Cycloartanes from *Euphorbia aellenii* Rech. f. and their Antiproliferative Activity

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Abstract

The cytotoxic chloroform fraction of *Euphorbia aellenii* afforded two cycloartane type triterpenes-cycloart-25-en-3β,24-diol (1) and 24-methylene-cycloartan-3β-ol (2)-for the first time from this plant. Preparation of cycloartane derivatives, 3β,24-O-diacetyl-cycloart-25-en as compound 3 and 3β-O-acetyl-24-methylene-cycloartan (4) were conducted by acetylation of 1 and 2, respectively. The structures of the isolated compounds were elucidated by spectroscopic methods and their activities evaluated by proliferation assay on human peripheral blood lymphocytes (PBLs). Comparing the results suggested that anti-proliferation effect of these compounds on PBLs might be due to the presence of free 3-OH group while masking the free OH groups by acetylation, could induce proliferation activity.

Keywords: *Euphorbia aellenii*; Proliferation assay; Cycloartanes; Structure-activity relationship (SAR); Protein kinase C (PKC).

Introduction

Cycloartanes (9, 19-Cyclolanostanes) are one of the main tetra-cyclic triterpene skeletons including the side chain and characteristic cyclopropane ring (1). They are the major key intermediates in the phytosterols biosynthesis (1). In addition, these compounds are used as specific chemotaxonomic markers in *Euphorbia* genus, which comprises well over 2000 species in tropical and temperate zones of asia and other parts of the world. In iran, 70 species are reported, 17 of which are endemic (2). Within our recent study on triterpenoids, present work describes the isolation and structure elucidation of two cycloartane type triterpenes from *Euphorbia aellenii* Rech. f. (Figure 1) along with their anti-proliferative activity on human peripheral lymphocytes and their structure-function studies on protein kinase C (PKC) that plays an important role, as an early event, in
T-cell activation (3).

**Experimental**

**General**

Column chromatographies (CC) were run by silica gel 63-200 µm, LiChroprep® Si 60 (25-40 µm) and RP-18 (40-63 µm, Merck). The size exclusion chromatography (SEC) was rendered on sephadex (LH-20, Sigma-Aldrich) and recycling preparative HPLC on LC-908 (Hitachi Company, Japan), equipped with YMC Pack-Sil column (250×20 mm i.d). The NMR spectra were recorded on Bruker AV-300 (1H), AV-600 (13C) and HR-ESI-MS on Waters Q-TOF Micro YA019 mass spectrometer.

**Plant material**

The aerial flowering parts of *Euphorbia aellenii* Rich. F. (euphorbiaceae) were collected from populations growing in Galil-e-Shirvan (Iran) and identified by Dr. Yasamin Naseh, herbaceous sciences research center at Ferdowsi University of Mashhad. A herbarium specimen No. 2024 is preserved in the herbarium of the faculty of pharmacy, Isfahan University of Medical Sciences (Iran).

**Isolation of cycloartanes**

The air-dried powdered plant (7 Kg) was macerated for four days with MeOH (20 L×3), at room temperature. Filtration and *in-vacuo* evaporation resulted in a green gum (500 g), which was partitioned between methanol and n-hexane. The defatted methanolic extract was concentrated, dissolved in water, and extracted sequentially with chloroform, ethyl acetate, n-butanol, and water. The resulted fractions (Fr.1-Fr.4) were compared *in-vitro* for cytotoxic activity (4), showing LD$_{50}$ value of 177.06 (96.90-255.61) and 770.66 (670.06 - 916.40) μg mL$^{-1}$ for Fr.1 and Fr.2, respectively while other fractions (Fr.3 and Fr.4) were marked as non-cytotoxic. The most active fraction (Fr.1, 240 g) was subjected to silica gel column, using Hexane/CHCl$_3$ (0 → 100), to render several fractions (Fr.1a-Fr.1f). Then, Fr.1b was eluted with Hexane/CHCl$_3$ 1:1 and chromatographed on silica gel (Hexane/EtOAc, 0 → 50) to come up with ten subfractions. Finally, 1 g of the fraction was eluted with Hexane/EtOAc 9:1, purified on recycling HPLC (Hexane/EtOAc 6:4, 4.0 mL/min), and consequently yielded two pure compounds: 1 (20 mg, $t_R$ 200 min) and 2 (10 mg, $t_R$ 220 min).

**Preparation of cycloartane derivatives**

A mixture of cycloartane type compound (10 mg), acetic anhydride (1 mL) and pyridine (0.5 mL) were stirred at room temperature for three days. The final point of reaction was controlled by TLC and the reaction mixture was poured over ice to decompose the remaining acetic anhydride. Thereafter, suspension was extracted twice with
ethyl acetate (2:1 v/v), and evaporated **in-vacuo**
to yield acetylated products (3 and 4), which was
confirmed by Mass and H-NMR spectra (5).

**Brine shrimp (artemia salina) cytotoxicity**
Shrimp eggs were added to a specific tank
containing artificial seawater, hatched within two
days and transferred to sample vials (1000, 100
and 10 μg/mL). 24 h later, keeping vials under
illumination, the surviving shrimps were counted
to obtain LC\textsubscript{50}, and 95% confident intervals (4).

**Proliferation assay**
Peripheral human blood lymphocytes were
incubated with different concentrations of
the test compounds (0.5, 5, and 50 µg/mL (in
triplicates) in supplemented RPMI-1640 along
with phytohemagglutinin (PHA) at 37°C in CO\textsubscript{2}
environment for 72 h. Further incubation for 18 h
after the addition of thymidine [\textsuperscript{3}H] (Amersham,
UK) was done and cells were harvested using
cell harvester (Innotech Dottikon, Switzerland).
Finally, proliferation level was determined by
the radioactivity count as CPM recorded from
the Beta-scintillation counter (Beckman coulter,
LS 6500, Fullerton, CA, USA) (6).

**Docking methodology**
Using the crystal structure of protein kinase
C (PKC), retrieved from the protein data bank
(PDB code: 1zrz), compounds (1-4) were
selected for docking process and optimized by
Polak-Ribiere conjugate gradient algorithm and
AM1 semi empirical method, implemented in
HyperChem. The optimized structures were
used as the input of auto dock tools and the
partial charges of atoms were calculated using
gasteiger-marsili procedure (7). Merging non-
polar hydrogens, rotatable bonds were assigned;
removing the heteroatom including water
molecules from protein, all missing hydrogens
were added. Thereafter, determining Kollman
united atom charges (8), non-polar hydrogens
were merged to their corresponding carbons and
as a final processing part in protein preparation,
desolvation parameters were assigned to each
atom. Using auto Grid tool, the grid maps (one
for each atom type in the ligand, and one for
 electrostatic interactions) were constructed
adequately large to include the active site of
protein as well as significant regions of the
surrounding surface. In all the cases, a grid map
of 60 points in each Cartesian direction apart from
a grid-point spacing of 0.375 Å (a quarter of the
carbon-carbon single bond) were generated. By
the ligand location in the complex, the maps were
centered on the ligand’s binding site, searching
favorable interactions with the functional
groups. Based on lamarckian genetic algorithm
(9), using the pseudo-solis and wets local search
method (10), Auto Dock Tools were employed
to produce both grid and docking parameter files
i.e. .gpf and .dpf files. Applying 2.0 Å clustering
tolerance to construct clusters of the closest
compounds, the initial coordinates of the ligand
were used as the reference structure. In addition,
for the internal validation phase, ligand structure
(corresponding HETATM and CONECT records)
was extracted from the pdb file of CDK\textsubscript{2} (2BTS).
Later, assigning bond orders, missed hydrogens
were added and a short minimization (100
steepest descent steps were taken, using MM\textsuperscript{+}
force field with a gradient convergence value of
0.05 Kcal/mol Å\textsuperscript{2}) to release any internal strain.
At last, docking results (PKC-ligand complexes)
were visualized using VMD1.8.6 (11).

**Statistical analysis**
The IC\textsubscript{50} values were calculated using Excel
based program and reported as mean ± SD of the
mean. Significance was attributed to p-values (p < 0.05) and the probability values obtained by
the student t-test between the sample and control
data.

**Results and Discussion**
Compound 1, white crystals with mp 180-
184°C, showed the molecular formula of
C\textsubscript{30}H\textsubscript{50}O\textsubscript{2} based on positive El-HR-MS m/z
442.3784 (calc. for C\textsubscript{30}H\textsubscript{50}O\textsubscript{2}: 442.3811, Δ 6.10
ppm), in accordance with the number and the
multiplicity of \textsuperscript{13}C-NMR spectra (Table 1). The
IR spectrum confirmed presence of hydroxyl
group (3373 cm\textsuperscript{-1}), double bond absorption (1650
and 756 cm\textsuperscript{-1}), C-O functions (1219, 1095 and
1026 cm\textsuperscript{-1}), and cyclopropane C-H (3018 cm\textsuperscript{-1})
together with C-H stretch bonds (2916 and 2848
\textsuperscript{1}H-NMR revealed five singlet methyls
at δ\textsubscript{H} 1.70 (s, Me\textsubscript{27}), 0.94 (s, 6H: Me\textsubscript{18}, Me\textsubscript{30}),
and 297.2587 [C_{22}H_{35}O]^+. In addition, 381.3153 [M-H$_2$O-C$_3$H$_7$]^+ together with 355.3018 [M-H$_2$O-
C$_5$H$_9$]^+, fragmented due to the elimination of parts of side chain (during a Mc Lafferty process), inferred presence of hydroxyl group in side-chain as is shown in Figure 2 (11). Regarding to these findings and the published data (11), compound 1 was identified as cycloart-25-en-3β,24-diol.

HR-EI-MS identified compound 2, as C$_{31}$H$_{50}$O with molecular ion peak at m/z 440.4015 (calc. for C$_{31}$H$_{50}$O: 440.4018, a 0.7 ppm). The IR spectrum confirmed absorption of hydroxyl group (3311 cm$^{-1}$), double bond peak (1640 and 771 cm$^{-1}$), C-O functions (1219 cm$^{-1}$) and C-H stretching at 3020 (cyclopropane ring), 2916 and 2848 cm$^{-1}$. Six degree of unsaturation suggested a double bond (Table 1) and consequently five rings in the molecule. The resonances encompassed thirty-one carbons including seven methyls, twelve methylenes, six methines and six quaternary carbons. 1H-NMR revealed five singlet methyls at $\delta$ 1.01 (d, $J = 3$ Hz, Me$_{25}$), 0.99 (d, $J = 3$ Hz, Me$_{24}$), 0.94 (s, 6H: Me$_{18}$, Me$_{20}$), 0.88 (s, Me$_{22}$), 0.86 (d, $J = 6$ Hz, Me$_{19}$) and 0.79 (s, Me$_{23}$), a pair of doublets in the up-field area at $\delta$ 0.30 and 0.53 ($J = 4.25$ Hz) indicative of cyclopropane ring characteristic of cycloartanes. A doublet of doublet proton at $\delta$ 3.26 (dd, $J_{ax,eq} = 11.0$, $J_{ax,ax} = 4.0$ Hz, H$_1$), indicative of equilaterial β orientated hydroxyl-group, and one pair of olefinic protons $\delta$ 4.64 (d, $J = 0.5$ Hz) and 4.69 (br-s) related to exocyclic terminal methylene. Based on these data, compound 2 was determined as 24-methylene-cycloartan-3β-ol (12), confirmed by the EI-MS fragments m/z 425 [M-CH$_3$], 407 [425-H$_2$O], 315 [M-side chain], 297[315-H$_2$O], 300[C$_{22}$H$_{39}$O] and 175[300-sidechain].

Compound 3 obtained by the acetylation of 1, was identified as 3β, 24-O-diacyl-cycloart-25-en through EI-MS molecular ion peak m/z 526 [M]+, 466 [M-CH$_3$COOH]+, 423[466-CH$_2$CO], 406[466-CH$_2$COOH]+ and 3β-H-NMR spectrum. The IR spectrum supported absorptions at 3020 (cyclopropane ring), 2936, 2868, 1736 (esteric
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[Chemical structures and spectra]

**Figure 2.** El-Mass fragmentation pattern of cycloart-25-en-3β,24-diol.

Carbonyl, 1650, 1456, 1373, 1244, 1026 and 758 cm

-1 without hydroxyl group peak at [3500-3300 cm

-1]. Likewise, the structure of compound 4 after acetylation of 2, was confirmed as 3β-O-acetyl-24-methylene-cycloartan on the bases of EI-MS molecular ion peak m/z 482 [M]+ and 422 [M-COOH]+, IR and NMR spectra. IR spectrum showed absorption at 3018 (Cyclopropane ring), 2916, 2848, 1736 (Esteric carbonyl), 1642, 1456, 1373, 1244, 1026 and 758 cm

-1 without any peak at hydroxyl area [3600-3200 cm

-1] and the signals of δH 4.64 (d, J = 0.5 Hz) and 4.69 brs, each one H related to external methylene, 3.26 (dd, Jax,ax = 11.0 ,jax,eq = 4.0 Hz, H) geminal to oxygenated carbon, 2.03 (s, acetate methyl), 1.01 (d, J = 3 Hz, Me27), 0.99 (d, J = 3 Hz, Me26), 0.94 (s, 6H: Me15, Me20), 0.88 (s, Me28), 0.86 (d, J = 6 Hz, Me25), 0.79 (s, Me18) together with 0.30 (d, 4.25 Hz) and 0.53 (J = 4.25 Hz) of cyclopropane ring were observed in 1H-NMR spectrum.

**Proliferation assay**

The anti-proliferation effect of the test compounds was determined by measuring the PHA-induced T-cell proliferation by determining radioactive thymidine incorporation. Comparison of positive, negative controls were included to assess the activity of test compounds. cycloart-25-ene-3β, 24-diol (1) showed dose-dependent decrease in lymphocyte proliferation with IC50: 12.1 ± 0.6 µg/mL. This result was in conformity with another study by
Figure 3. Proliferation assay on peripheral blood lymphocytes of cycloartanes in *Euphorbia aellenii*: cycloart-25-en-3β,24-diol (1), 24-methylene-cycloartan-3β-ol (2), 3β, 24-O-diacetyl-cycloart-25-en (3), 3β-O-acetyl-24-methylene-cycloartan (4). T-cells were stimulated by phytohemagglutinin (PHA) in the presence of three different concentrations of compounds. Significance differences between the means of compounds as compared to the control (PHA +ve) were calculated by using one-way ANOVA at p < 0.05.

Smith-Kielland (14) which showed cytotoxic activity against Ehrlich ascites tumor cells in mice. Likewise, 24-methylene-cycloartan-3β-ol (2), presented dose dependent inhibitory effect with IC50: 10.4 ± 0.1 µg/mL agreed with other published data supporting pain-relieving activity, and anti-inflammatory effect by TPA-induced ear oedema in mice (15). Masking free OH groups of 1 and 2 by acetylation, anti-proliferative effect decreased significantly (IC50 > 50 µg/mL). On the other hand, in the case of 3 with two acetyloxy groups (3-OAc and 24-OAc), proliferation of PBLs increased by 23-25% at the low concentration (0.5 µg/mL) in comparison with PHA (5 µg/mL) as positive control. However of the higher concentration 50 and 5 μg mL−1 of 66-70% and 36-39% increase in proliferation were absorb. These results suggested that the proliferation stimulatory activity on PBLs is related to the presence of 24-OAc function while anti-proliferation effect induced by free 3-OH group (Figure 3).

**Docking results**

In the internal validation phase of docking, bis(indolyl) maleimide was docked onto the PKC and the lowest energy pose for docking is shown in Figure 4. Superimposing the experimental and predicted conformations, the RMSD was achieved as 1.02 Å, considered as a successful docking (16) of such ligands with PKC. Thereafter, the proposed mechanism of the action was validated by docking the compounds (1-4) in the binding site (Figure 5). All of four compounds tended to accept similar orientations, and docked into the active site of PKC. In compounds (1-2) forming hydrogen-bond interactions between 3-OH and Pro-332, could explain the antiproliferative effect of T-cell derived PKC *in-vitro*. Therefore, based on this structure-function study, the presence of 3-OH could be correlated with the ability to deactivate PKC and inhibited T-cell proliferation. Likewise, hydrogen bond interaction between 24-O-acetyl group (3) and Asp-330 of PKC active site could be responsible for induction of lymphocyte proliferation derived PKC *in-vitro*.

**Conclusion**

For the first time Cycloart-25-en-3β,24-diol (in high quantity) and 24-methylene-cycloartan-3β-ol could be isolated from *Euphorbia aellenii*. Their immunomodulatory effects suggested that the proliferation stimulatory activity on PBLs is related to the presence of 24-OAc function while anti-proliferation effect induced by free 3-OH group. The SAR studies on PKC confirmed these results and provided further support for the role of PKC in transduction of activation signals in T-cells.
Asp-330 of PKC active site could be responsible for induction of lymphocyte proliferation. T-cell proliferation. Likewise, hydrogen bond interaction between 24-O-acetyl group (3) and (green structure) with that from AutoDock model (red structure).

Docking results and predicted conformations, the RMSD was achieved as 1.02 Å, considered as a successful and the lowest energy pose for docking is shown in Figure 4. Superimposing the experimental surface (SES) and conformational comparison of bisindolylmaleimide from crystal structure Figure 4.

antiproliferative effect of T-cell derived PKC forming hydrogen-bond interactions between 3-OH and Pro-532, could explain the validated by docking the compounds (1-4) in the binding site (Figure 5). All of four compounds docking (16) of such ligands with PKC. Thereafter, the proposed mechanism of the action was study, the presence of 3-OH could be correlated with the ability to deactivate PKC and inhibited activation by tumor promoters: role of protein kinase C. J. Immunol. (1987) 138: 3100-3107.

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