On the Active Principles of the Spurge Family (Euphorbiaceae) XI. [1] The Skin Irritant and Tumor Promoting Diterpene Esters of *Euphorbia tirucalli* L. Originating from South Africa

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Skin Irritation, 4-Deoxyphorbol, Cocarcinogenesis, Tumor Promoters, Occupational Cancer

The irritant and tumor-promoting constituents of latex of *Euphorbia tirucalli* L. originating from South Africa were isolated. They were identified as irritant ingenane and tigliane type diterpene esters derived from unsaturated aliphatic acids and acetic acid and the polyfunctional diterpene parent alcohols 4-deoxyphorbol, phorbol and ingenol, respectively.

The irritant and tumor-promoting esters of 4-deoxyphorbol are predominant and were fully characterized chemically and biologically. They are positionally isomeric 12,13-acylates, acetates *e.g.* Euphorbiafactors Ti_1–Ti_4. As acyl groups they carry homologous, highly unsaturated aliphatic acids of the general structure $CH_3-(CH_2)_m-(CH=CH)_n-COOH$ ($m=2,4$; $n=1,2,3,4,5$; $N=2n+m+2$). Corresponding diesters of 4-deoxy-4α-phorbol are also present which are biologically inactive. Comparison of structures and biological activities of 12,13-diesters of 4-deoxyphorbol indicates that – for a distinct total number of C-atoms *(N)* in the acyl moiety – an increasing number of conjugated double bonds *(n)* may increase the irritant but decrease the tumor-promoting activity. Replacement of the hydroxyl function at C-4 (phorbol-12,13-diesters) by hydrogen (corresponding 4-deoxyphorbol-12,13-diesters) does not essentially alter biological activities. Epimerization of 4-deoxyphorbol-12,13-diesters at C-4 abolishes biological activities.

The specific chemical properties demonstrated for the diterpene ester irritants contained in the latex of *E. tirucalli* and hence in all plant parts may be useful in trials to abolish the potential risk of cancer involved especially in occupational mass production and handling of the plant. Some of the structure activity relations of the *Euphorbia* factors isolated made them excellent tools in experimental cancer research for the analysis of mechanisms of tumorigenesis.

Introduction

Euphorbia tirucalli L. (Euphorbiaceae) is an almost leafless succulent tree, up to nine meters high. The green, cylindric, finely striated branches form brush-like dense masses ("pencil tree", [2]). Botanists appear to favor Eastern Africa as the region from which *E. tirucalli* originated, but it is widespread throughout all tropical regions of the world due to its ease of propagation from cuttings [2–4]. The frequency with which it was introduced into new areas and its subsequent naturalization there gave rise to many variants within the species *E. tirucalli* [4]. They may differ in their pattern of diterpenes [5].

E. tirucalli or parts thereof have been utilized by man for centuries. The plant is frequently used as a hedge plant ("rubber hedge", "milk hedge", [2]), for various, folk medicinal or fetish purposes, as an insecticide and as a fish poison [6, 7]. The roots are reported to be used as an emetic in treatment of snake bites [8]. The yellowish milky juice of *E. tirucalli* exuding in copious quantities from any wound of the stem or branches is highly irritant and vesicant to mucous membranes [6–9]. During the second world war the latex of *E. tirucalli* was processed for the production of rubber ("rubber euphorbia", [2]). Currently, large scale plantations of *E. tirucalli* are being investigated as photosynthetic source for biomass to produce energy, *e.g.* gasoline [10] or charcoal [11]. In Brasil, Indonesia and India the latex was used to remove warts. In India and Malaysia latex preparations were used as antitumor or anticancer drugs [12]. Nowadays, because of its odd and attractive form of growth, *E. tirucalli* is widely used as ornamental plant in rooms, gardens and parks [3].

In chemical investigations of the latex of *E. tirucalli* the triterpenes euphol, tirucallol and taraxasterol [13–16], several plant acids [17], hydrocarbons [18] and an isochinoline derivative [19] were isolated and characterized. Most of them are biologically inert.

* Part of dissertation, see l.c. [31]

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The latex of *E. tirucalli* exerts toxicological effects similar to those of Croton Oil, the seed oil of *Croton tiglium* L. (Euphorbiaceae). Thus irritant and tumor promoting activities were demonstrated in acetone extracts of latex assayed in the mouse [20]. The wide distribution of *E. tirucalli* in tropical regions and its manyfold utilizations prompted our investigations aimed at the purification and isolation of the unknown irritant and tumor promoting principles from the latex of *E. tirucalli* originating from South Africa and their chemical and biological characterization as reported briefly [21–23].

The current technological efforts towards generation of biomass and some important findings with respect to the mechanism of tumor promotion [24] both involving *E. tirucalli* prompted us to publish the entire scope of our chemical and toxicological endeavours regarding this plant.

Material and Methods

Plant material

The latex of *E. tirucalli* L., preserved with methanol, was collected in stands of the tree in Umgeni Valley, Natal, Republic of South Africa following the standardized collection procedure described elsewhere [25, 26]. The tree was identified by Prof. Dr. A. W. Bayer, University of Pietermaritzburg.

Analytical methods

The methods and machinery of multiplicative distribution have been described previously [25]. Merck silica gels HF 254 and PF 254 were used for thin layer chromatography (TLC). The spots were detected under UV-light at 254 nm and visualized by heating up to 110 °C after spraying with vanillin/sulfuric acid. Column chromatography was carried out with Merck silica gel 0.05–0.20 mm, deactivated with 13% of water. Gas chromatography was performed with a Packard Gas Chromatograph 420 using as stationary phase 5% DEGS on Chromosorb W 80/100 mesh for the analysis of carboxylic acid methyl esters and 1% silicon GEXE 60 on Chromosorb W/AW-DMCS for the analysis of triterpenes.

Spectra

Mass spectra were measured with a CEC 21-110 B mass spectrometer, IR spectra with a Perkin Elmer spectral photometer 521, UV spectra with a Beckman DK 2a far UV spectrometer in methanol and ^{1}H-NMR spectra with a Varian HA-100 or a Jeol JNM-C-60 HL spectrometer (the spectra were measured usually in $CDCl_3$ with TMS, $\delta = 0.00$ ppm, as internal standard).

Biological assays (for details see [25, 26])

Skin irritant activities were determined quantitatively as irritation dose 50 (ID_{50}) on the ear of NMRI mice, using as pilot data the irritation unit (IU) determined on the ear of SIM mice. ID_{50} data relevant for monitoring the fractionation of the latex are given in the separation scheme (Chart).

The tumor promoting activity was determined in the standard assay on the back skin of NMRI mice, using a single, subcarcinogenic dose of $i = 100$ nmol of 7,12-dimethylbenz[a]anthracene (DMBA, dissolved in 0.1 ml of acetone) as initiator. Doses p of materials to be assayed were administered twice weekly for 36 or 48 weeks. As positive controls, appropriate doses of 12-O-tetradecanoylphorbol-13-acetate (TPA, dissolved in 0.1 ml of acetone) were used. For negative controls, groups of animals received acetone followed by tumor promoting agent or DMBA followed by acetone. The promoting activity is expressed as the average tumor rate T_r (*i.e.* number of tumor bearing animals/survivors) and as the average tumor yield T_y (number of skin tumors/survivors) taken from the same experiment. In order to monitor the status of general health in the experimental groups, weight and survival rates S_R were routinely recorded. All tumors generated were examined macroscopically and those suspicious of malignant growth were diagnosed histologically according to standardized procedures [25]. – More experimental details together with survival rates for the acetone extract, selected fractions of its fractionation and factors isolated are given in Table I. The time course of promoting activity was plotted in Figs. 4 and 5 (see Results).

Terminology and abbreviations

Molecularly uniform, irritant, diterpenoid constituents of the latex are assigned as *Euphorbia* factors "Ti_x", mixtures thereof as "MF_x". Non-irritant diterpenoid constituents ($ID_{50} > 50$ nmol/mouse ear) are designated as compounds "α-Ti_x", mixtures thereof as "MC_x". 12-O-tetradecanoylphorbol-13-

Table I. Tumor-promoting activity in the back skin of NMRI mice of selected fractions of the separation procedure, of *Euphorbia* factors and of compounds, with TPA as reference[a].

	Application		Tumor rate (T_R)				Survival rate (S_R%)		Histologic diagnosis	
Fraction[b] *Euphorbia* factor[b] compound[b] (Exp. No.)	Single dose *p* [µg]	Duration of application [weeks]	Tumor bearers/survivors at week				at week		Tumors in treated area	
			12	24	36	48	24	48	Total/mice investigated histologically	Malignant tumors in total[c]
Acetone extract (185)	2500	48	5/24	9/20	8/15	5/10	71	36	25/11	0
Hydrophilic fraction (196)	250	48	1/27	3/24	6/19	4/15	81	54	16/9	1 PEC
Hydrophobic fraction (186)	2500	42	0/27	1/20	0/17	–	71	–	0	0
Fraction ET-1 (520)	12.3	48	0/28	5/24	10/22	13/20	86	71	10/4	1 PEC
Fraction ET-2 (283)	12.3	48	0/28	10/27	11/18	5/14	96	50	18/7	2 PEC
MF_2 (585)[d]	12.3	48	2/28	11/27	10/24	12/22	96	79	13/4	0
MC_2 (606)[e]	11.0	48	0/28	0/28	0/27	0/27	100	96	0	0
Ti_1 (565)	10.0	48	0/28	5/26	10/26	10/24	93	86	16/5	0
4-Deoxy-DPA (630)	10.0	36	1/27	13/27	15/26	–	96	–	47/12	1 PEC
TPA (179)	1.23	48	1/28	4/26	9/21	12/18	93	64	21/9	1 PEC, 1 ABDAM
TPA (503)	6.16	48	13/28	21/28	22/27	18/24	100	86	70/14	3 PEC

[a] Twenty-eight NMRI mice/experiment (males/females = 1/1); in experiment no. > 276 only females were used; as initiator a single dose i = 100 nmol of 7,12-dimethylbenz[a]anthracene (DMBA) was administered.
[b] See Chart, Tables II and III.
[c] PEC: squamous cell carcinoma; ABDAM: adenoid basaliom.
[d] Mixture MF_2 (see Table IV): 12-O-acetyl-4-deoxyphorbol-13-(2,4,6-decatrienoate) (34%), 12-O-acetyl-4-deoxyphorbol-13-(2,4,6,8-dodecatetraenoate) (66%).
[e] Mixture MC_2 (see Table IV): 12-O-acetyl-4-deoxy-4α-phorbol-13-(2,4,6,8,10-tetradecapentaenoate), 12-O-acetyl-4-deoxy-4α-phorbol-13-(2,4,6,8-dodecatetraenoate).

acetate, TPA; 12-O-acetylphorbol-13-tetradecanoate, APT; 12-O-decanoylphorbol-13-acetate, DPA; 12-O-decanoyl-4-deoxyphorbol-13-acetate, 4-deoxy-DPA; 7,12-dimethylbenz[a]anthracene, DMBA; thin layer chromatography, TLC; gas liquid chromatography, GLC.

Separation procedure (see Chart)

All stages of the separation procedure were carried out under extensive exclusion of oxygen.

Acetone extract. The methanolic preparation of latex (3500 g) was shaken in portions of 350 g with 3.5 l of acetone under a nitrogen atmosphere for 15 hours. The insoluble material was filtered off and the extraction procedure was repeated twice under identical conditions. Evaporation of the solvent from the combined extracts yielded 730 g of dry acetone extract (ID_{50}: 0.03 µg/ear).

Hydrophilic fraction I. 360 g of the acetone extract were separated by the O'Keeffe distribution procedure in a battery with $z = 11$ elements, ($V = 300$ ml/300 ml) using one feeding of 40 g of acetone extract per cycle in the solvent system petroleum ether/methanol/water = 15/10/0.4 (Chart). The combined upper phases contained the hydrophobic fraction (314 g; 87.4%; ID_{50}: 5.0 µg/ear). From the combined lower phases the hydrophilic fraction I was obtained (42 g; 12%; ID_{50}: 0.003 µg/ear).

Hydrophilic fraction II. 40 g of the hydrophilic fraction I was subjected to a second O'Keeffe distribution in the solvent system tetrachloromethane/methanol/water = 2/1/0.15 employing an automatic Counter-Double-Current-Distribution-Instrument ($z = 100$ elements, $V = 50$ ml/50 ml, $n = 100$ transfers). Repetitive feedings (1 g, two feedings per cycle) were performed in the center of the battery. By means of this distribution procedure the hydrophilic

fraction I was resolved into three fractions: a non-irritant polar fraction which left the battery in the upper phase, a non-irritant unpolar fraction which left the battery in the lower phase and the hydrophilic fraction II which remained in the battery containing all the irritant material (Chart). The non-irritant side fractions yielded 17.7 g (5.3%; ID_{50}: > 100 µg/ear), the hydrophilic fraction II afforded 22 g (6.7%; ID_{50}: 0.001 µg/ear).

Fractions ET-1 and ET-2: 22 g of the hydrophilic fraction II were subjected to a third O'Keeffe distribution using the same automatic battery and procedure (see above, n = 100 transfers, 1 g per feeding) in the solvent system tetrachloromethane/methanol/water = 2/1/0.1. The hydrophilic fraction II was separated into two irritant fractions (Chart) exhibiting a single spot each in TLC. The irritant fraction ET-1 from the combined lower phases yielded 10.6 g (3.2%; ID_{50}: 0.001 µg/ear), the irritant fraction ET-2 from the combined upper phases 9.0 g (2.7%; ID_{50}: 0.001 µg/ear).

Craig distribution of ET-1 (see also Fig. 1): 13.5 g of fraction ET-1 were subjected to a Craig distribution using petroleum ether/tetrachloromethane/methanol/water = 2/0.4/1.75/0.1 (z = 1020; V = 12 ml/10 ml; single withdrawal procedure; n = 7000 transfers). According to the bands shown in the distribution diagram (Fig. 1), the fractions r in the apparatus were combined to yield sections. Three sections (r = 0–225; r = 751–1020; ϱ = 1–6000) contained non-irritant material (ID_{50} > 100 µg/ear). The non-irritant section r = 0–225 contained polar material (TLC). Base-catalyzed transesterification (10^{-2} M sodium methoxide in methanol) of this material yielded a single polar product, which has been characterized as its acetyl derivative (see below). The non-irritant section r = 751–1020 did not contain diterpenoid compounds as indicated by TLC (staining with vanillin/sulfuric acid). The withdrawn section ϱ = 1–6000 represented a mixture of decomposition products (TLC) and was not further investigated. The three non-irritant sections comprised 9.7 g (2.3%). Irritant activity was found in sections r = 226–440, r = 441–600, and r = 601–750 (see also Fig. 1). From these sections and subsections thereof the irritant Euphorbia factors Ti_2–Ti_4, a non-irritant compound α-Ti_4 (see Chart and Table II) and mixtures of Euphorbia factors MF_1–MF_4 and of non-irritant compounds MC_1–MC_3 were isolated (see Table III) after column chromatography on silica gel using the solvent system ether/petroleum ether = 4/1.

Craig distribution of fraction ET-2 (see also Fig. 2): 10 g of fraction ET-2 were separated by means of Craig distribution using the same machinery and solvent system as for ET-1 (n = 6800 transfers). According to the bands shown in the distribution diagram (Fig. 2), the fractions r were combined to yield non-irritant (ID_{50} > 100 µg/ear) sections (r = 0–124; r = 581–1020; ϱ = 1–5800) compris-

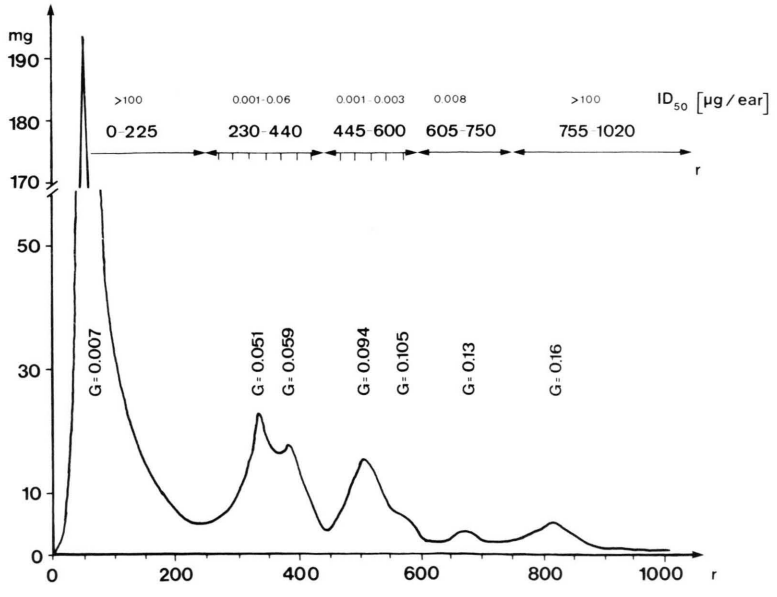


Fig. 1. Craig distribution of fraction ET-1 of latex of *E. tirucalli* originating from South Africa. Solvent system: petroleum ether/tetrachloromethane/methanol/water = 2/0.4/1.75/0.1; z = 1020 elements, V = 12 ml/10 ml; T = 20 °C; n = 7000 transfers; single withdrawal procedure. The ordinate represents the weight of every 5th fraction of the battery; withdrawn fractions are not recorded; G = distribution number of maximum of the bands. Sections (arrows) and subsections with ranges of irritant activities (ID_{50}), refering to the most and least irritant subsections are recorded in the upper part of the figure.

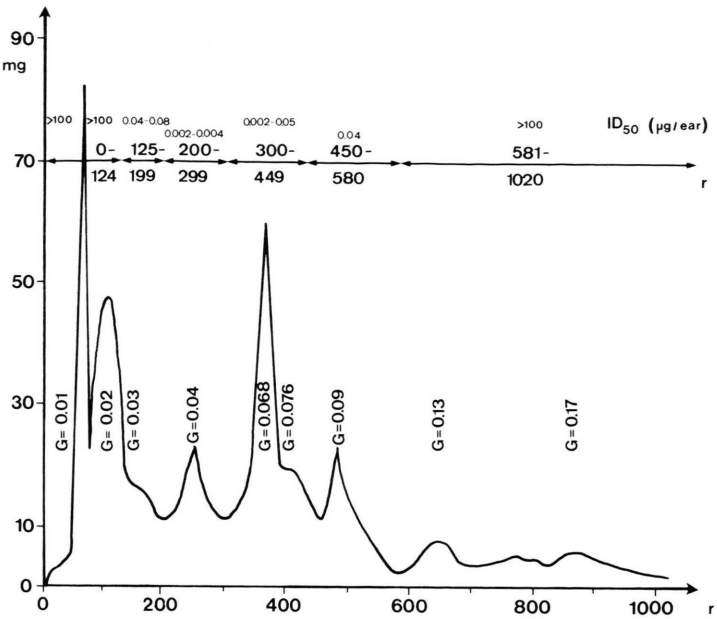


Fig. 2. Craig distribution of fraction ET-2 of latex of *E. tirucalli* originating from South Africa (n = 6800 transfers). All other details identical with those described in legend of Fig. 1.

ing 4.1 g (1.1%). The non-irritant section r = 581–1020 and the withdrawn section ϱ = 1–5800 represented mixtures of decomposition products (TLC) and were not further investigated. Base-catalyzed transesterification (10^{-2} M sodium methoxide in methanol) of the section r = 0–124 yielded two polar products, which have been identified as their acetylation products (see below). Four sections contained almost all irritant activity: sections r = 125–199; r = 200–299; r = 300–449 and r = 450–580 (see Fig. 2). From these sections and subsections thereof the irritant Euphorbia factor Ti_1 and the non-irritant compound α-Ti_1 (Chart and Table II) as well as the mixtures of Euphorbia factors MF_5 and MF_6 and of non-irritant compounds MC_4 were isolated (see Table III) after column chromatography on silica gel using the solvent system ether/petroleum ether = 4/1.

Isolation and identification of triterpenes from the hydrophobic fraction (see Chart)

86% of the material in the hydrophobic fraction represent a mixture of the triterpenes Euphol, Tirucallol and Taraxasterol (R_f = 0.6 on silica gel HF_{254}, chamber saturation, in ethyl acetate/chloroform = 3/2). They were separated by column chromatography on neutral Al_2O_3, deactivated by 3% of H_2O using a gradient solvent system of petroleum ether/diethylether = 10/1 to 4/1 and identified after crystallization by determination of the melting points, euphol (m.p. 108–111 °C), tirucallol (m.p. 130–132 °C) and taraxasterol (m.p. 218–220 °C) according to [13–15]. In addition, they were characterized by their gas chromatographic retention times, euphol (t_R = 30 min), tirucallol (t_R = 30.6 min) and taraxasterol (t_R = 33 min) according to [16], using a glass column (1 m × 4 mm), nitrogen support 30 ml/min, temperature program 5 °C/min, temperature range 80–260 °C.

Physical and chemical characterization from fractions ET-1 and ET-2 of Euphorbia factors, of compounds and of inseparable mixtures

The physical and chemical characterization of the *Euphorbia* factors Ti_1 (**2**), Ti_2 (**3**), Ti_3 (**4**), Ti_4 (**5**) and of the compounds α-Ti_1 (**9**), α-Ti_4 (**10**) (see also Table II) is described in [23].

Stored and exposed to air and daylight Ti_1–Ti_4, α-Ti_1 and α-Ti_4 decomposed to yield material with lower R_f-values as demonstrated by TLC analysis. In the decomposed material, 4-deoxy-4α-phorbol (**6**) was still detectable after base-catalyzed hydrolysis. Storage of Ti_1–Ti_4, α-Ti_1 and α-Ti_4 in dilute solutions in

acetone under a nitrogen atmosphere at −70 °C and under exclusion of daylight guarantees a reasonable stability.

Physical and chemical characterization of mixtures of Euphorbia factors MF_1–MF_6 (see also Table III)

Mixture MF_1: MS: *m/z* = 538, 512 (parent ions). UV λ_{max}: 267, 309 nm. NMR: 1-H: 7.60; H-olef.: 7.5–5.5; 7-H: 5.50; 12-H: 5.45; 20-H_2: 4.00; 10-H: 3.25; CH_3-CO: 2.10; 19-H_3: 1.75 ppm.

Mixture MF_2: MS: *m/z* = 564, 538 (parent ions). UV λ_{max}: 309, 342 nm. NMR: 1-H: 7.60; H-olef.: 7.5–5.5; 7-H: 5.50; 12-H: 5.42; 20-H_2: 4.00; 10-H: 3.25; CH_3-CO: 2.12; 19-H_3: 1.75ppm.

Mixture MF_3: MS: *m/z* = 566, 540, 514 (parent ions). UV λ_{max}: 267, 311 nm. NMR: 1-H: 7.58; H-olef.: 7.5–5.5; 7-H: 5.50; 12-H: 5.45; 20-H_2: 4.00; 10-H: 3.25; CH_3-CO: 2.12; 19-H_3: 1.75 ppm.

Mixture MF_4: MS: *m/z* = 592, 566 (parent ions). UV λ_{max}: 304, 342 nm. NMR: 1-H: 7.60; H-olef.: 7.5–5.5; 7-H: 5.50; 12-H: 5.42; 20-H_2: 4.00; 10-H: 3.25; CH_3-CO: 2.11; 19-H_3: 1.74 ppm.

Mixture MF_5: MS: *m/z* = 538, 512 (parent ions). UV λ_{max}: 268, 308 nm. NMR: 1-H: 7.58; H-olef.: 7.5–5.6; 7-H: 5.50; 12-H: 5.46; 20-H_2: 4.00; 10-H: 3.25; CH_3-CO: 2.11; 19-H_3: 1.74 ppm.

Mixture MF_6: MS: *m/z* = 590, 564, 538 (parent ions). UV λ_{max}: 304, 336, 356 nm. NMR: 1-H: 7.56; H-olef.: 7.5–5.5; 7-H: 5.50; 12-H: 5.44; 20-H_2: 4.00; 10-H: 3.25; CH_3-CO: 2.11; 19-H_3: 1.74 ppm.

*Preparation and identification of 12-O-acetyl-4-deoxy-4α-phorbol (**7**)*

Base-catalyzed transesterification (2.5×10^{-3} M sodium methoxide in methanol) of mixtures MF_1–MF_4 for 72 hours at 4 °C, subsequent extraction of the neutralized reaction mixtures with ethyl acetate and purification by TLC yielded (**7**), R_f = 0.35 (dichloromethane/methanol = 10/1). The spectroscopic data of **7** were identical with that of an authentic sample of 12-O-acetyl-4-deoxy-4α-phorbol (see [23]).

*Preparation and identification of 12-O-(2,4,6-decatrienoyl)-4-deoxy-4α-phorbol (**8**) and homologous 12-O-acyl-4-deoxy-4α-phorbols*

Base-catalyzed transesterification (5×10^{-3} M sodium methoxide in methanol) of Ti_1 (**2**) or mixtures MF_5 and MF_6 for 6 hours at room temperature and subsequent extraction of the neutralized reaction mixtures with ethyl acetate and purification by TLC yielded **8** or homologous 12-O-acyl-4-deoxy-4α-phorbols, R_f = 0.35 (dichloromethane/methanol = 10/1). For spectroscopic identification see [23].

Physical and chemical characterization of mixtures of compounds MC_1–MC_4 (see also Table III)

Mixture MC_1: MS: *m/z* = 538, 512 (parent ions). UV λ_{max}: 268, 304 nm. NMR: 1-H: 7.05; H-olef.: 7.4, 6.8–5.5; 12-H: 5.50; 7-H: 5.13; 20-H_2: 3.95; 10-H: 3.50; CH_3-CO: 2.07; 19-H_3: 1.77 ppm.

Mixture MC_2: MS: *m/z* = 590, 564 (parent ions). UV λ_{max}: 342, 360 nm. NMR: 1-H: 7.05; H-olef.: 7.4, 6.8–5.5; 12-H: 5.5; 7-H: 5.12; 20-H_2: 3.95; 10-H: 3.50; CH_3-CO: 2.07; 19-H_3: 1.75 ppm.

Mixture MC_3: MS: *m/z* = 566, 540, 514 (parent ions). UV λ_{max}: 268, 311 nm. NMR: 1-H: 7.05; H-olef.: 7.5–5.6; 12-H: 5.52; 7-H: 5.15; 20-H_2: 3.95; 10-H: 3.5; CH_3-CO: 2.07; 19-H_3: 1.78 ppm.

Mixture MC_4: MS: *m/z* = 590, 564, 538 (parent ions). UV λ_{max}: 304, 336, 356 nm. NMR: 1-H: 7.02; H-olef.: 7.5–5.6; 12-H: 5.54; 7-H: 5.13; 20-H_2: 3.95; 10-H: 3.51; CH_3-CO: 2.07; 19-H_3: 1.78 ppm.

Base-catalyzed transesterification of mixtures MC_1–MC_3 (2.5×10^{-3} M sodium methoxide in methanol; 72 hours; 4 °C) yielded 12-O-acetyl-4-deoxy-4α-phorbol (**7**) (see above). Base catalyzed transesterification of MC_4 (5×10^{-4} M sodium methoxide in methanol; 6 hours; room temperature) yielded a mixture of 12-O-acyl-4-deoxy-4α-phorbols (see above).

Gas chromatography of carboxylic acid methyl esters: Euphorbia factors and mixtures MF_1–MF_6 of *Euphorbia* factors, compounds and mixtures MC_1–MC_4 of compounds each were transesterified (10^{-2} M sodium methoxide in methanol). The mixtures of carboxylic acid methyl esters, obtained from the reaction mixtures by TLC, were hydrogenated (in ethanol, Pd/charcoal, 6 hours). The hydrogenation was stopped by filtration of the catalyst. The mixtures of hydrogenated carboxylic acid methyl esters were resolved by gas liquid chromatography and identified by cochromatography of authentic samples (see Tables II and III).

*Preparation of 4-deoxy-4α-phorbol (**6**):* 20 mg of *Euphorbia* factor Ti_1 (**2**) were dissolved in 2 ml

10^{-2} M sodium methoxide in methanol. After 12 h at room temperature the solution was neutralized with acetic acid, evaporated and the residue extracted with butanol. After purification by TLC (dichloromethane/methanol = 10/1) 12 mg of resinous **6** were obtained, R_f: 0.25. MS: *m/e* = 348 (parent ion), 330, 312, 294. NMR (d_5-pyridine): 1-H: 7.26 (m); 7-H: 5.70 (m); 12-H: 4.86 (d, J = 10 Hz); 20-H_2: 4.35 (s); 10-H: 3.70 (m); 5a-H: 3.70 (m); 4-H: 2.80 (m); 5b-H: 2.70 (d, J = 14 Hz); 8-H: 2.25 (m); 11-H: 2.04 (m); 19-H_3: 1.80 (m); 18-H_3: 1.62 (d, J = 6 Hz); 16-H_3, 17-H_3: 1.42 (s); 14-H: 1.06 (d, J = 5 Hz) ppm; UV: λ_{max}: 236, 310 nm; ε_{max}: 6640, 70. IR (KBr): 3400 (-OH); 1695 (-CO-); 1635 cm^{-1} (C = C).

Preparation and identification of 12-O-decanoyl-4-deoxyphorbol-13-acetate (4-deoxy-DPA, **12**): 10 mg of Ti_1 (**2**) were dissolved in 25 ml ethanol. Hydrogenation was performed in the presence of 2 mg Pd/C catalyst for 15 min. The reaction was stopped by filtering of the catalyst. After purification by TLC (chloroform/ethyl acetate = 3/2) 7.5 mg of **12** were obtained, R_f = 0.38 (ethyl acetate/chloroform = 3/2). MS: *m/e* = 544 (parent ion) 526, 484, 373, 330, 312. UV: λ_{max} 230, 315 nm; ε_{max}: 4400, 120.

*Preparation and identification of phorbol (**13**) from the irritant sections r = 125–199 and r = 200–299 (ET-2):* 100 mg of the combined sections r = 125–199 and r = 200–299 of the Craig distribution of fraction ET-2 (see Fig. 2) were dissolved in 10 ml of 10^{-2} M sodium methoxide in methanol. After 12 h at room temperature, the solution was neutralized, evaporated and the residue extracted with butanol. After purification by TLC (dichloromethane/methanol = 10/1) 55 mg of phorbol (**13**) were obtained, R_f = 0.15 (dichloromethane/methanol = 10/1). The spectroscopic data of **13** were identical with that of an authentic sample [27]. **13** was further characterized by acetylation to phorbol-12,13,20-triacetate (**14**) (see [27]).

*Preparation and identification of ingenol (**15**) from the irritant section r = 601–750 (ET-1):* 20 mg of the section r = 601–750 of the Craig distribution of fraction ET-1 (see Fig. 1) were dissolved in 10 ml of 10^{-2} M sodium methoxide in methanol. After 12 hours at room temperature, the solution was neutralized with acetic acid, evaporated and the residue was extracted with ethyl acetate. After purification by TLC (dichloromethane/methanol = 10/1) 8 mg of ingenol (**15**) were obtained, R_f = 0.35. **15** was characterized by acetylation to ingenol-3,5,20-triacetate (**16**). The spectroscopic data of **16** were identical with that of an authentic sample (see [28]).

*Preparation and identification of 4-deoxy-4α-phorbol (**6**) from the non-irritant section r = 0–225 (ET-1):* 50 mg of the section r = 0–225 of the Craig distribution of fraction ET-1 were dissolved in 5 ml 10^{-2} M sodium methoxide in methanol. After 12 h at room temperature, the solution was neutralized with acetic acid, evaporated and the residue extracted with butanol. After purification by TLC (dichloromethane/methanol = 10/1), 26 mg of resinous 4-deoxy-4α-phorbol (**6**) were obtained (R_f = 0.35). **6** was characterized by acetylation to 4-deoxy-4α-phorbol-12,13,20-triacetate (**11**) (see [23]).

*Preparation and identification of phorbol (**13**) and 4-deoxy-4α-phorbol (**6**) from the non-irritant section r = 0–124 (ET-2):* 50 mg of the section r = 0–124 of the Craig distribution of fraction ET-2 were dissolved in 5 ml of 10^{-2} M sodium methoxide in methanol. Following the procedure described above, 14 mg of phorbol (**13**) and 10 mg of 4-deoxy-4α-phorbol (**6**) were obtained. **13** and **6** were characterized by acetylation to phorbol-12,13,20-triacetate (**14**, [27]) and 4-deoxy-4α-phorbol-12,13,20-triacetate (**11**, [23]).

Results

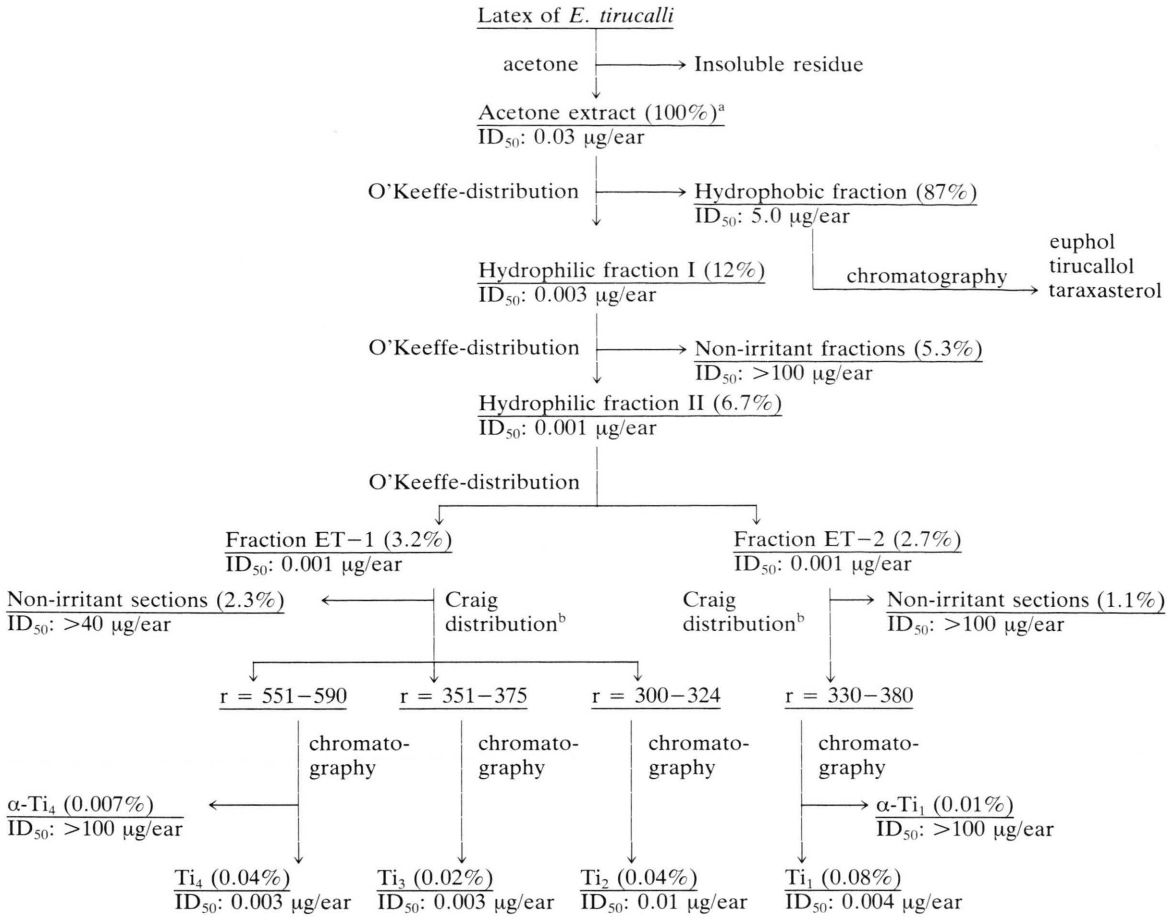
Isolation of Euphorbia factors, of compounds and of inseparable mixtures thereof

A separation procedure was established (Chart) guided throughout by the assay for irritant activity on the mouse ear [27, 29].

Extraction of the methanolic latex preparation with acetone yielded the *acetone extract,* which was separated into two fractions by means of an O'Keeffe distribution. After further purification (see Materials and Methods) the *hydrophobic fraction* yielded the inactive triterpenes Euphol, Tirucallol and Taraxasterol. The *hydrophilic fraction I* contained essentially all the irritant activity (Chart) and was subjected to a second O'Keeffe distribution, yielding the highly irritant *hydrophilic fraction II,* in addition to non-irritant side fractions. By a third O'Keeffe distribution the *hydrophilic fraction II* was separated into the irritant fractions *ET-1* and *ET-2* (Chart). Both fractions were subjected to multistage Craig distributions yielding various non-irritant and irritant sections (Chart; Figs. 1 and 2).

From the irritant sections, the irritant *Euphorbia* factors Ti_1, Ti_2, Ti_3 and Ti_4 (Chart 1 and Table II),

Chart: Separation scheme for latex of the South-African *Euphorbia tirucalli*



[a] Percentages given at individual stages of the fractionation refer to the weight of the acetone extract.
[b] Sections yielding mixtures of *Euphorbia* factors or of compounds are omitted from the scheme and summarized in Table III.

Table II. Characterization of *Euphorbia factors* and of *compounds* of the tigliane type from Craig distributions of ET-1 and ET-2 from latex of the South African *E. tirucalli*.

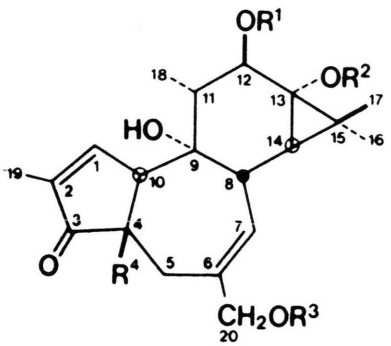
Factor/ compound	ID_{50}[a] [nmol/ear]	R_f[b]	Molecular ion [*m/z*]	UV (CH_3OH) λ_{max}(nm); ε_{max}		GLC Carboxylic acid by hydrogenation[c]	Parent alcohol
Ti_1	0.008	0.35	538	304	26760	decanoate	4-deoxyphorbol
α-Ti_1	> 100	0.40	538	304	19670	decanoate	4-deoxy-4α-phorbol
Ti_2	0.02	0.40	538	306	25000	decanoate	4-deoxyphorbol
Ti_3	0.006	0.40	590	357	34000	tetradecanoate	4-deoxyphorbol
Ti_4	0.005	0.40	592	332	27000	tetradecanoate	4-deoxyphorbol
α-Ti_4	> 100	0.45	592	330	29000	tetradecanoate	4-deoxy-4α-phorbol

[a] References TPA ID_{50}: 0.016 nmol/ear; 4-deoxy-DPA ID_{50}: 0.09 nmol/ear.
[b] TLC on silica gel HF 254 (chamber saturation); solvent system ethyl acetate/chloroform = 3/2. All factors or compounds show extinction of fluorescence under UV-light (254 nm) and stain brown with vanillin/sulfuric acid.
[c] Gas liquid chromatography of carboxylic acid methyl esters obtained from factors and compounds by base-catalyzed transesterification and subsequent hydrogenation. Identification according to authentic references.

and the non-irritant compounds α-Ti_1 and α-Ti_4 (Chart and Table II) were isolated by column chromatography. Beside pure factors and non-irritant compounds the mixtures MF_1–MF_6 of *Euphorbia* factors and the mixtures MC_1–MC_4 of non-irritant compounds were isolated (Table III).

Chemical characterization of Euphorbia factors, of compounds and of inseparable mixtures thereof

The elucidation of the chemical structures of the *Euphorbia* factors Ti_1–Ti_4 and of the compounds α-Ti_1 and α-Ti_4 (Table II) has been reported briefly [20]. Their structures are compiled in Fig. 3 together with other compounds. *Euphorbia* factor Ti_1 is 12-O-[(2*Z*, 4*E*)-2,4,6-decatrienoyl]-4-deoxyphorbol-13-acetate (**2**) and the compound α-Ti_1 its 4-epimer 12-O-[(2*Z*, 4*E*)-2,4,6-decatrienoyl]-4-deoxy-4α-phorbol-13-acetate (**9**). Ti_2 represents 12-O-acetyl-4-deoxyphorbol-13-[(2*Z*, 4*E*)-2,4,6-decatrienoate] (**3**), Ti_3 12-O-acetyl-4-deoxyphorbol-13-(2,4,6,8,10-tetradecapentaenoate) (**4**). Ti_4 is 12-O-acetyl-4-deoxyphorbol-13-(2,4,6,8-tetradecatetraenoate) (**5**) and the compound α-Ti_4 its 4-epimer 12-O-acetyl-4-deoxy-4α-phorbol-13-(2,4,6,8-tetradecatetraenoate) (**10**; Table II and Fig. 3).



4-deoxyphorbol **1** : $R^1 = R^2 = R^3 = R^4 = H$

Ti_1 **2** : $R^1 = CO-(CH\overset{Z}{=}CH)-(CH\overset{E}{=}CH)-(CH=CH)-(CH_2)_2-CH_3$; $R^2 = COCH_3$; $R^3 = R^4 = H$

Ti_2 **3** : $R^1 = COCH_3$; $R^2 = CO-(CH\overset{Z}{=}CH)-(CH\overset{E}{=}CH)-(CH=CH)-(CH_2)_2-CH_3$; $R^3 = R^4 = H$

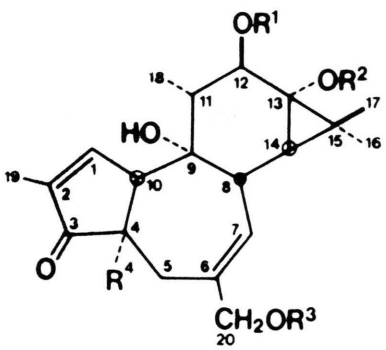
Ti_3 **4** : $R^1 = COCH_3$; $R^2 = CO-(CH=CH)_5-(CH_2)_2-CH_3$; $R^3 = R^4 = H$

Ti_4 **5** : $R^1 = COCH_3$; $R^2 = CO-(CH=CH)_4-(CH_2)_4-CH_3$; $R^3 = R^4 = H$

4-deoxy-DPA **12** : $R^1 = CO-(CH_2)_8-CH_3$; $R^2 = COCH_3$; $R^3 = R^4 = H$

phorbol **13** : $R^1 = R^2 = R^3 = H$; $R^4 = OH$

14 : $R^1 = R^2 = R^3 = COCH_3$; $R^4 = OH$



4-deoxy-4α-phorbol **6** : $R^1 = R^2 = R^3 = H$

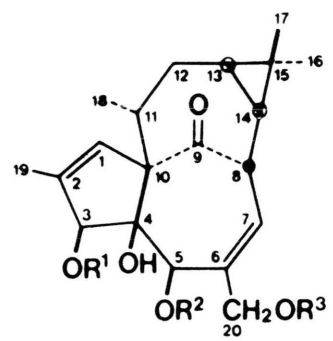
7 : $R^1 = COCH_3$; $R^2 = R^3 = H$

11 : $R^1 = R^2 = R^3 = COCH_3$

8 : $R^1 = CO-(CH=CH)_3-(CH_2)_2-CH_3$; $R^2 = R^3 = H$

α-Ti_1 **9** : $R^1 = CO-(CH\overset{Z}{=}CH)-(CH\overset{E}{=}CH)-(CH=CH)-(CH_2)_2-CH_3$; $R^2 = COCH_3$; $R^3 = H$

α-Ti_4 **10** : $R^1 = COCH_3$; $R^2 = CO-(CH=CH)_4-(CH_2)_4-CH_3$; $R^3 = H$



ingenol **15** : $R^1 = R^2 = R^3 = H$

16 : $R^1 = R^2 = R^3 = COCH_3$

Fig. 3. Chemical structures of diterpene parent alcohols and of corresponding Euphorbia factors, compounds and derivatives thereof.

The mixtures of factors and compounds are compiled in Table III. The mixtures MF_1-MF_6 represent 12,13-diesters of the diterpene parent 4-deoxyphorbol (**1**), the mixtures of compounds MC_1-MC_4 12,13-diesters of the epimeric diterpene parent 4-deoxy-4α-phorbol (**6**; [23]). Both epimeric diterpene esters exhibit characteristic differences in their NMR spectra with respect to the chemical shifts of 1-H (7.60 ppm vs 7.05 ppm), 7-H (5.5 ppm vs 5.15 ppm) and 10-H (3.25 ppm vs 3.50 ppm). The signals at 5.45–5.55 ppm indicate that the secondary hydroxyl function at C-12 as well as the vicinal tertiary hydroxyl function at C-13 of the epimeric diterpene alcohols are esterified (see below). One carboxylic moiety is acetic acid as revealed by the signals at 2.0–2.2 ppm in the NMR spectra. The second acid moiety represents long chain carboxylic acids carrying carbonyl conjugated double bonds as indicated by UV maxima between 267 and 356 nm of the mixtures (Table III) and by gas chromatographic analysis of the fatty acid methyl esters obtained upon base-catalyzed transesterification and hydrogenation of the mixtures (Table III). The signals between 3.95 and 4.00 ppm in the NMR spectra of mixtures MF_1-MF_6 and MC_1-MC_4 indicate a free hydroxyl function at C-20.

Table III. Characterization of *mixtures* MF_1-MF_6 of *Euphorbia factors* and of *mixtures* MC_1-MC_4 of *compounds* from Craig distributions of fractions ET-1 and ET-2 of latex of the South African *E. tirucalli*.

Fraction	Section	Mixtures[a] of factors/ compounds	Yield[b] [%]	ID_{50}[c] [μg/ear]	R_f[d]	Parent alcohol	Molecular ions *m/z*	UV λ_{max} [nm]	GLC[e] Carboxylic acids by hydrogenation	relative amounts
	275–299	MF_1	0.02	0.02	0.4	4-deoxyphorbol	512	267	octanoate	4.6
							538	309	decanoate	1
	325–350	MF_2	0.05	0.004	0.4	4-deoxyphorbol	538	309	decanoate	1
							564	342	dodecanoate	1.9
	450–475	MF_3	0.03	0.007	0.4	4-deoxyphorbol	514	–	octanoate	2.5
							540	267	decanoate	1.5
							566	311	dodecanoate	1
ET-1	525–550	MF_4	0.02	0.003	0.4	4-deoxyphorbol	566	304	dodecanoate	1
							592	342	tetradecanoate	10
	300–324	MC_1	0.004	> 100	0.45	4-deoxy-4α-phorbol	512	268	octanoate	2.5
							538	304	decanoate	1
	376–399	MC_2	0.006	> 100	0.45	4-deoxy-4α-phorbol	564	342	dodecanoate	1
							590	360	tetradecanoate	3.2
	450–475	MC_3	0.002	> 100	0.45	4-deoxy-4α-phorbol	514	–	octanoate	
							540	268	decanoate	n.d.[f]
							566	311	dodecanoate	
	300–329	MF_5	0.02	0.01	0.35	4-deoxyphorbol	512	268	octanoate	1
							538	308	decanoate	2.8
	406–450	MF_6	0.06	0.02	0.35	4-deoxyphorbol	538	309	decanoate	4.6
ET-2							564	340	dodecanoate	2.7
							590	356	tetradecanoate	1
	406–450	MC_4	0.007	> 100	0.4	4-deoxy-4α-phorbol	538	304	decanoate	
							564	336	dodecanoate	n.d.[f]
							590	356	tetradecanoate	

[a] Obtained by column chromatography of subsections of Craig distributions of fractions ET-1 and ET-2 of the latex of *Euphorbia tirucalli* (see Figs. 1 and 2).
[b] Percentages refer to the weight of the acetone extract.
[c] References TPA ID_{50}: 0.01 μg/ear; 4-deoxy-DPA ID_{50}: 0.05 μg/ear.
[d] TLC on silica gel HF_{254} (chamber saturated); solvent system: ethyl acetate/chloroform = 3/2. All mixtures show extinction of fluorescence under UV-light (254 nm) and stain brown with vanillin/sulfuric acid.
[e] Identification of the carboxylic acid methyl esters obtained from mixtures of factors or compounds by transesterification and subsequent hydrogenation by comparison of the retention times with those of authentic references (see Methods).
[f] n.d. not determined.

Selective transesterification with $NaOCH_3/CH_3OH$ removes the acetyl group in positions 13 of the mixtures MF_5, MF_6 and MC_4, yielding 12-monoacylates of 4-deoxy-4α-phorbol (**6**) as indicated by the chemical shift of the vicinal 12-H to higher field (see [23, 30]). In the case of MF_5 and MF_6 the diterpene parent epimerizes concomitantly with transesterification as evidenced by the upfield shift of 1-H and 7-H and the downfield shift of 10-H in the NMR spectra of the 12-monoesters [23]. Transesterification of mixtures MF_1-MF_4 and MC_1-MC_3 yields 12-O-acetyl-4-deoxy-4α-phorbol (**7**), demonstrating that the long chain acyloxy residue is located at C-13. Thus mixtures of factors MF_1-MF_4 comprise 12-O-acetyl-4-deoxyphorbol-13-acylates, mixtures MF_5 and MF_6, 12-O-acyl-4-deoxyphorbol-13-acetates, whereas mixtures MC_1-MC_3 represent 12-O-acetyl-4-deoxy-4α-phorbol-13-acylates and mixture MC_4, 12-O-acyl-4-deoxy-4α-phorbol-13-acetates (Table III). An overview of the diterpene constituents isolated and identified from latex of *E. tirucalli*, ordered systematically with respect to their structures, is given in Table IV. It may be seen from the Table that the esters of 4-deoxy- as well as of 4-deoxy-4α-phorbol are acetates, acylates with long chain acyl moieties of the general structures $CH_3\text{-}(CH_2)_m\text{-}(CH{=}CH)_n\text{-}COOH$ and an overall chain length of $N = 2n + m + 2$. They may be positionally isomeric (*e.g.* MF_1/MF_5 and MC_2/MC_4).

The combined active sections r = 125–199 and r = 200–299 from the Craig distribution of fraction *ET-2* (Fig. 2) consist of unsaturated esters of phorbol (**13**), the active section r = 600–750 of the Craig distribution of fraction *ET-1* of unsaturated esters of ingenol (**15**) as revealed by transesterification and identification of their diterpene parents as phorbol-12,13,20-triacetate (**14**, [27]) and ingenol-3,5,20-triacetate (**16**, [28]), respectively. For the detailed investigation of the positions and the chemical nature of the unsaturated acid moieties carried by the phorbol- (**13**) and ingenol- (**15**) esters, see reference [31]. The diterpene parents phorbol (**13**) and ingenol (**15**) represent 0.8% and 0.006% of the acetone extract, respectively, as compared to 4-deoxyphorbol (**1**) comprising 1.8%.

All the *Euphorbia* factors and compounds isolated from latex of *E. tirucalli* are very labile. This results mainly from the tendency of oxidative polymerization of the unsaturated acid moieties providing also an explanation for the finding that the intact diter-

Table IV. Structural overview of the diterpene constituents isolated and identified from latex of *Euphorbia tirucalli* originating from South Africa.

Euphorbia factor, compound, mixtures	Parent alcohol	Acid moieties in ester groups at C-12	C-13	Structure of the long chain acyl moiety[a] N	m	n
Ti_2 (**3**) Ti_3 (**4**) MF_1 MF_2	4-deoxyphorbol	CH_3CO	$CH_3-(CH_2)_m-(CH{=}CH)_n-CO$	10 14 8,10 10,12	2 2 2,2 2,2	3 5 2,3 3,4
Ti_4 (**5**) MF_3 MF_4	4-deoxyphorbol	CH_3CO	$CH_3-(CH_2)_m-(CH{=}CH)_n-CO$	14 8,10,12 12,14	4 4,4,4 4,4	4 1,2,3 3,4
MC_1 MC_2	4-deoxy-4α-phorbol	CH_3CO	$CH_3-(CH_2)_m-(CH{=}CH)_n-CO$	8,10 12,14	2,2 2,2	2,3 4,5
MC_3 α-Ti_4 (**13**)	4-deoxy-4α-phorbol	CH_3CO	$CH_3-(CH_2)_m-(CH{=}CH)_n-CO$	8,10,12 14	4,4,4 4	1,2,3 4
Ti_1 (**2**) MF_5 MF_6	4-deoxyphorbol	$CH_3-(CH_2)_m-(CH{=}CH)_n-CO$	CH_3CO	10 8,10 12,14	2 2,2 2,2	3 2,3 4,5
α-Ti_1 (**9**) MC_4	4-deoxy-4α-phorbol	$CH_3-(CH_2)_m-(CH{=}CH)_n-CO$	CH_3CO	10 10,12,14	2 2,2,2	3 3,4,5

[a] General structure $CH_3-(CH_2)_m-(CH{=}CH)_n-COOH$ with an overall chain length of $N = 2n + m + 2$.

pene parent alcohols may be obtained by transesterification of the polar non-irritant sections r = 0–225 of fraction ET-1 (Fig. 1) and r = 0–124 of fraction ET-2 (Fig. 2). In addition, part of the sensitivity of the *Euphorbia* factors Ti_1–Ti_4 towards acidic and alkaline conditions is due to the easy and irreversible epimerization at C-4 yielding 12,13-diesters of 4-deoxy-4α-phorbol (**6**) (see Chart and Tables II and IV).

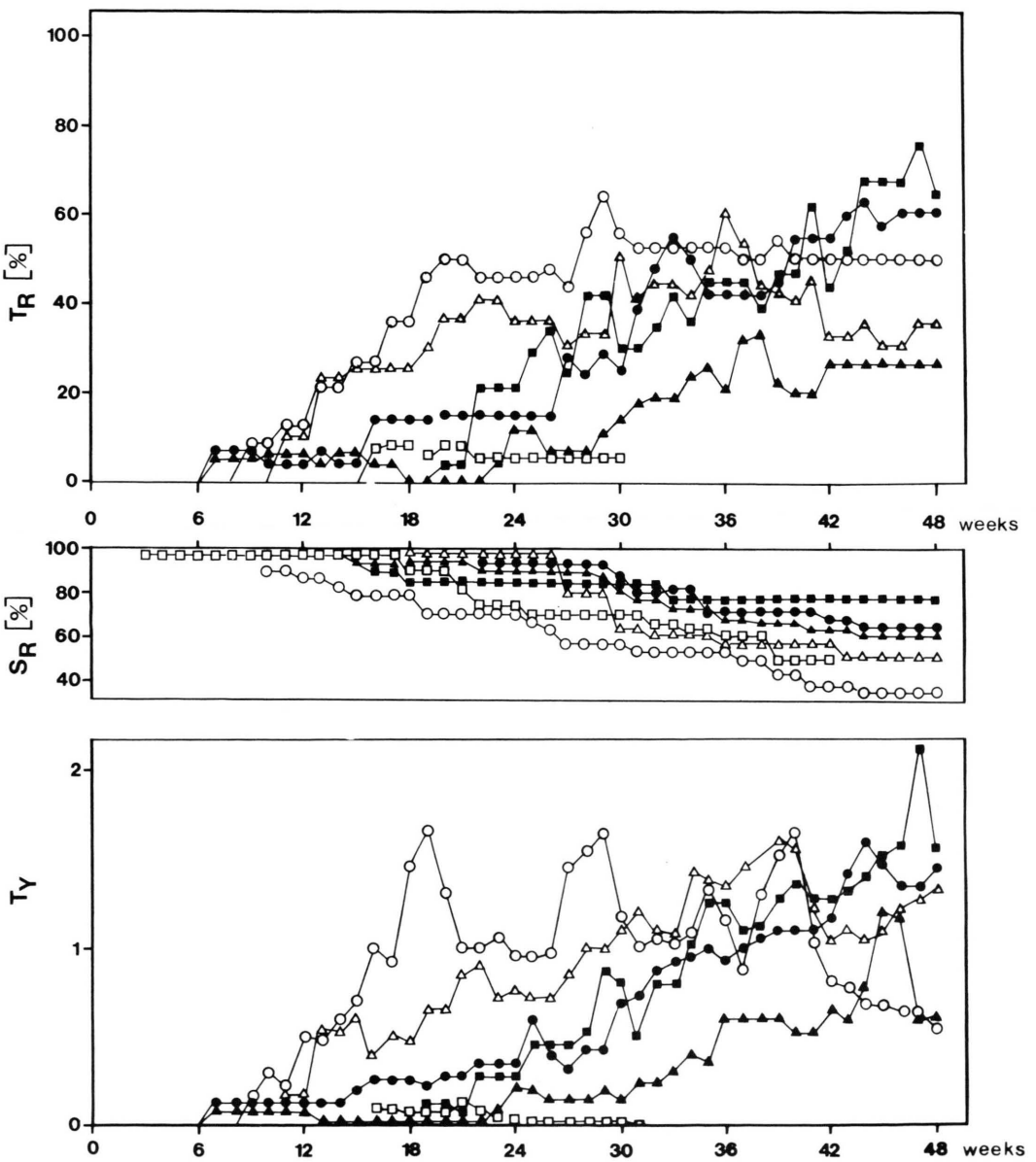


Fig. 4. Time course of the tumor promoting activities of fractions of the separation procedure (see also Chart and Table I) in the standardized assay for tumor promoting activity on the back skin of 14 ♂ and 14 ♀ NMRI mice up to 48 weeks; initiation: one single dose i = 100 nmole of DMBA; promotion: twice a week one single dose of the compound to be tested. Acetone extract, p = 2.5 mg (○, Exp. No. 185); Hydrophobic fraction, p = 2.5 mg (□, 186); Hydrophilic fraction, p = 0.25 mg (▲, 196); Fraction ET-1, p = 12.3 μg (■, 520); fraction ET-2, p = 12.3 μg (△, 283); TPA, p = 1.23 μg (●, 179).

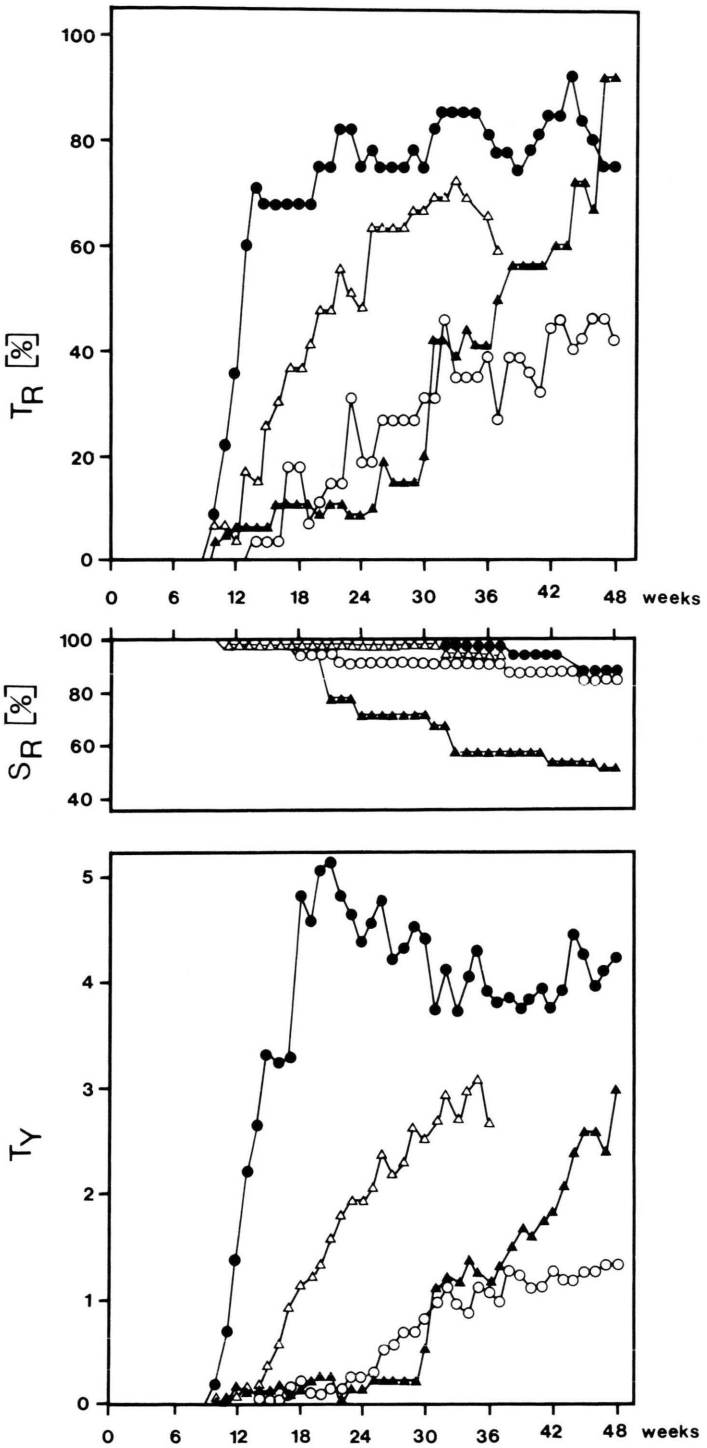


Fig. 5. Time course of the tumor promoting activities in the standardized assay for tumor-promoting activity on the back skin of 28 ♀ NMRI mice up to 36 weeks; initiation: one single dose i = 100 nmol of DMBA; promotion: twice a week a single dose of the compound to be tested. Croton oil factor A_2, DPA, p = 20 nmol (▲, Exp. No. 241); croton oil factor A_1, TPA, p = 10 nmol (●, 503); Ti_1, p = 19 nmol (○, 565); 4-deoxy DPA, p = 18 nmol (△, 630).

Biological activities of fractions of the separation procedure and of the Euphorbia factors Ti_1–Ti_4

The irritant activities increase concomitantly with increasing purification of the active principles up to the pure *Euphorbia* factors (see Chart and Tables II and III). The factors Ti_1–Ti_4 represent highly active irritants as compared to the standard irritant and tumor promoter TPA (Chart and Table II). By selective hydrogenation of the decatrienoyl moiety in *Euphorbia* factor Ti_1 (**2**, Table II), 12-O-decanoyl-4-deoxyphorbol-13-acetate [4-deoxy-DPA; (**12**), Table II, footnote a] is obtained showing about 1/10 of the irritant activity of its unsaturated analogue Ti_1 (see also Table V). The epimeric compounds α-Ti_1 and α-Ti_4 are completely inactive as irritants (Chart and Table II). Mixtures of factors exhibit irritant activities of a similar degree as the pure factors (Table III).

In contrast to the irritant activities of fractions of the separation procedure their tumor promoting activities *decrease* with increasing purification of the factors (Table I and Fig. 4). The *acetone extract* at a single dose of $p = 2.5$ mg displays a tumor promoting activity similar to that obtained by TPA (at $p = 1.23$ μg as a positive control. The *hydrophilic fraction* exhibits a weak to moderate tumor response with one tenth of the dose p of the acetone extract, corresponding to an approximately tenfold purification, whereas the *hydrophobic fraction* at $p = 2.5$ mg is inactive (Fig. 4 and Table I). The highly irritant fractions *ET-1* ($p = 12.3$ μg) and *ET-2* ($p = 12.3$ μg), require the tenfold single dose p to evoke tumor promoting activities comparable with those of TPA ($p = 1.23$ μg). In the tumor promoting assays of the irritant fractions generally lower survival rates (Table I) are observed than in assays with pure *Euphorbia* factors (see below and Table I and Fig. 5).

The *Euphorbia* factor Ti_1 ($p = 10.0$ μg; 19 nmol) exhibits a promoting activity comparable to that of TPA at about 1/8 of the dose of Ti_1 ($p = 1.23$ μg; 2 nmol). Its activity is lower than that of DPA (12-O-decanoylphorbol-13-acetate) [29] at equimolar dose levels ($p = 20$ nmol). As a tumor promoter ($p = 18$ nmol) 4-deoxy-DPA (**12**; Fig. 3) is more potent than Ti_1 when tested in approximately equimolar doses (Table I and Fig. 5). The mixture of *Euphorbia* factors MF_2 (see Table III) displays a tumor response similar to that of Ti_1 in an approximately equal single dose p (Table I). The mixture of the two non-irritant 4-deoxy-4α-phorbol-12,13-diesters (MC_2, see Table IV) turns out to be completely inactive as a tumor promoter (Table I). In all assays in which tumor promoting activity was found, most of the numerous tumors obtained were benign papillomas besides a few malignant tumors (Table I).

Discussion

The present investigation was aimed at the isolation and the chemical and biological characterization of the irritant principles of latex of *E. tirucalli* originating from South Africa. Biologically active and inactive diterpene constituents were identified either in non-separable mixtures or as molecularly uniform factors Ti_1–Ti_4 or epimeric compounds (α-Ti_1 and α-Ti_4). Whereas most of the *Euphorbia* latices, investigated so far, appear to contain either tigliane type *or* ingenane type diterpenes [32], it is remarkable that from *E. tirucalli*, diterpene esters of both types [of phorbol (**13**) and of 4-deoxyphorbol (**1**), as well as of ingenol (**15**)] were obtained. The esters of 4-deoxyphorbol (**1**) dominate.

Subsequent to the first identification of the chemical structure of the diterpene parent 4-deoxyphorbol [23] derivatives thereof have been found in various Euphorbiaceae [33–36]. Sapatoxin A [35] isolated from *Sapium indicum* is identical with *Euphorbia* factor Ti_1. 12-O-[(2*Z*, 4*E*)-2,4-octadienoyl]-4-deoxyphorbol-13-acetate was isolated from *E. tirucalli* latex collected in Colombia [37]. It is also present in latex from *E. tirucalli* as characterized in a mixture (MF_5) with *Euphorbia* factor Ti_1. However, in the Colombian latex the latter, homologous unsaturated diesters could not be detected [37]. Variabilities of this kind, perhaps indicative for the existence of chemical races, are more pronounced in the diterpene profiles of latices of *E. tirucalli* collected in South Africa, in Madagascar, and from a green house cultivation in Heidelberg [38].

The identification of the 4-epimeric, inactive esters α-Ti_1 and α-Ti_4 and of the mixture of similarly inactive 4-deoxy-4α-phorbol esters (MC_1–MC_4) raises the problem of their origin. Most probably they are artefacts generated from 4-deoxyphorbol (**1**) or -esters due to their facile and irreversible conversion to 4α-epimers (**6**) [23, 31]. Identification of 4-deoxy-4α-phorbol and/or its esters, therefore, may be taken as an indication for the presence of 4-deoxyphorbol (**1**) and -esters. – Esters of 4-deoxy-4α-phorbol (**6**) have

Table V. Irritant and tumor-promoting activities of structurally related 4-deoxyphorbol- and phorbol-12,13-diesters regarding unsaturation in the long chain ester group and epimerism or lack of hydroxylgroup at C-atom 4.

Factor/ compound	Irritation ID_{50} [nmol/ear]	Tumor response after 24 weeks[a] single dose p [%]	tumor rate [%]	tumor yield [pap. surv.]	Structure of the long chain fatty acid[b] N	m	n
4-deoxy DPA (**12**)	0.09	18	48	2.0	10	8	0
Ti_1 (**2**)	0.008	19	19	0.4	10	2	3
α-Ti_1 (**9**)	> 100	n.d.	n.d.	n.d.	10	2	3
DPA[c]	0.02	20	25	0.35	10	8	0
TPA	0.016	20	75	4.4	14	12	0
APT[c]	0.01	n.d.	n.d.	n.d.	14	12	0
Ti_2 (**3**)	0.02	n.d.	n.d.	n.d.	10	2	3
Ti_3 (**4**)	0.006	n.d.	n.d.	n.d.	14	2	5
Ti_4 (**5**)	0.005	n.d.	n.d.	n.d.	14	4	4

[a] Data taken from Table I.
[b] General structure $CH_3-(CH_2)_m-(CH=CH)_n-COOH$ with an overall chain length of $N = 2n + m + 2$.
[c] Taken from reference [29].

been isolated also from the seed oil of *Croton tiglium* [27] and of *Sapium indicum* [39].

Some remarkable structure activity relations may be taken from some of the isolated structures selected and compiled in Table V. Thus, it is evident that the presence of a conjugated system of C = C double bonds in the long chain acyl moieties may increase the irritant activity as compared to the saturated ester with the same numbers of C-atoms N (compare 4-deoxy DPA/Ti_1, APT/Ti_3 and APT/Ti_4). In such esters the tumor-promoting activity decreases with increasing number of C = C-bonds in the acyl chain (compare 4-deoxy DPA/Ti_1 of Table V with different examples reported in the literature, *e.g.* references [21, 22, 31, 40]). Moreover, the hydroxyl function at C-4 apparently is not essential for irritant and tumor promoting potencies (compare 4-deoxy DPA/DPA, Table V). However, if tigliane type diterpenes carry a hydroxyl-function at C-4, it has to be free to exhibit irritant and tumor promoting potency [41]. Epimerization at C-4 regarding the hydrocarbon, compare Ti_1 to α-Ti_1 (Table V), leads to a complete loss of irritant and tumor promoting potency. This finding supports the previous interpretation that in tigliane diterpenes trans-connection of the five- and the seven-membered rings is a structural element necessary for biological activity [41, 42].

For the assessment of the potential risk of cancer in using *E. tirucalli* (*e.g.* as an ornamental plant, for folk medicinal drugs and for future resources of biomass), the chemical and toxicological properties of the irritant constituents of the plant are important. Thus, to eliminate toxicological problems, especially with occupational exposure during mass production and handling of the plant, the chemical instability of the irritants identified, which is due to oxidative polymerization in ester moieties and/or to 4-epimerization in the diterpene moiety leading to biologically inactive entities, may be used. Moreover, the structure activity relations regarding irritant and promoting activities as a function of the unsaturation of the acyl chain in the diterpene esters of the plant proved to be stimulating in the analysis of the mechanism of tumorigenesis [43, 44].

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