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STUDIES ON INHIBITORS OF SKIN-TUMOR PROMOTION. INHIBITORY EFFECTS OF TRITERPENES FROM *COCHLOSPERMUM TINCTORIUM* ON EPSTEIN-BARR VIRUS ACTIVATION

BILO DIALLO, M. VANHAELEN,* R. VANHAELEN-FASTRÉ,

Department of Pharmacognosy, U.L.B. Campus de la Plaine B205-4, Bd. Triomphe, 1050 Bruxelles, Belgium

TAKAO KONOSHIMA, MUTSUO KOZUKA,

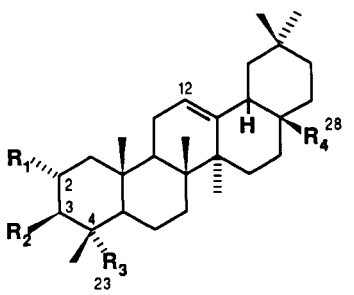
Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607, Japan

and HARUKUNI TOKUDA

Department of Microbiology, Faculty of Medicine, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606, Japan

ABSTRACT.—Arjunolic acid, an oleanene-type triterpene isolated from the rhizome of *Cochlospermum tinctorium*, its triacetate derivative, and their methyl esters were tested using the short-term in vitro assay on EBV-EA activation in Raji cells induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Their inhibitory effects on skin tumor promotors were found to be greater than those of previously studied natural products.

In continuation of our search for biologically active constituents from the rhizomes of *Cochlospermum tinctorium* A. Rich. (syn. *Cochlospermum niloticum* Oliv.) (Cochlospermaceae) (1,2), combined chromatographic methods allowed the purification of three pentacyclic triterpenoids. Among the three, one major constituent was elucidated as arjunolic acid, 2α,3β,23-trihydroxyolean-12-en-28-oic acid [**1**]; this triterpene was previously isolated from *Terminalia arjuna* (3), *Mitragyna ciliata* (4), *Tristania conferta* (5), and *Psidium guaijava* (6); its presence in *Dryobalanops aromatica* was also presumed (7). Identification of **1** was achieved using ^{1}H-nmr, ^{13}C-nmr, and ms data of its triacetate and methyl ester derivatives. These data have not been published until now and were compared to those reported for methyl asiatate (methyl 2α,3β,23-trihydroxyursan-12-en-28-oate) and for methyl 2α,3α,23-trihydroxyolean-12-en-28-oate (8).



1 $R_1=R_2=R_3=OH$, $R_4=COOH$
2 $R_1=R_2=R_3=OH$, $R_4=COOMe$
3 $R_1=R_2=R_3=OAc$, $R_4=COOH$
4 $R_1=R_2=R_3=OAc$, $R_4=COOMe$

As the inhibitory activity of several related triterpenes on skin tumor promoters was recently reported (9), arjunolic acid and three derivatives, compounds **2**–**4**, obtained by methylation and/or acetylation were tested using the short-term in vitro assay of Epstein-Barr virus early antigen (EBV-EA) activation in Raji cells induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The four triterpenes showed stronger inhibitory effects than those of triterpenes studied earlier (9). Triacetate **3** and its methyl ester **4** exhibited complete inhibition on EBV-EA activation at 5×10^2 mol ratio and strong inhibitory effects even at 1×10^2 mol ratio (Table 1). As all values of activation were identical to that of negative control (negative control = 0.1; positive control with TPA 20 ng/ml = 34.5), it was concluded that the four compounds did not exhibit any activation.

TABLE 1. Inhibitory Effects of Arjunolic Acid **1** and Derivatives **2**, **3**, and **4** on Epstein-Barr Virus Activation.[a]

Compound	Concentration (mol ratio compound/TPA)				Early Antigen (EA) Activation[b]
	1000	500	100	10	
1	18.5 (60.0) ± 2.1	38.4 (>80) ± 4.0	85.9 (>80) ± 3.2	100.0 (>80) ± 0	0.1
2	11.4 (>80) ± 1.6	47.1 (>80) ± 3.7	79.4 (>80) ± 3.0	100.0 (>80) ± 0	0.1
3	0.0 (40.0) ± 0	0.0 (50.0) ± 2.0	34.3 (>80) ± 2.6	42.7 (>80) ± 1.8	0.1
4	0.0 (30.0) ± 0	0.0 (60.0) ± 1.8	26.6 (>80) ± 3.0	45.1 (>80) ± 2.1	0.1

[a]Table entries are percentage required to control; percent viability is in parentheses. Each value represents the average of three determinations ± SD; positive control (TPA: 32 pM) = 100.

[b]Each value in this column represents the % of EA activation by each compound alone (32 nM); negative control (*n*-butyric acid: 4 mM) = 0.1; positive control (*n*-butyric acid 4 mM + TPA 20 ng/ml) = 34.5.

In the series of the oleanene-type triterpenes, it has been observed that compounds having a 2α,3β-OH group and their acetylated derivatives were more cytotoxic than those having only a 3β-OH or 3β-OAc substitution (9). However these latter compounds were found to be more cytotoxic than arjunolic acid [**1**] and its triacetate derivative. Therefore, it was assumed that the 23-OH or 23-OAc substitution, shown by arjunolic acid and its derivatives, significantly reduces the cytotoxicity of the oleanene-type triterpenes on the Raji cells. Furthermore, the inhibitory effects of the acetate derivatives were remarkably enhanced. As the methylation of the COOH group of the acetylated derivative **3** did not significantly modify its activity or its cytotoxicity, it is unlikely that the decrease of the hydrophilic character is the only feature that explains its enhanced potency.

The inhibitory activities of **3** and **4** are greater than that of 7-*O*-acetylafromosin, which was reported to have a potency of inhibitory effect more than 10 times higher than those of retinoic and glycyrrhetinic acids (10,11). From these results, it is hoped that **3** and **4** will have valuable potency as antitumor-promoters. In vivo initiation-promotion tests are now in progress.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All nmr spectra (^{1}H nmr at 250 MHz; ^{13}C nmr, broad band, and DEPT at 62.89 MHz) were recorded on a Bruker WP 250 instrument with TMS as internal reference; ms spectra were recorded with a VG Micromass 7070F apparatus (EI at 70 eV). Analytical tlc was carried out with precoated Si gel 60 F_{254} plates from E. Merck (layer thickness: 0.25 mm).

PLANT MATERIAL.—Rhizomes of *C. tinctorium* were collected in Guinea, Mamou, Middle-Guinea, in April 1984. The plant was identified at the 'Station Autonome de Sérédou', Macenta, Guinea, where a voucher specimen (number 1265) has been deposited.

EXTRACTION AND ISOLATION.—Powdered dry rhizomes (500 g) were extracted exhaustively with cold MeOH by percolation. The MeOH extract was evaporated to yield a syrup which was mixed with cellulose (100 g). This mixture was percolated successively with petroleum ether (1 liter), Et_2O (2.5 liters), $CHCl_3$ (0.5 liters), $CHCl_3$-Me_2CO (1:1) (350 ml), $CHCl_3$-MeOH (9:1, 200 ml, 8:2, 200 ml, 1:1, 450 ml). The $CHCl_3$-MeOH (1:1) fraction was evaporated and was partitioned with $CHCl_3$-MeOH-H_2O (5:3.5:1.5, 600 ml). The $CHCl_3$ layer was evaporated and the residue (2.6 g) was subjected to cc on Si gel (100 g); the column elution was achieved with $CHCl_3$ containing increasing amounts of MeOH. Compound **1** was detected in the $CHCl_3$-MeOH (9:1) fractions; it was further purified by preparative tlc on Si gel in $CHCl_3$-EtOAc-HOAc (8:0.5:1.5) and finally on a C_{18} Si gel column eluted with MeOH-H_2O (6:4, 8:2) to remove pigments. Crystallization from EtOH/Me_2CO/H_2O afforded **1** (75 mg): tlc on Si gel, R_f = 0.51, $CHCl_3$-MeOH (9:1).

ACETYLATION OF ARJUNOLIC ACID [**1**].—The triacetate **3** was prepared by acetylation with Ac_2O in pyridine at room temperature for 12 h followed by purification using tlc on Si gel in $CHCl_3$-MeOH (9.5:0.5): tlc on Si gel, R_f = 0.7, $CHCl_3$-MeOH (9.5:0.5).

METHYLATION OF ARJUNOLIC ACID [**1**] AND COMPOUND **3**.—The methyl esters of **1** and **3** were prepared by methylation with CH_2N_2 in Et_2O followed by purification using tlc on Si gel in $CHCl_2$-MeOH (9:1 and 9.5:0.5) to yield, re-

spectively, pure **2** and **4**: tlc on Si gel, $R_f = 0.64$, $CHCl_3$-MeOH (9:1) for **2**; $R_f = 0.9$, $CHCl_3$-MeOH (9.5:0.5) for **4**.

SPECTRAL CHARACTERIZATION OF **2**.—Eims *m/z* (rel. int.) $[M]^+$ 502 (4.5), $[M-OH]^+$ 485 (0.8), $[M-HCO_2Me]^+$ 442 (3.8), 407 (3.1), 262 (71.5), 249 (9.3), 248 (5.3), 239 (1.6), 203 (100); ^1H nmr ($CDCl_3$, 250 MHz) δ 0.72 (3H, s, H-26), 0.90, 0.92, 1.02 (12H, 3s, H-24, -25, -29, -30), 1.12 (3H, s H-27), 2.88 (1H, dd, $J = 13.5$ Hz, $J = 4.0$ Hz, H-18), 3.61 (3H, s, COOMe), 5.28 (1H, broad t, H-12).

SPECTRAL CHARACTERIZATION OF **3**.—Eims *m/z* (rel. int.) $[M]^+$ 614 (0.3), $[M-HCOOH]^+$ 568 (2.5), 526 (0.2), 510 (0.3), 494 (1.3), 466 (1.1), 452 (0.8), 434 (1.4), 367 (3.6), 307 (3.6), 248 (100), 235 (8.9), 233 (4.5), 203 (76); ^1H nmr ($CDCl_3$, 250 MHz) δ 0.76 (3H, s, H-26), 0.88, 0.91, 0.93, 1.08 (12H, 4s, H-24, -25, -29, -30), 1.12 (3H, s, H-27), 1.98, 2.02, 2.08 (9H, 3s, 2-, 3-, 23-OAc), 2.83 (1H, dd, $J = 13.5$ Hz, $J = 4.0$ Hz, H-18), 3.58 (1H, d, $J = 11.8$ Hz, H_A-23), 3.84 (1H, d, $J = 11.8$ Hz, H_B-23), 5.07 (1H, d, $J = 10.3$ Hz, H-3), 5.14 (1H, m, H-2), 5.28 (1H, broad t, H-12).

SPECTRAL CHARACTERIZATION OF **4**.—Eims *m/z* (rel. int.) $[M]^+$ 628 (15.6), $[M-15]^+$ 613 (2.2), $[M-HOAc]^+$ 568 (17.8), 553 (2.2), 526 (2.2), 5.08 (9.6), 493 (3.7), 448 (5.9), 367 (3.7), 307 (2.2), 262 (100), 248 (14.8), 203 (98.5); ^1H nmr ($CDCl_3$, 250 MHz) δ 0.73 (3H, s, H-26), 0.89, 0.90, 0.92, 1.01 (12H, 4s, H-24, -25, -29, -30), 1.11 (3H, s, H-27), 1.98, 2.02, 2.08 (9H, 3s, 2-, 3-, 23-OAc), 2.86 (1H, dd, $J = 13.5$ Hz, $J = 4.0$ Hz, H-18), 3.58 (1H, d, $J = 11.8$ Hz, H_A-23), 3.62 (3H, s, COOMe), 3.85 (1H, d, $J = 11.8$ Hz, H_B-23), 5.07 (1H, d, $J = 10.3$ Hz, H-3), 5.14 (1H, m, H-2), 5.28 (1H, broad t, H-12); ^{13}C nmr ($CDCl_3$, 62.89 MHz) δ 14.08 (C-24), 17.07 (C-25 or C-26), 17.10 (C-26 or C-25), 18.21 (C-6), 20.94, 21.04, 21.26 ($3 \times CH_3$-CO), 23.26 (C-16), 23.72 (C-11), 23.86 (C-30), 26.01 (C-27), 27.86 (C-15), 30.92 (C-20), 32.45 (C-7), 32.59 (C-22), 33.31 (C-29), 34.12 (C-21), 38.16 (C-10), 39.61 (C-8), 41.56 (C-18), 41.90 (C-14), 42.21 (C-4), 43.88 (C-1), 46.11 (C-19), 46.94 (C-17), 47.91 (C-9), 47.97 (C-5), 51.71 ($COOCH_3$), 65.60 (C-2), 70.14 (C-23), 75.20 (C-3), 122.12 (C-12), 144.19 (C-13), 170.50, 170.60, 170.98 ($3 \times$ Ac), 178.35 (C-28).

BIOLOGICAL ACTIVITIES.—The assays of inhibitory effects of the compounds on EBV-EA activation were performed according to the short-term assay, details of which were reported in previous papers (9,12).

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