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# Synthesis and Cytotoxic Activity of Conjugates of Mitochondrial-Directed Cationic Compound F16 with Ursane-Structure Triterpenic Acids Containing a Polyhydroxylated A-ring †

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**Abstract:** We chemically linked corosolic and asiatic acids and a synthetic polyoxygenated analogue of ursolic acid via an alkyl linker to the cationic mitochondrial targeting compound F16 (4-(1H-indole-3-ylvinyl)-*N*-methylpyridinium iodide). The conjugates were tested for cytotoxic activity against two human lung adenocarcinoma cell lines, H1299 and A549, and non-cancerous mouse embryonic fibroblast cells. The results showed that conjugation of polyoxygenated triterpene acids with the terminal cationic fragment F16 in the C-28 side chain enhanced cytotoxicity (30–35 fold) compared to the original natural ursolic acid. However, the presence of hydroxyl or acetyl functions in the A-ring of F16 conjugates of corosolic or asiatic acids resulted in a significant decrease in cytotoxicity compared to their structural analogue, the F16 derivative of ursolic acid.

**Keywords:** ursolic acid; conjugates; polyhydroxylated A-ring; delocalized lypophilic cations; F16; anti-cancer agents



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## 1. Introduction

Pentacyclic triterpenoids of the ursane series, including ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) and its oxygenated structural analogues, corosolic and asiatic acids, are found in many medicinal plants and various fruits, berries, and vegetables (Figure 1) [1–3].

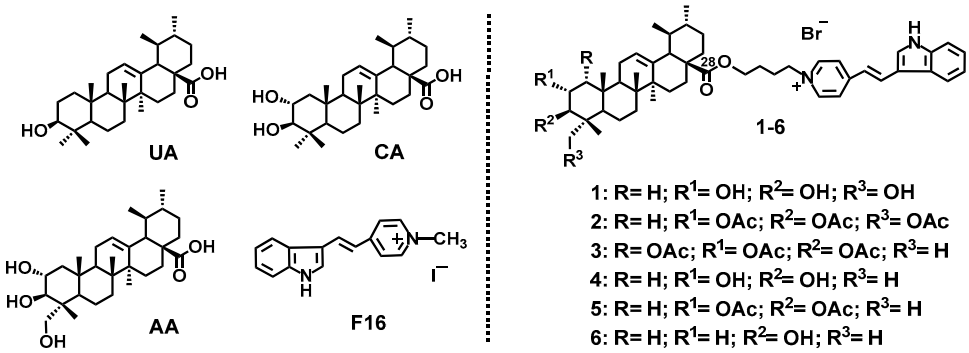



**Figure 1.** Molecular structures of ursolic acid (**UA**), corosolic acid (**CA**), asiatic acid (**AA**), **F16** ([E-4-(1H-indol-3-ylvinyl)-*N*-methylpyridinium iodide]) and F16–triterpenoid conjugates (**1**–**6**).

These phytochemicals possess a broad spectrum of beneficial biological properties, particularly their multifunctional anti-cancer activity and their capacity to trigger the mitochondrial pathway of apoptosis in a range of tumor cell types [4–9]. Furthermore, as

an integral component of the diet, these triterpenoids could be used as preventive measures against human oncological diseases. However, their high hydrophobicity limits the penetration of triterpenic acids through cell membranes, inhibiting their ability to reach the intended target and exhibit the necessary therapeutic effects in animal models. To overcome this, extensive research has been conducted over the last few decades focusing on the chemical modification of triterpenic acids, which, unfortunately, does not always yield the expected results [5,6]. For instance, their characteristic anti-tumor activity might disappear, or there might be a substantial increase in their toxicity to normal cells. Recently, to enhance the biological potential and bioavailability of pentacyclic triterpenes (such as betulin, betulinic and ursolic acids), we synthesized their derivatives that contain, at the end of the C-28 side chain, the fragment of the membranotropic delocalized cationic compound 4-(1-*H*-indol-3-ylvinyl)-*N*-methylpyridinium iodide (F16) [10]. This small cationic molecule is known for its ability to selectively accumulate in the mitochondrial matrix of various tumor cell lines [11]. These hybrid molecules demonstrated a significant synergistic enhancement of anti-tumor action against different human tumor cell lines (U937, Jurkat, K562). On the other hand, the data available in the literature today on the biological activity of oxygenated triterpenoids indicate that the configuration and quantity of hydroxyl or acetyl groups in the triterpenoid core of ursolic, corosolic and asiatic acid derivatives can significantly influence the biological activity and selectivity of these compounds concerning the tested cell lines [12–16]. For instance, natural triterpenoids with a polyhydroxylated A-ring, isolated from the plant *Euphorbia sieboldiana*, demonstrated potent anti-cancer effects on HeLa cells through the generation of active oxygen forms and blocking of the NF-κB signaling pathway [12]. Introducing two hydroxyl groups at positions C-1 and C-2 of the ursolic acid triterpenoid core significantly amplified its antibacterial activity and altered the mechanism of antibacterial action of these triterpenoids [13]. Benzylamide derivatives of asiatic acid have demonstrated potent anticancer activity against the HL-60 cell line ($IC_{50}$ = 0.47 μM) while exhibiting no cytotoxicity against normal human endothelial HUVEC cells [14]. Among a large series of conjugates of pentacyclic triterpenoids with rhodamine B, asiatic acid has emerged as a leading structure for the development of anticancer, mitochondria-targeted agents, displaying high cytotoxic activity coupled with high selectivity and the ability to overcome drug resistance. The potential of asiatic acid derivatives was further confirmed in preclinical models of human tumors [15,16].

Given these promising results, it was of interest to synthesize and explore the anticancer effects of F16 derivatives of asiatic acid **1** and **2** and the tri-hydroxylated analogue of ursolic acid **3**. The cytotoxicity of these hybrid compounds was tested by us on the lung adenocarcinoma cell lines A549 and H1299 and benign mouse embryonic fibroblasts MEFs, compared with the previously described F16 derivatives of ursolic and corosolic acids **4**–**6**. Ursolic acid was used as a reference compound.

## 2. Materials and Methods

### *2.1. Chemistry*

Ursolic, oleanolic and asiatic acids were purchased from Acros Organics (Geel, Belgium). Corosolic acid was obtained from ursolic acid as previously described followed by chromatographic purification of the crude product [17]. Acetates of asiatic and corosolic acids were easily synthesized by employing the well-established method [8]. Polyhydroxy derivative of ursolic acid **12** was synthesized according to the method in [13].

The bromalkyl esters **8**, **9**, **13**, **17**, **18** and conjugates **1**–**5** were synthesized as previously described [18,19]. The physico-chemical and spectral characteristics of the compounds were in full agreement with the data presented in [19].

General Procedure for the Synthesis of the Conjugates **1**–**5**

A solution of the corresponding triterpenoic acid (488.7 mg, 1.0 mmol), $K_2CO_3$ (138 mg, 1.0 mmol) and 1,4-dibrombutane (0.48 mL, 4.0 mmol) in dry DMF-$CH_3CN$ (3:1, 8 mL) was stirred at 50 °C for 3 h. The reaction mixture was poured into water (40 mL) and extracted

with $CHCl_3$. The organic layer was washed with $H_2O$ and dried over $MgSO_4$. The residue after evaporation of the solvent in vacuo was subjected to column chromatography ($SiO_2$, hexane/EtOAc 30:1 → 1:1) to yield bromalkyl esters **8**, **9**, **13**, **17** and **18,** each as a white powder. Yield: 68–88%.

The resulting compounds **8**, **9**, **13**, **17** and **18** were reacted with 4-(1H-indole-3-ylvinyl)-N-methylpyridine by refluxing in DMF at 85 °C in an argon atmosphere for 12 h. The mixture was cooled to room temperature and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel, using $CH_2Cl_2$/MeOH 30:1 → 10:1, to give pure compounds **1**–**5** as brown powders. Yield: 78–82%.

### *2.2. Biology*

#### 2.2.1. Cell Lines and Culture Conditions

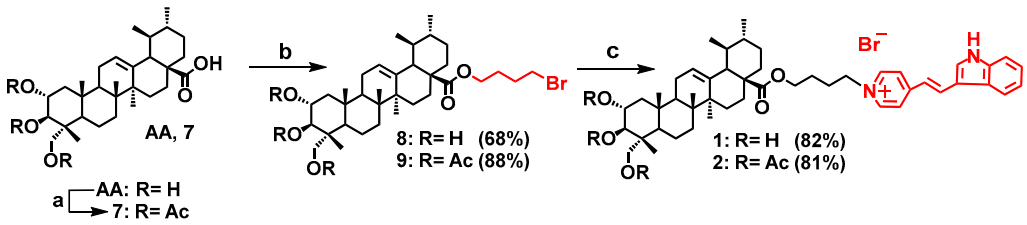
All cell lines used in this study (human non-small lung adenocarcinoma—H1299 and A549, mouse embryonic fibroblasts—MEFs) were purchased from ATCC. Cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 µg/mL gentamycin and 2 mM of glutamine at 37 °C in a 5% $CO_2$ atmosphere.

#### 2.2.2. Cytotoxic Assay of Conjugates (MTT Assay)

To carry out the MTT assay, 3500 cells per were well planted overnight in 96-well plates. A day after the seeding, conjugates **1**–**6** dissolved in DMSO were added in the different concentrations. DMSO was used as a control. After 48 h, 10 µL of 5 mg/mL Thiazolyl Blue (Paneko, Russia) solution was added to each well and cells were kept for 3 h at 37 °C in a $CO_2$ incubator. After removing the thiazol-containing medium, 150 µL of isopropyl alcohol (supplemented with 40 mM HCl and 0.1% NP-40) was added to dissolve the MTT-formazan salt. The absorbance at 570 and 630 nm (reference) was measured using BioRad iMark microplate reader (BioRad, Hercules, CA, United States). Three biological replicates were used for the experiment. The results were processed in Excel software (version number: 14.0.6023.1000 (32-bit)). $IC_{50}$ values were calculated by using the AAT Bioquest online calculator (https://www.aatbio.com/tools/ic50-calculator, accessed on 15 May 2023) and are represented as the mean ± SD.

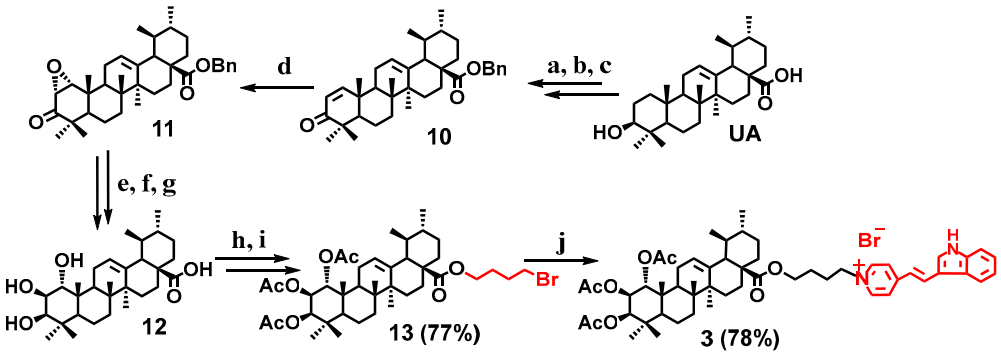
## 3. Results and Discussion

F16 conjugates with asiatic acid **1** and **2** were synthesized from available asiatic acid, which was transformed into acetate **7.** Next, triacetate **7** and the starting asiatic acid were converted into bromoalkyl esters **8** and **9** in an alkaline medium using a fourfold molar excess of 1,4-dibromobutane. The reaction of esters **8** and **9** with (E)-4-(1*H*-indol-3-ylvinyl)pyridine in DMF at 85 °C gave conjugates **1** and **2** in 81–82% yield after purification by column chromatography on silica gel (Scheme 1).




**Scheme 1.** Synthesis of conjugates **1** and **2**: **a** AcCl, THF, pyridine, DMAP, rt; **b** 1,4-dibromobutane, $K_2CO_3$, $CH_3CN$, DMF, 50 °C; **c** (E)-4-(2-(1*H*-indol-3-yl)vinyl)pyridine, DMF, 85 °C, 12 h.

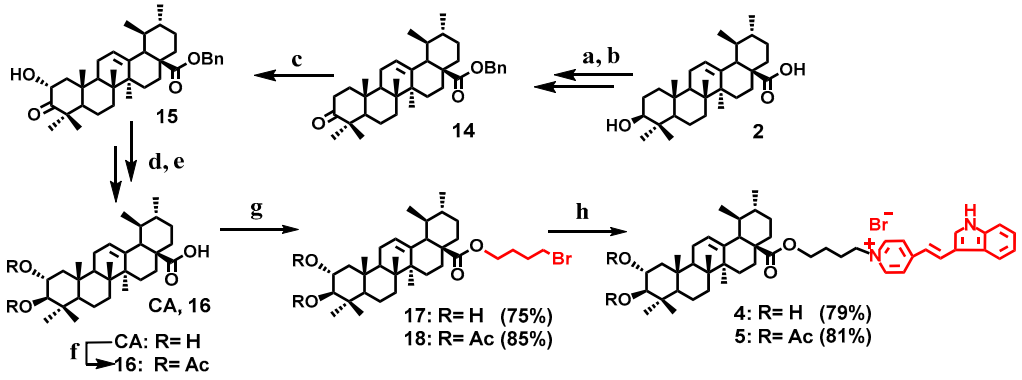
The starting compound for the synthesis of hybrid molecule **3** was triterpenic acid **12** with a tri-hydroxylated A-ring, which was obtained from available ursolic acid according to a previously developed multistage scheme (Scheme 2) [13].




**Scheme 2.** Synthesis of conjugate **3**: **a** $CrO_3$, $H_2SO_4$, acetone, 0 °C; **b** BnCl, $K_2CO_3$, DMF, 50 °C, 2 h; **c** PhSeCl, EtOAc, *m*CPBA, pyridine, rt; **d** 30 % $H_2O_2$, 10% NaOH, MeOH, rt; **e** $NaBH_4$, MeOH–THF, rt; **f** $HClO_4$, $H_2O$, acetone, rt; **g** Pd-C/10%, $H_2$, MeOH–THF, rt; **h** AcCl, THF, pyridine, DMAP, rt; **i** 1,4-dibromobutane, $K_2CO_3$, $CH_3CN$, DMF, 50 °C; **j** (E)-4-(2-(1*H*-indol-3-yl)vinyl)pyridine, DMF, 85 °C, 12 h.

The synthesis involved the production of ursolic acid derivative **10**, containing an enone fragment in the A-ring, and stereoselective epoxidation of the enone double bond using 30% $H_2O_2$ in an alkaline medium, resulting in 1α,2α-epoxyketone **11** as the only product. Interaction of epoxyketone **11** with $NaBH_4$, stereoselective opening of the epoxy ring using $HClO_4$ in acetone, and subsequent removal of the benzyl protection yielded a polyoxygenated analogue of ursolic acid **12** with a 1α,2β,3β-trihydroxylated A-ring. Further modification of acid **12** involved acetylation of hydroxyl groups, etherification of the carboxyl function using an excess of 1,4-dibromobutane in a $K_2CO_3$/DMF solution, and interaction of the bromoalkyl ether **13** with the neutral **F16** precursor (Scheme 2).

The corosolic acid used in the synthesis of conjugates **4** and **5** was derived from ursolic acid using a method based on the stereoselective electrophilic attack of *m*-chloroperbenzoic acid in the presence of $H_2SO_4$ on the C-2 atom of the benzyl ester of ursonic acid **14** (Scheme 3) [17].




**Scheme 3.** Synthesis of conjugates **4** and **5**: **a** $CrO_3$, $H_2SO_4$, acetone, 0 °C; **b** BnCl, $K_2CO_3$, DMF, 50 °C, 2 h; **c** *m*CPBA, $H_2SO_4$, MeOH–$CH_2Cl_2$, 0 °C; **d** $NaBH_4$, MeOH–THF, rt; **e** Pd-C/10%, $H_2$, MeOH–THF, rt; **f** AcCl, THF, pyridine, DMAP, rt; **g** 1,4-dibromobutane, $K_2CO_3$, $CH_3CN$, DMF, 50 °C; **h** (E)-4-(2-(1*H*-indol-3-yl)vinyl)pyridine, DMF, 85 °C, 12 h.

The reduction in the 3-keto function in 2α-hydroxyketone **15** with $NaBH_4$ predominantly resulted in the desired 2α,3β-diol, which, after chromatographic isolation on a column with $SiO_2$ and the removal of benzyl protection, yielded the required corosolic acid. Further transformations of corosolic acid and its acyl derivative **16** into bromoalkyl esters **17** and **18** and the synthesis of conjugates **4** and **5** were conducted under conditions previously described for compounds **1**–**3**.

The structures of all compounds were determined using IR, $^1$H NMR, $^{13}$C NMR, and MS spectra. The physicochemical and spectral characteristics of asiatic, corosolic, and

polyhydroxylated analogues of ursolic acid, as well as their acetates, conformed to the literature data [8,13,17,19]. Moreover, the relative configuration of the epoxy ring and the orientation of hydroxyl functions in ring A in compounds **10**–**13** and **15**–**18** were confirmed using DEPT, HMBC, HSQC, COSY and NOESY experiments.

The synthesized conjugates **1**–**5** were examined for cytotoxic activity against two human lung adenocarcinoma cell lines H1299 and A549 and non-cancerous MEFs (mouse embryonic fibroblasts). The cytotoxicity of the previously synthesized ursolic acid **F16**-derivative **6** was also examined on these cell lines for comparison (Table 1) [18].

**Table 1.** Cytotoxic activity of **F16** conjugates with triterpenoids **1**–**6** against human lung adenocarcinoma H1299 and A549 and non-cancerous mouse fibroblasts MEFs: $IC_{50}$ values from MTT tests after 48 h treatment are given in μM.

| No. | H1299 | A549 | MEF | SI [1] | SI [2] |
|---|---|---|---|---|---|
| **1** | 13.51 ± 1.81 | 9.03 ± 0.59 | 20.04 ± 6.73 | 1.48 | 2.21 |
| **2** | 8.51 ± 2.05 | 3.80 ± 1.86 | 5.85 ± 0.22 | 0.68 | 1.54 |
| **3** | 3.57 ± 0.20 | 4.81 ± 2.15 | 3.79 ± 0.32 | 1.06 | 0.79 |
| **4** | 4.01 ± 1.69 | 1.87 ± 0.14 | 6.76 ± 1.58 | 1.69 | 3.62 |
| **5** | 3.58 ± 0.30 | 3.04 ± 0.34 | 5.79 ± 0.49 | 1.62 | 1.91 |
| **6** | 2.80 ± 0.25 | 2.40 ± 0.30 | n.d. | – | – |
| **UA** | 97.60 ± 5.63 | 68.93 ± 19.63 | 50.39 ± 16.92 | 0.51 | 0.74 |

[1,2] The selectivity indices (SI) are defined as SI = $IC_{50}$ (MEF)/$IC_{50}$ H1299 or SI = $IC_{50}$ (MEF)/$IC_{50}$ A549.

From the results presented in the table, it is evident that introducing a terminal cationic fragment into the C-28 side chain of the examined triterpenic acids resulted in significant enhancement of cytotoxicity compared to ursolic acid, regardless of the presence of acetylated or free hydroxyl groups in the A-ring. In particular, the **F16** conjugate with corosolic acid **4** was 24 and 36 times more effective than the original ursolic acid against the A549 and H1299 tumor cell lines, respectively, while the **F16** derivative of acetylated corosolic acid **5** exceeded the anti-tumor effect of ursolic acid by 23–27 times. However, the additional presence of hydroxyl or acetyl functions in the A-ring led to some reduction in the cytotoxic effect compared to the **F16** derivative of ursolic acid, contrary to known facts in the literature [14–16]. For instance, regarding the H1299 tumor cell line, the cytotoxicity $IC_{50}$ values for conjugates **1**, **4**, and **6** were, respectively 13.51 μM, 4.01 μM, and 2.80 μM. Natural ursolic acid and the studied conjugates did not show selectivity against tumor cells and mouse fibroblasts except for the **F16** derivative of corosolic acid **4**, which demonstrated a selectivity difference between tumor and healthy cells with a selectivity index in the range of 1.7–3.6.

## 4. Conclusions

In the present work, we have demonstrated the cytotoxic effects of conjugates of the membrane-penetrating cation **F16** with several polyoxygenated triterpenoids of the ursane structure on lung adenocarcinoma tumor lines A549 and H1299 and on healthy mouse fibroblast cells MEFs. All synthesized conjugates surpassed natural ursolic acid in terms of cytotoxic action by many times. Among the hybrid compounds, the **F16** derivative of corosolic acid, along with a high antitumor effect, showed a selectivity index between tumor and healthy cells in the range of 1.7–3.6.

**Author Contributions:** Validation and writing—review and editing, A.S.; performing the chemistry experiments, D.N. and E.D. The manuscript was prepared through the contributions of D.N. and E.D. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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