Cytotoxic Triterpenoids from the Leaves of *Euphorbia pulcherrima*

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Abstract: Two cytotoxic triterpenes have been isolated from *Euphorbia pulcherrima*. Their structures and stereochemistry have been established from NMR, IR, and EI-mass spectroscopy. The compounds were identified as 9,19-cycloart-23-ene-3β,25-diol and 9,19-cycloart-25-ene-3β,24-diol. Cytotoxicity evaluation was performed using Ehrlich ascites tumor cells. While cycloartenol induced no cytotoxic activity against Ehrlich ascites tumor cells, both isolated triterpenes exhibited cell inactivating effects. The IC_{50} is approximately 7.5 μM, while the IC_{90} is approximately 13.5 μM for 9,19-cycloart-25-ene-3β,24-diol. The 3β,25-diol compound is 50 % less active.

Key words: Triterpenes, cycloartenol, *Euphorbia pulcherrima*, Euphorbiaceae, cytotoxicity, Ehrlich ascites.

Introduction

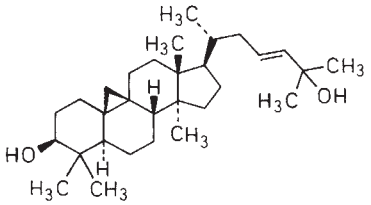
The use of natural products in the treatment of disease has been an important component of medical therapy for centuries. Plants of the family Euphorbiaceae have been used to treat cancers, tumors, and warts for hundreds of years, and references to their use have appeared in the literature of many countries (1).

We report here the isolation and characterization of two stereoisomeric cyclic triterpenes **1** and **2** from leaves of *Euphorbia pulcherrima* Willd. Their synthesis from cycloartenol is also described. The isolated triterpenes show significant inhibitory activity against Ehrlich ascites tumor cells in culture. As the content of these triterpenoids in *E. pulcherrima* is very small (about 0.05 parts per thousand), it was necessary to find other sources. From *Artocarpus integrifolia* Forst. (2) we have isolated the same triterpenes in small quantities and we have also isolated cylcoartenone and cylcoartenol.

Materials and Methods

Plant material

Leaves of *Euphorbia pulcherrima* were obtained from a local plant nursery. A voucher specimen (ISK/0993/1) has been deposited in the Department of Pharmacognosy, Institute of Pharmacy, University of Oslo.



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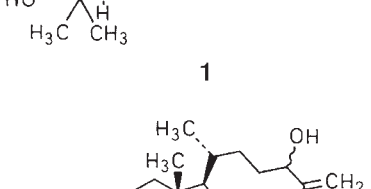

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Cell culture

Ehrlich ascites tumor cells, originally isolated from murine cancer mammae were adapted to suspension culture and kept in exponential growth by daily dilution with Eagle's minimum essential medium with Earle's salts supplemented with 10 % heat-inactivated fetal calf serum, 100 IU/ml streptomycin-penicillin, and HEPES buffer (pH 7.3) to a final concentration of 15 mM, and non-essential amino acids (all from Gibco, Paisley, Scotland). Cells were exposed to drugs for 24 hours at 37 °C with appropriate controls. Cell growth was evaluated from cell counts using a hemocytometer, and the number of living cells was determined by the Trypan blue dye exclusion test. The cells were subcultured at a density of 2×10^5 cells/ml in 5 ml medium. Cholesterol and mevalonic acid used in drug combination studies were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell cytotoxicity assay

Ehrlich ascites tumor cells were seeded into separate flasks at a cell density of 2×10^5 cells/ml. Compounds dissolved in 96 % ethanol were added to give desired concentrations. The ethanol concentration did not exceed 0.5 % in any experiment.

Cells were incubated with compounds for 24 h in an orbital shaker incubator holding 37 °C. After incubation aliquots were removed and cell toxicity evaluated by differential counting of trypan blue stained cells.

Chromatography

For medium pressure chromatography (MPLC), a Büchi MPLC column, 4.9 × 92 cm filled with silica gel (230–400 mesh, Merck, Darmstadt, Germany) and coupled to a Büchi B-681 pump (Büchi, Flawil, Switzerland) was employed. Centrifugally accelerated radial chromatography was performed on a Chromatotron model 7924T (Harrison Research, Palo Alto, CA, USA) using 2-mm layers of silica gel PF_{254} (Merck). For HPTLC, silica gel $60F_{254}$ plates (Merck) were used, and spots were visualized by spraying with anisaldehyde reagent (18 : 1 : 1 anisaldehyde : H_2SO_4 : CH_3COOH), 6 % in ethanol.

Extraction and purification

Air-dried leaves of *E. pulcherrima* (300 g) were powdered and percolated at room temperature with 3 l petroleum ether (bp 60–80 °C) for 24 hours. Concentration of the extract in vacuo yielded 18 g of residue. Of this, 13 g were dissolved in toluene, filtered, and chromatographed (MPLC) with petroleum ether (2 l), toluene (2 l), toluene + 5 % ethyl acetate (4 l), toluene + 7 % ethyl acetate (4 l), and 1 l portions of 10 %, 12.5 %, 15 %, 20 %, and 40 % ethyl acetate in toluene. Fractions of 250 ml were collected, and the cytotoxicity of the fractions was monitored by tests using Ehrlich ascites tumor cells in suspension culture. Three cytotoxic fractions were collected: A (86.5 mg with 10 % ethyl acetate in toluene), B (45 mg with 12.5 % ethyl acetate in toluene) and C (210 mg with 20 % ethyl acetate in toluene).

The active fractions were further purified by centrifugally accelerated radial chromatography with hexane-ethyl acetate (6 : 1, v/v) as eluent. Fifteen fractions of 10–15 ml each were collected and monitored by HPTLC (hexane-ethyl acetate, 2 : 1). The fractions were also tested for cytotoxicity.

Four of the fractions obtained from A showed significant cytotoxic activity. The most active of these fractions crystallized on evaporation of solvent (6.7 mg) and showed one spot on HPTLC (R_f = 0.46). The substance was identified as the triterpenoid 9,19-cycloart-25-ene-3β,24-diol (**2**) by comparison of the spectral data of the substance and its acetate (^{1}H-NMR, ^{13}C-NMR, IR, EI-mass) with the literature values (3–6).

Among the fractions from C, 6 showed significant cytotoxic activity. The most active fraction was crystallized by evaporation (9.1 mg) and showed one spot on HPTLC (R_f = 0.37). The substance was similarly identified as 9,19-cycloart-23-ene-3β,25-diol (**1**).

MPLC fraction B contained a mixture of these two compounds.

Synthesis of **1** *and* **2**

Cycloartenol: Reduction of cycloartenone (7.53 g) with $LiAlH_4$ in ether gave the α- and β-epimers in a 26 : 74 ratio, respectively, according to the ^{1}H-NMR spectrum showing the C3-H as two well separated double doublets. The assignment of the β-isomer was based on comparison of both the ^{1}H- and ^{13}C-NMR spectra in the literature (7). The other product formed was assumed to be the α-epimer since it formed cycloartenone upon oxidation. Flash chromatography (silica gel, 230–400 mesh, 80 g : 4 g crude product, hexane-ethyl acetate 8 : 2) gave the pure β-isomer. In a separate experiment, it was found much easier to separate the two epimers as their acetates. ^{1}H-NMR (270 MHz, $CDCl_3$) of cycloartenol: δ = 3.28 (dd, J_1 = 4.4, J_2 = 10.9 Hz, C3-H, β-epimer), 3.48 (dd, J_1 = 6.9, J_2 = 13.8 Hz, C3-H, α-epimer). Yield: 5.08 g, 67 % based on starting cycloartenone, 91 % based on separated α-epimer. The latter epimer could be recycled by oxidation followed by a new reduction.

Cycloartenol acetate was made in quantitative yield using pyridine and acetic anhydride. Flash chromatography (silica gel, 230–400 mesh, 80 g : 6 g crude acetate, hexane-ethyl acetate 9 : 1) gave the pure product.

Cycloart-25-ene-3β,24-diol and *cycloart-23-ene-3β,25-diol* were made in a modification of a previous synthesis (8). To a solution of 5.58 g (11.9 mmol) cycloartenyl acetate in 450 ml pyridine was added 1.30 g hematoporphyrine. The solution was kept at room temperature by means of a water bath and oxygen was bubbled through via a glass sinter. The mixture was irradiated using a 250 W immersion high pressure mercury lamp with a pyrex filter. The reaction was monitored by TLC (silica gel, hexane-ethyl acetate, 97.5 : 2.5) and was stopped when all starting material had disappeared (2 h). Most of the pyridine was evaporated on a rotary evaporator (not to dryness), 400 ml absolute ether was added and the product reduced by the portionwise addition of 3.46 g (91.2 mmol) $LiAlH_4$. Stirring for 2 h at room temperature followed by the successive addition of 3.5 ml water, 3.5 ml 15 % NaOH (aq) and 10.5 ml water with vigorous stirring, filtering and evaporation gave a dark oil. According to ^{1}H-NMR of the crude mixture, the two compounds had formed in a 35 : 65 ratio, respectively. Flash chromatography (silica gel, 230–400 mesh, 80 g : 3 g crude product, hexane-ethyl acetate 75 : 25) separated the two compounds. Spectroscopic and physical data were in accord with the literature (3–6).

Results

Cell inactivating effect

The isomeric compounds 9,19-cycloart-23-ene-3β,25-diol (**1**) and 9,19-cycloart-25-ene-3β,24-diol (**2**) were dissolved in ethanol. The cytotoxic activity was assayed using three different concentrations (Fig. **1**). Both isolated triterpenes exhibited cell inactivating effects. The IC_{50} is approximately 7.5 μM, while the IC_{90} is approximately 13.5 μM for 9,19-cycloart-25-ene-3β,24-diol. The 3β,25-diol compound is 50 % less active. Acetylation of 9,19-cycloart-23-ene-3β,25-diol did not change the activity of the substance, whereas acetylation of 9,19-cycloart-25-ene-3β,24-diol resulted in inactivation of the substance (Fig. **1**, open circles).

The triterpenes probably arise biogenetically by oxidation of cycloartenol which is an intermediate in the biosynthesis of plant steroids, and is a common constituent of *Euphorbia* (9). Cycloartenol showed no activity against Ehrlich ascites tumor cells up to a concentration of 100 μg/ml (Fig. **1**, open triangles).

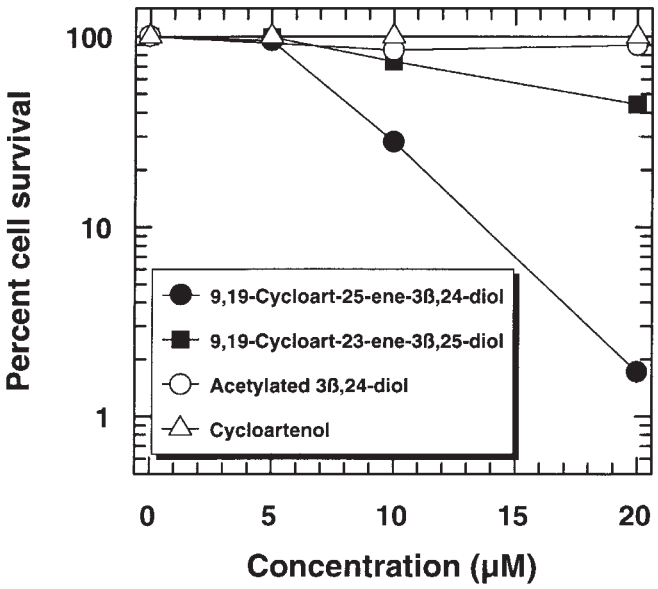

Fig. 1 The effect of 9,19-cycloart-23-ene-3β,25-diol (■) or 9,19-cycloart-25-ene-3β,24-diol (●) on cell survival of Ehrlich ascites tumor cells. Cells were grown in suspension and cell densities assayed following 24 hour drug treatment. Additionally, the effect of acetylation of 9,19-cycloart-25-ene-3β,24-diol (○) and cycloartenol (△) on cell survival of Ehrlich ascites tumor cells was also investigated following 24 hour drug treatment.

Irreversible effect of 9,19-cycloart-25-ene-3β,24-diol

Ehrlich ascites tumor cells were treated for 4 hours, the cells were centrifuged, washed with PBS, and finally resuspended in medium for a further 20 hour incubation (Fig. **2**). The results show that the effect of 9,19-cycloart-25-ene-3β,24-diol is irreversible.

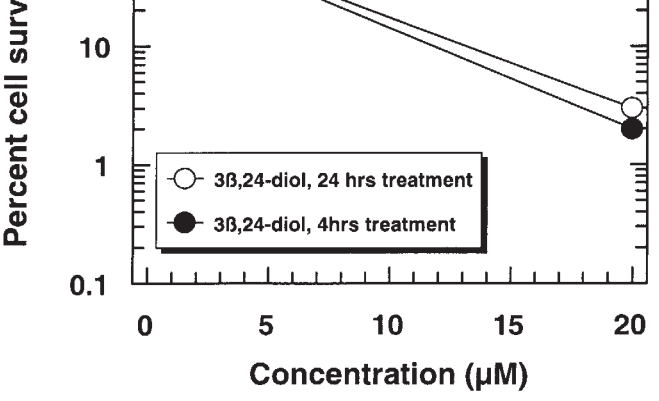

Fig. 2 The effect of varying treatment times of 9,19-cycloart-25-ene-3β,24-diol on cell survival of Ehrlich ascites tumor cells. Cells growing in suspension were treated with drug for either 24 h (○) or for 4 h (●) with a subsequent wash and resuspension in fresh medium for an additional 20 h.

Effects of cholesterol and mevalonic acid

As indicated above, the cytotoxic effect of 9,19-cycloart-25-ene-3β,24-diol is irreversible. Neither addition of cholesterol up to 15 μM nor mevalonic acid (1 mM) reversed cytotoxicity induced by the cycloartenol derivative (Table **1**).

Table 1 Survival of Ehrlich ascites tumor cells incubated for 24 h with cholesterol or mevalonic acid in combination with 9,19-cycloart-25-ene-3β,24-diol relative to survival following treatment with 9,19-cycloart-25-ene-3β,24-diol alone. Survival following treatment with 3β,24-diol alone was 10.7 %.

3β,24-diol alone	Cholesterol			Mevalonic acid
10 μM	5 μM	10 μM	15 μM	1 mM
1	0.86 ± 0.06	0.99 ± 0.04	0.94 ± 0.04	0.94 ± 0.11

Discussion

Our results show that the stereoisomeric triterpenes isolated from the leaves of *Euphorbia pulcherrima* are cytotoxic towards Ehrlich ascites tumor cells. These tetracyclic triterpenes have not earlier been isolated from this genus, but from other *Euphorbia* species, such as *E. cyparissias* and *E. antiquorum* (10, 11).

Valisolalao et al. (12) have isolated 9,19-cycloart-25-ene-3β,24-diol from the fungal Chinese drug *Poria cocos*. They found no biological activity against HTC (cultured hepatoma) cells, however. It has been reported (13) that the same substance shows antibacterial activity towards *Staphylococcus aureus* and *Escherichia coli*.

Acetylation of the diol 9,19-cycloart-23-ene-3β,25-diol (**1**) does not result in the formation of a 25-acetoxy group (14) as indicated by the NMR spectra of the diol and its monoacetate. The other diol, 9,19-cycloart-25-ene-3β,24-diol (**2**), is isomeric with the former, but in contrast it readily formed a diacetate.

The cytotoxic effect of substance **1** did not change after acetylation in contrast to substance **2** which lost its cytotoxic effect after acetylation (Fig. **1**). From this it can be inferred that the OH group in the 3-position is not essential for the cytotoxic effect. The cytotoxic effect of the cycloartenol derivative 9,19-cycloart-25-ene-3β,24-diol is irreversible (Fig. **2**) and the effect cannot be reversed by cholesterol or mevalonic acid (Table **1**). This implies that the primary effect of the substance cannot be on HMG-CoA reductase. This is in contrast to the report of Defay et al. (15) who found 100% inhibition of HMG-CoA reductase from human lymphocytes.

Cycloartenol has previously been identified as the main triterpene alcohol from latex of *E. pulcherrima* (16). Cycloartenol, the putative precursor of **1** and **2**, induced, as expected, no cytotoxic activity.

Further experimentation to elucidate the mechanism and specificity of the cytotoxic effect induced by 9,19-cycloart-25-ene-3β,24-diol is currently in progress.

Acknowledgements

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