acetate, 3:1) to afford **29** as a white solid (189 mg, 50%) Mp: 102.3–105.0 °C. ν_{max}/cm^{-1} (KBr): 3083.62, 3060.48, 2946.70, 2925.48, 2871.49, 2215.81, 1718.26, 1637.27, 1579.41, 1496.49, 1452.14, 1162.87. ^{1}H NMR (400MHz, $CDCl_3$): δ= 7.69 (d, *J* = 16.15 Hz, 1H, H-3'), 7.54-7.52 (m, 2H, H-2''and H-6''), 7.41-7.39 (m, 3H, H-3'', H-4'' and H-5''), 6.51 (s, 1H, H-3), 6.43 (d, *J* = 16.17 Hz, 1H, H-2'), 5.27 (t, J = 2.65 Hz, 1H, H-12), 4.10 (d, J = 10.93 Hz, 1H, H-23), 4.05 (d, *J* = 10.99 Hz, 1H, H-23), 3.61 (s, 3H, COO$\underline{CH_3}$), 1.29 (s, 3H), 1.11 (s, 3H), 1.08 (s, 3H), 0.93 (d, *J* = 6.04 Hz, 3H), 0.85 – 0.84 (m, 6H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 177.95 (C28), 166.75, 152.77 (C3), 145.50, 138.72 (C13), 134.16, 130.50, 128.91 (2C), 128.17 (2C), 127.06 (C2), 124.91 (C12), 117.48 (CN), 117.42, 69.70, 56.22, 52.88, 52.66, 51.46, 49.23, 48.01, 43.81, 42.34, 40.89, 38.81, 38.77, 36.51, 33.44, 30.53, 28.11, 24.54, 24.05, 23.85, 21.13, 19.06, 18.44, 17.54, 17.08, 16.28 ppm. DI-ESI-MS m/z: 610.33 ($[M+H]^+$); Calcd. For $C_{40}H_{51}NO_4$: C, 78.78; H, 8.43; N, 2.30. Found: C, 78.82; H, 8.20; N, 2.46%.

4.1.30 Methyl 2-cyano-23-(2'-Methyl-1H-imidazole-carbonyloxy)-A(1)-norurs-2,12-diene-28-oate (30)

Accordingly to the method described for **22**, using compound **16** (280 mg, 0.58 mmol), dry THF (11 mL) and CBMI (277.55 mg, 1.46 mmol, 2.5 eq.) at reflux temperature under nitrogen atmosphere for 8 hours. The crude solid was purified by flash column chromatography (petroleum ether/ethyl acetate, 2:1→1:1) to afford **30** as a white solid (204.1 mg, 59%). Mp: 104.1–107.2 °C. ν_{max}/cm^{-1} (KBr): 3166.54, 2948.63, 2925.48, 2215.81, 1747.19, 1724.05, 1455.99, 1384.64, 1249.65. ^{1}H NMR (400MHz, $CDCl_3$): δ = 7.27 (s, 1H, H-5'), 6.87 (s, 1H, H-4'), 6.47 (s, 1H, H-3), 5.27 (t, *J* = 3.0 Hz, 1H, H-12), 4.29 (d, *J* = 11.04 Hz, 1H, H-23), 4.18 (d, *J* = 11.01 Hz, 1H, H-23), 3.60 (s, 3H, COO$\underline{CH_3}$), 2.63 (s, 3H, CH_3 Imidazole), 1.30 (s, 3H), 1.13 (s, 3H), 1.04 (s, 3H), 0.94 (d, *J* = 5.68 Hz, 3H), 0.86 (d, *J* = 6.89 Hz, 3H), 0.84 (s, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 177.94 (C28), 151.40 (C3), 149.37 (OCO), 147.98, 138.73 (C13), 128.33, 127.99 (C2), 124.76 (C12), 117.68, 117.01 (CN), 72.34, 55.96, 52.84, 52.72, 51.48, 49.22, 47.99, 43.91, 42.30, 40.90, 38.82, 38.76, 36.50, 33.45, 30.51, 28.10, 24.51, 24.02, 23.70, 21.13, 18.95, 18.39, 17.43, 17.13, 16.85, 16.20 ppm. DI-ESI-MS m/z: 588.63 ($[M+H]^+$); Calcd. For $C_{36}H_{49}N_3O_4$: C, 73.56; H, 8.40; N, 7.15. Found: C, 73.46; H, 8.79; N, 6.86%.

4.1.31 Methyl 2-cyano-23-methanesulfonyloxy-A(1)-norurs-2,12-diene-28-oate (31)

Accordingly to the method described for **15**, using compound **16** (250 mg, 0.52 mmol), dry CH_2Cl_2 (10.7 mL), triethylamine (145.40 µL; 1.04 mmol, 2 eq.) and methanesulfonyl chloride (80.65 µL, 1.04 mmol, 2 eq.) at room temperature, in anhydrous conditions for 4 hours. The crude solid was purified by flash column chromatography (petroleum ether/ethyl acetate, 4:1→3:1) to afford **31** as a white solid (179.2 mg, 62%). Mp: 109.50–112.10 °C. ν_{max}/cm^{-1} (KBr): 2948.63, 2925.48, 2871.49, 2217.74, 1722.12, 1455.99, 1359.57, 1176.36, 960.38. ^{1}H NMR (400MHz, $CDCl_3$): δ = 6.43 (s, 1H, H-3), 5.27 (t, *J* = 2.54 Hz, 1H, H-12), 4.05 (d, *J* = 9.84, 1H, H-23), 4.00 (d, *J* = 9.98 Hz, 1H, H-23), 3.60 (s, 3H, COO$\underline{CH_3}$), 3.02 (s, 3H, S-CH_3), 1.28 (s, 3H), 1.12 (s, 3H), 1.10 (s, 3H), 0.94 (d, *J* = 5.88 Hz, 3H), 0.87 (d, *J* = 6.44 Hz, 3H), 0.83 (s, 3H) ppm. ^{13}C NMR (100MHz, $CDCl_3$): δ = 177.94 (C28), 150.86 (C3), 138.79 (C13), 128.01 (C2), 124.76 (C12), 117.08 (CN), 74.09, 55.95, 52.87, 52.79, 51.47, 49.07, 48.00, 43.72, 42.38, 40.88, 38.81, 38.79, 37.56, 36.50, 33.27, 30.53, 28.10, 24.49, 24.04, 23.87, 21.15, 19.03, 18.43, 17.52, 17.20, 15.96. DI-ESI-MS m/z: 558.28 ($[M+H]^+$); Calcd. For $C_{32}H_{47}NO_5S.0.25H_2O$: C, 68.35; H, 8.51; N, 2.49; S, 5.70. Found: C, 68.55; H, 8.51; N, 2.67; S, 5.30%.

4.2 Biology

4.2.1 Cells and reagents

MCF-7, HT-29, Jurkat, PC-3, A375, MIA PaCa-2, HeLa and BJ cell lines were obtained from American Type Culture Collection (USA). Dulbecco's Modified Eagle Medium (DMEM), RPMI 1640 Medium, Dulbecco's Phosphate Buffered Saline (DPBS) and L-Glutamine were obtained from Biowest. Minimum Essential Medium (MEM), penicilin/streptomicin solution and Fetal Bovine Serum (FBS) were obtained from Gibco. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder and the XTT cell proliferation kit were purchased from Applichem Panreac. Sodium pyruvate solution 100 mM and Trypsin / EDTA were obtained from Biological Industries. Sodium bicarbonate solution 7.5% and Glucose solution 45% were purchased from Sigma-Aldrich Co.

Asiatic acid and its derivatives were suspended in DMSO at 20 mM as stock solutions that were stored at -80 °C. To obtain final assay concentrations, the stock solutions were diluted in culture medium. The final concentration of DMSO in working solutions was always equal or lower than 0.5%. Cisplatin was obtained from Sigma Aldrich.

Primary antibodies against $p21^{cip1/waf1}$ (sc-397), Bcl-2 (sc-509), Bax (sc-493), Cyclin E (sc-247) and Cyclin D_3 (sc-182) were obtained from Santa Cruz Biotechnology, inc. Primary antibodies against for $p27^{kip1}$ (#610242), Bid (#550365) and poly-(ADP-ribose)-polymerase (PARP) (#556493) were obtained from BD Biosciences. Primary antibodies against caspase 3 (#9662) and caspase 8 (#9746S) were purchased from Cell Signaling. Primary antibody against α-Actin (#69100) was obtained from MP Biomedicals. Secondary antibodies anti-

mouse (P0260) and anti-rabbit (NA934) were obtained from Dako and from Amersham Biosciences, respectively.

4.2.2 Cell culture

HT-29, PC-3, A375, MIA PaCa-2 and HeLa cells were routinely maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. MCF-7 cells were maintained in MEM supplemented with 10% heat-inactivated FBS, 0.1% penicillin/streptomycin, 2 mM L-Glutamine, 1 mM sodium pyruvate, 0.01 mg/mL of insulin, 10 mM glucose and 1x MEM-EAGLE Non Essential Aminoacids. BJ cells were routinely maintained in DMEM supplemented with 10% heat-inactivated FBS, 110 mg/L sodium pyruvate, 1% penicillin/streptomycin and 1.5 g/L sodium bicarbonate. Jurkat cells were cultivated in RPMI 1640 supplemented with 10% FBS, 1% penicillin/streptomycin and 2 mM L-Glutamine. All cell lines were incubated in a 5% CO_2 humidified atmosphere at 37 °C.

4.2.3 Cell viability assay

The antiproliferative activities of compounds against MCF-7, HT-29, PC-3, A375, MIA PaCa-2, HeLa and BJ cell lines were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, $8 \times 10^2 - 1 \times 10^4$ cells per well were plated in 96-well plates in 200 µL of medium and were left to grow. After 24h, the culture medium was removed and replaced by new medium (200µL) containing the tested compounds at different concentrations, in triplicate. After 72h of incubation, 100µL of MTT solution (0.5mg/ml) replace the supernatant in each well. Following 1h of incubation, the supernatant was removed and 100 µL of DMSO were added to each well. Relative cell viability was measured by absorbance at 550 nm on an ELISA plate reader (Tecan Sunrise MR20-301, TECAN, Salzburg, Austria).

The antiproliferative activities of compounds against Jurkat cells were determined by XTT assay. Briefly, Jurkat cells were seeded at a density of 4×10^3 cells per well in 96 well plates in 100 µL of medium. After 24h of incubation, 100 µL of medium containing the tested compounds at different concentration, in triplicate, were added. Following 72h of incubation, 100µL of XTT solution were added to each well and the plates were incubated for a further 4 hours at 37 °C. Relative cell viability was measured by absorbance at 450nm on an ELISA plate reader (Tecan Sunrise MR20-301, TECAN, Salzburg, Austria).

4.2.4 Cell cycle assay

HeLa cells were seeded at a density of 1×10^5 cells per well in 6-well plates with 2 mL of medium and incubated at 37 °C for 24h. Cells were then treated with compound **24** at the specified concentrations for 24h. Cells were harvested by mild trypsinization, centrifuged, washed twice with PBS and then stained with Tris-buffered saline (TBS) containing 50 mg/mL PI, 10 mg/mL DNase-free RNase and 0.1% Igepal CA-630 for 1 hour at 4 °C in darkness. Cell cycle was assessed by flow cytometry using a fluorescent activated cell sorter (FACS). FACS analysis was carried out at 488 nm in an Epics XL flow cytometer (coulter Corporation, Hialeah, FL). Data from 1×10^4 cells were collected and analysed using the multicycle software (phoenix Flow Systems, San Diego, CA).

4.2.5 Annexin V-FITC/PI flow cytometry assay

Apoptosis was assessed by evaluation of the annexin – V binding to phosphatidylserine (PS), which was externalized early in the process of apoptosis. HeLa cells were plated in 6-well plates at a density of 1×10^5 cells/well and incubated at 37 °C for 24h. Cells were then treated with compound **24**, at specified concentrations, for 24h. Cells were harvested by mild trypsinization, collected by centrifugation and suspended in 95µL of binding buffer (10mM HEPES/NaOH, pH 7.4, 10mM NaCl, 2.5mM $CaCl_2$). Cells were the stained with annexin V-FITC conjugate for 30 min at room temperature and protected from the light. Then, 500 µL of binding buffer were added to each vial of cells. Approximately 2 min before FACS analysis, 20 µL of 1mg/mL PI solution were added to each vial and the samples were analysed by flow cytometry. Data from 1×10^4 cells were collected and analyzed.

4.2.6 Morphology analysis by phase – contrast microscopy

In this experiment, 1×10^5 HeLa cells per well were seeded in 6-well plates with 2mL of medium and incubated at 37 °C for 24 h. Cells were then treated with compound **24** at specified concentrations for 24 h. The morphological changes were observed using a inverted phase – contrast microscope (Olympus IMT-2) with an objective of 40× and a digital camera *Fujifilm* A2O5S (*Fuji Foto Film,* CO. LTD.)

4.2.7 Hoechst 33258 Staining

HeLa cells were seeded at a density of 1×10^5 cells per well in 6-well plates with 2mL of medium and incubated at 37 °C for 24h. Cells were then treated with compound **24** at the specified concentrations for 24h. The culture medium was removed and cells were harvested by mild trypsinization, collected by centrifugation and washed twice with PBS. The cells were then stained with 500 µL of Hoechst 33258 solution (2 µg/mL in PBS) for 15 min at room temperature and protected from the light. Subsequently the Hoechst 33258 solution was removed and cells were washed twice with PBS, resuspended in 10µL of PBS and then mounted in a slide. The morphological modifications were analysed by fluorescence microscopy using a fluorescence microscope (DMRB Leica Microsystems, Weltzar, germany) with a DAPI filter.

***4.2.8* Preparation of total protein extract**

To prepare the total protein extracts, HeLa cells (5.6×10^4) were seeded in 100-mm plates and incubated at 37 °C for 24h. Cells were then treated with specified concentrations of compound **24** for 24h. Subsequently HeLa cells were washed with ice cold PBS and resuspended in ice cold lysis buffer [20mM Tris/ acetate, pH 7.5, 270 mM sucrose, 1 mM EDTA, pH 8.8, 1 mM EGTA, pH 8.8, 1% Triton X-100 and 1% protease inhibitor cocktail (Sigma- Aldrich)]. The samples were homogenized by sonication, incubated on ice for 15 min and centrifuged at 12000 rpm for 5 min at 4 °C. The protein content in supernatants was determined using the bicinchoninic acid (BCA) assay kit (Pierce Biotechnology, Rockford).

4.2.9 Western blotting

The protein extracts were separated on 10% or 15% SDS-polyacrilamide gel and then transferred to polyvinyl nitrocellulose transfer membranes (BioRad Laboratories, Richmond). Transferred membranes were blocked with Tris

Buffered saline (TBS) buffer (20 mM Tris, pH 7.5, and 132 mM NaCl) containing 0.1% Tween and 5% of BSA or nonfat dry milk during 1h at room temperature. The membranes were then incubated with a primary specific antibody overnight at 4 °C. Following incubation, membranes were washed five times (five minutes each) with TBS – 0.1% Tween and incubated with the appropriate secondary antibody for 1h at room temperature. After incubation, membranes were washed five times (five minutes each) with TBS – 0.1% Tween. The membranes were treated with Immobilon ECL Western Blotting Detection Kit Reagent (Millipore) and developed after exposure to an autoradiography film in a film cassette.

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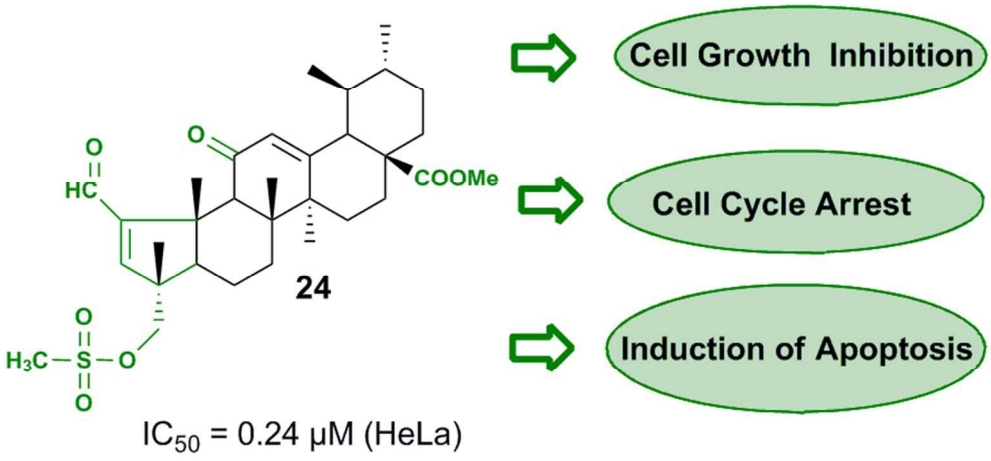

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