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ON THE ACTIVE PRINCIPLES OF THE EUPHORBIACEAE, XII.[1] HIGHLY UNSATURATED IRRITANT DITERPENE ESTERS FROM *EUPHORBIA TIRUCALLI* ORIGINATING FROM MADAGASCAR

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ABSTRACT.—The latex of *Euphorbia tirucalli* originating from Madagascar contains as irritant constituents ingenane- and tigliane-type diterpene esters derived from the parent alcohols ingenol and phorbol. The main irritant constituents are isomeric 12,13-acetates, acylates of phorbol as well as 3-acylates of ingenol. 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=2,3,4,5; total number N of C-atoms=2n+m+2). The lack of 4-deoxyphorbol esters in this latex as compared to latex of South African origin is probably indicative of the existence of chemical races of *E. tirucalli*. In the acyl moiety of phorbol esters investigated in detail, an increasing number of C-atoms or an increasing number of double bonds at a fixed number of C-atoms leads to an increase of irritant activity. As compared to their saturated analogs, corresponding unsaturated phorbol esters exhibit similar irritant activities. On the other hand, by an increasing number of conjugated double bonds in the acyl moieties of phorbol esters, the promoting activity is decreased, thus indicating that irritant activity is a necessary, but insufficient, requirement for promoting activity of phorbol esters. An assessment of a potential carcinogenic risk involved in mass production and handling of the plant should point to the very weak tumor-promoting activity and the chemical instability demonstrated for the diterpene constitutents in the latex and hence in all plant parts.

Euphorbia tirucalli L. is a succulent tree of the plant family Euphorbiaceae (1,2) with a long history of utilization by man (1-4). Demonstration of the tumor-promoting potency of an Me_2CO extract of latex of *E. tirucalli* (5) led us to investigate the isolation and characterization of the active principles. This endeavor revealed that latex of *E. tirucalli* of South African origin contains as its main active principles unsaturated acylates, acetates of 4-deoxyphorbol (1). A simultaneous investigation of another latex preparation collected from *E. tirucalli* in Madagascar revealed a different diterpene profile (6). Here, we report on the isolation of the active principles of the Malagasy latex and their chemical and biological characterization. From the latter, important conclusions may be drawn with regard to chemotaxonomy, to assessment of a potential risk of cancer, and to mechanistic aspects of tumorigenesis.

EXPERIMENTAL

PLANT MATERIAL.—The latex of *E. tirucalli* preserved with MeOH was collected in Madagascar. The plant was identified by Mr. Schomerus, Ampanihy-Ouest, Madagascar, and by Prof. Dr. W. Rauh, Botanical Institute, University of Heidelberg. The standardized collection procedure is described elsewhere (7).

SPECTRA.—Mass spectra were measured with a CEC 21-110 B mass spectrometer, ir spectra with a Perkin-Elmer spectraphotometer 521, uv spectra with a Beckman DK 2a far uv spectrometer in MeOH, 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 (δ=0.00 ppm) as internal standard.

ANALYTICAL METHODS.—The methods and machinery of multiplicative distribution have been described previously (8). Merck silica gel HF 254 and PF 254 were used for tlc. The spots were detected under uv light at 254 nm and visualized by heating up to 110° after spraying with vanillin/H_2SO_4. Column chromatography was performed with Merck silica gel 0.05-0.20 mm, deactivated with 13% of H_2O. Gc was performed with a Packard Gas Chromatograph 420, using as stationary phase 5% DEGS on Chromosorb W 80/100 mesh.

[1]For part XI, see Fürstenberger and Hecker (1).

[2]Taken from the dissertation of G. Fürstenberger (6).

TABLE 1. Characterization of Mixtures MF_1-MF_8 of *Euphorbia* factors from Craig Distributions of Latex of *Euphorbia tirucalli* Originating from Madagascar

Fraction	Section	Mixture[a] of factors	Yield[b] [%]	ID_{50}[c]	Rf[d] [μg/ear]	Parent alcohol	Molecular ion [*m/z*]	uv λ max (nm)	glc[e] Carboxylic acids by hydrogenation	Relative amounts
ET-1	126-139	MF_1	0.02	n.d.	0.35	phorbol	528	264	octanoate	3.4
							554	307	decanoate	1
	201-225	MF_2	0.03	0.02	0.35	phorbol	582	310	dodecanoate	n.d.
							608	336	tetradecanoate	
	301-340	MF_3	0.06	0.01	0.40	ingenol	496	310	decanoate	2.6
							522	334	dodecanoate	1
	341-375	MF_4	0.08	0.07	0.40	ingenol	522	330	dodecanoate	2.9
							548	352	tetradecanoate	1
	401-425	MF_5	0.08	n.d.	0.40	ingenol	498	n.d.	decanoate	n.d.
							524		dodecanoate	
	426-480	MF_6	0.02	0.06	0.40	ingenol	524	310	dodecanoate	4.1
							550	336	tetradecanoate	1
ET-2	306-329	MF_7	0.03	0.06	0.3	phorbol	528	260	octanoate	13.2
							554	304	decanoate	1
	441-475	MF_8	0.03	0.03	0.3	phorbol	556	260	decanoate	n.d.
							582	310	dodecanoate	
							608	336	tetradecanoate	

[a]Obtained by column chromatography of subsections of Craig distributions of fractions ET-1 and ET-2 of the latex of *E. tirucalli*.

[b]Percentages refer to the weight of the Me_2CO extract.

[c]Reference TPA, 0.01 μg/ear; n.d. = not determined.

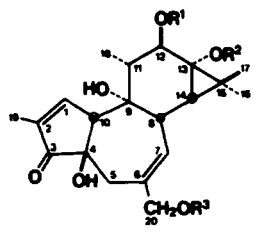
[d]Tlc on silica gel HF 254 (chamber saturation); solvent system EtOAc-$CHCl_3$ (3:2). All mixtures of factors show extinction of luorescence under uv-light (254 nm) and stain brown with vanillin/H_2SO_4.

[e]Glc of carboxylic acid methyl esters obtained from mixtures of factors by base-catalyzed transesterification and subsequent hydrogenation; identification according to authentic references.

Solvent systems used for O'Keeffe distributions: system A, petroleum ether-MeOH-H_2O (15:10:0.4); system B, CCl_4-MeOH-H_2O (2:1:0.1) and for Craig distributions: system C, petroleum ether-MeOH-CCl_4-H_2O (2:1.75:0.4:0.1).

BIOLOGICAL ASSAYS.—Skin irritant activities were determined as irritant dose 50 (ID_{50}) (1,9).

The tumor-promoting activity was determined in the standard assay on the back skin of NMRI mice (1,8,9). Experimental details and the time course of promoting activity, together with survival rates for some of the factors isolated, are given in Table 2 and Figure 2.



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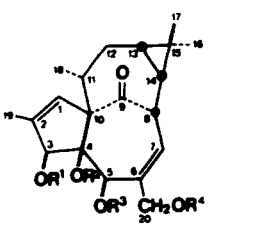

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phorbol	**1**	$R^1=R^2=R^3=H$
Ti_5	**2**	$R^1=CO\text{-}(CH\overset{Z}{=}CH)\text{-}(CH\overset{E}{=}CH)\text{-}(CH_2)_2\text{-}CH_3$; $R^2=COCH_3$; $R_3=H$
Ti_6	**3**	$R^1=CO\text{-}(CH\overset{Z}{=}CH)\text{-}(CH\overset{E}{=}CH)\text{-}(CH=CH)\text{-}(CH_2)_2\text{-}CH_3$; $R^2=COCH_3$; $R^3=H$
Ti_7	**4**	$R^1=CO\text{-}(CH=CH)_5\text{-}(CH_2)_2\text{-}CH_3$; $R^2=COCH_3$; $R^3=H$
Ti_8	**5**	$R^1=CO\text{-}(CH=CH_4)\text{-}(CH_2)_4\text{-}CH_3$; $R^2=COCH_3$; $R^3=H$
Ti_9	**6**	$R^1=COCH_3$; $R^2=CO\text{-}(CH=CH)_5\text{-}(CH_2)_2\text{-}CH_3$; $R^3=H$
	7	$R^1=COCH_3$; $R^2=R^3=H$
	8	$R^1=CO\text{-}(CH=CH)_n\text{-}(CH_2)_m\text{-}CH_3$ (m=2,4; n=2,3,4); $R^2=R^3=H$
	9	$R^1=CO\text{-}(CH\overset{Z}{=}CH)\text{-}(CH\overset{E}{=}CH)\text{-}(CH_2)_2\text{-}CH_3$; $R^2=R^3=H$
	10	$R^1=CO\text{-}(CH\overset{Z}{=}CH)\text{-}(CH\overset{E}{=}CH)\text{-}(CH=CH)\text{-}(CH_2)_2\text{-}CH_3$; $R^2=R^3=H$
ingenol	**11**	$R^1=R^2=R^3=R^4=H$
	12	$R^1=R_3=R_4=COCH_3$; $R^2=H$
	13	$R^1=CO\text{-}R$; $R^2=H$; $R^3=R^4=C(CH_3)_2$
	14	$R^1=R^2=H$; $R^3=R^4=C(CH_3)_2$

FIGURE 1. Chemical structure of diterpene parent alcohols and of corresponding *Euphorbia* factors, and other derivatives thereof.

TERMINOLOGY AND ABBREVIATIONS.—Molecularly uniform, irritant, diterpenoid constituents of the latex are designated as *Euphorbia* factors "Ti_x," mixtures thereof as "MF_x." 12-*O*-Tetradecanoylphorbol-13-acetate is TPA; 12-*O*-octanoylphorbol-13-acetate (OPA); 12-*O*-decanoylphorbol-13-acetate (DPA); 12-*O*-acetylphorbol-13-tetradecanoate (APT); 7,12-dimethylbenz[*a*]anthracene (DMBA).

EXTRACTION, FRACTIONATION AND ISOLATION PROCEDURES.—Of the latex preparation, 514 g was extracted with Me_2CO under N_2 yielding 105 g of Me_2CO soluble material (ID_{50}: 0.1 μg/ear). By means of an O'Keeffe distribution (n=34 transfers) using solvent system A, the Me_2CO (98 g=100%) was separated into the non-irritant hydrophobic fraction (85 g; 87.4% ID_{50}: 50 μg/ear) and the irritant hydrophilic fraction I (10.2 g; 10.5%; ID_{50}: 0.003 μg/ear). The separation of nonirritant fractions (2.7 g: 4.1%; ID_{50}: 36 μg/ear) from the hydrophilic fraction I by an O'Keeffe distribution (n=100 transfers) using solvent system B afforded the hydrophilic fraction II (5.9 g; 6.3%; ID_{50}: 0.001 μg/ear). By an O'Keeffe distribution (n=100 transfers) using solvent system B, the hydrophilic fraction II was resolved into two irritant fractions ET-1 (2.6 g; 2.9%; ID_{50}: 0.005 μg/ear) exhibiting two spots in tlc and ET-2 (3.0 g; 3.4%; ID_{50}: 0.01 μg/ear) exhibiting a single spot in tlc.

Craig distribution of fraction ET-1.—Fraction ET-1 (2.2 g) was subjected to a Craig distribution (z=1020; V=12 ml/10 ml; n=4160 transfers) using solvent system C. According to the bands in the distribution diagram (not shown), the fractions r in the apparatus were combined to yield sections. Almost all irritant activity was found in sections r=126-139, r=140-160, r=201-225, r=301-340, r=341-375, r=401-425, and r=426-480. From these sections, an *Euphorbia* factor Ti_9 (section r=140-160; 39 mg; 0.04%; ID_{50}: 0.02 μg/ear) and mixtures of *Euphorbia* factors MF_1 and MF_6 (see Table 1) were obtained after column chromatography on silica gel using the solvent system Et_2O-petroleum ether (4:1).

Craig distribution of fraction ET-2.—Fraction ET-2 (19.3 g) was separated by means of Craig distribution (z=1020; V=12 ml/10 ml; n=8100) using solvent system C. According to the bands in the distribution diagram (not shown), the fractions r in the apparatus were combined to yield sections. Almost all irritant activity was found in sections r=215-305, r=306-329, r=331-385, r=386-404, r=441-475, and r=476-493. From the sections, four *Euphorbia* factors Ti_5 (section r=215-305; 29 mg; 0.03%; ID_{50}: 0.05 μg/ear), Ti_6 (r=331-385; 3 mg; 0.003%; ID_{50}: 0.04 μg/ear), Ti_7 (r=386-404; 20 mg; 0.02%; ID_{50}:

0.02 μg/ear), and Ti_8 (r=476-493; 49 mg; 0.05%; ID_{50}: 0.01 μg/ear) and mixtures of *Euphorbia* factors MF_7 and MF_8 (see Table 1) were obtained from column chromatography on silica gel using the solvent system Et_2O-petroleum ether (4:1).

PHYSICAL AND CHEMICAL CHARACTERIZATION OF THE *EUPHORBIA* FACTORS.—The physical and chemical characterization of the *Euphorbia* factors Ti_5-Ti_9 is described in Fürstenberger and Hecker (10).

Stored and exposed to air and daylight, Ti_5-Ti_9 decomposed to yield material with lower Rf-values (tlc). Storage of the factors in dilute solutions in Me_2CO under a N_2 atmosphere at −70° and under exclusion of daylight guarantees a reasonable stability.

PHYSICAL AND CHEMICAL CHARACTERIZATION OF MIXTURES OF *EUPHORBIA* FACTORS MF_1, MF_2, MF_7 AND MF_8.—*Mixture* MF_1.—Ms *m/z* 554, 528 (parent ions); uv (MeOH) λ max 267, 307 nm; ^{1}H nmr 1-H, 7.60 (m); H-olefin, 7.5-5.6; 7-H, 5.70 (m); 12-H, 5.55 (d, J=10 Hz); 20-H_2, 4.04 (s); 8-H, 10-H, 3.26 (m); CH_3-CO, 2.12 (s); 19-H_3, 1.76 (m) ppm.

Mixture MF_2.—Ms *m/z* 608, 582 (parent ions); uv (MeOH) λ max 310, 336 nm; ^{1}H nmr 1-H, 7.58 (m); 7-H, 5.70 (m); H-olefin, 7.4-5.6; 12-H, 5.50 (d, J=10 Hz); 20-H_2, 4.00 (s); 8-H, 10-H, 3.28 (m); CH_3-CO, 2.10 (s); 19-H_3, 1.76 (m) ppm.

Mixture MF_7.—Ms *m/z* 554, 528 (parent ions); uv (MeOH) λ max 260, 304 nm; ^{1}H nmr 1-H, 7.58 (m); H-olefin, 7.4-5.5; 7-H, 5.66 (m); 12-H, 5.42 (d, J=10 Hz); 20-H_2, 4.00 (s) 8-H, 10-H, 3.26 (m); CH_3-CO, 2.11 (s); 19-H_3, 1.75 (m) ppm.

Mixture MF_8.—Ms *m/z* 608, 582, 556 (parent ions); uv (MeOH) λ max 260, (310), 336 nm; ^{1}H nmr 1-H, 7.56 (m); H-olefin, 7.4-5.6; 7-H, 5.60 (m); 12-H, 5.46 (d, J=10 Hz); 20-H_2, 4.00 (s); 8-H, 10-H, 3.26 (m); CH_3-CO, 2.10 (s); 19-H_3, 1.76 (m) ppm.

Preparation and identification of phorbol (**1**) *from mixtures* MF_1, MF_2, MF_7, *and* MF_8.—Base-catalyzed transesterification (10^{-2}M sodium methoxide in MeOH) of mixtures MF_1, MF_2, MF_7, and MF_8 for 12 h and subsequent extraction of the neutralized reaction mixtures with n-BuOH yielded **1**. Rf=0.33 (CH_2Cl_2-MeOH, 10:1). The spectroscopic data of **1** were identical with that of an authentic sample (9).

Preparation of 12-O-acetylphorbol (**7**) *from mixtures* MF_1 *and* MF_2.—Base-catalyzed transesterification ($2.5 \cdot 10^{-3}$M sodium methoxide in MeOH) for 72 h at 4° and subsequent extraction of the neutralized reaction mixtures with EtOAc afforded **7**. Rf=0.2 (EtOAc). The spectroscopic data of **7** were identical with that of an authentic sample of 12-*O*-acetylphorbol (11).

Preparation and identification of 12-O-acylates of phorbol (**8**) *from mixtures* MF_7 *and* MF_8.—Base-catalyzed transesterification ($5 \cdot 10^{-3}$M sodium methoxide in MeOH) of MF_7 and MF_8 for 6 h and subsequent extraction of the neutralized reaction mixture with EtOAc and purification by tlc yielded mixtures of 12-*O*-acylates of phorbol (**8**); Rf=0.3 (CH_2Cl_2-MeOH, 10:1).

Transesterification product of MF_7.—^{1}H nmr 12-H, 4.90 (d, J=10 Hz). All other data is as in the nmr spectrum of MF_7 except the signal of CH_3CO. The 12-*O*-acylates of phorbol (**8**) from mixture MF_7 represented 12-*O*-[2*Z*,4*E*-(2,4) octadienoyl]phorbol (**9**) and 12-*O*-(2,4,6-decatrienoyl)phorbol (**10**) as determined by comparison of spectral data of the mixture with that of authentic (**9**) and (**10**) (10).

Product of transesterification of MF_8.—^{1}H nmr 12-H, 4.88 (d, J=10 Hz). All other data is as in the nmr spectrum of MF_8 except the CH_3CO signal.

PHYSICAL AND CHEMICAL CHARACTERIZATION OF MIXTURES MF_3-MF_6.—*Mixture* MF_3.—Ms *m/z* 496, 522 (parent ions); ^{1}H nmr H-olefin, 7.85-5.7; 3-H, 5.61 (s); 20-H_2; 4.15 (s); 5-H, 4.04 (s); 19-H_3; 1.80 (d, J=2 Hz); 16-H_3, 17-H_3, 1.07, 1.03 (2s); 18-H_3, 0.98 (d, J=7 Hz) ppm.

Mixture MF_4.—Ms *m/z* 522, 548 (parent ions); ^{1}H nmr H-olefin, 7.83-5.7; 3-H, 5.61 (s); 20-H_2, 4.15 (s); 5-H, 4.04 (s); 19-H_3, 1.80 (d, J=2 Hz); 16-H_3, 17-H_3, 1.07, 1.03 (2s); 18-H_3, 0.98 (d, J=7 Hz) ppm.

Mixture MF_5.—Ms *m/z* 498, 524 (parent ions); ^{1}H nmr H-olefin, 7.85-5.7; 3-H, 5.62 (s); 20-H_2, 4.15 (s); 5-H, 4.04 (s); 19-H_3, 1.80 (d, J=2 Hz); 16-H_3, 17-H_3, 1.07, 1.03 (2s); 18-H_3, 0.98 (d, J=7 Hz) ppm.

Mixture MF_6.—Ms *m/z* 524, 550 (parent ions); ^{1}H nmr H-olefin, 7.8-5.7; 3-H, 5.60 (s); 20-H_2, 4.15 (s); 5-H, 4.04 (s); 19-H_3, 1.80 (d, J=2 Hz); 16-H_3, 17-H_3, 1.07, 1.06 (2s); 18-H_3, 0.98 (d, J=7 Hz) ppm.

Preparation and identification of ingenol (**11**) *from mixtures* MF_3MF_6.—Base-catalyzed transesterification of mixtures MF_3-MF_6 (each 10^{-2}M sodium methoxide in MeOH for 12 h) and subsequent extraction (EtOAc) of the neutralized reaction mixture yielded ingenol (**11**). Rf=0.35 (CH_2Cl_2-MeOH, 10:1). Compound **11** was characterized by acetylation to ingenol-3,5-20-triacetate (**12**). The spectroscopic data of **12** are identical with that of an authentic sample (12).

Preparation and identification of ingenol-5,20-acetonide (**13**) *from mixtures* MF_3-MF_6.—(a) Preparation of the acetonides from mixtures MF_3-MF_6.

TABLE 2. Tumor-promoting Activity in the Back Skin of NMRI Mice of *Euphorbia* Factors with TPA as Reference[a]

Application			Tumor rate (T_x)				Survival rate (S_R %)		Histologic diagnosis	
Euphorbia factor[b] (Exp. No.)	Single dose p [μg]	Duration of application [weeks]	Tumor bearers/survivors at week				at week		Tumors in located area	
			12	24	36	48	24	48	Total/mice investigated histologically	Malignant tumors in total[c]
TPA (1205)	0.15	48	0	0	0	0	96	79	0/4	0
TPA (719)	1.5	48	4/28	16/28	21/27	16/24	100	86	10/2	0
TPA (503)	6.2	48	13/28	21/28	22/27	18/24	100	86	70/14	3 PEC
Ti_5 (746)	29	48	0/28	3/27			96		9/7	0
Ti_8 (744)	4.9	48	0/28	2/28			100		7/3	0
Ti_8 (1263)	1.2	48	0/28	0/28			100		0/4	0
Ti_7 (745)	9.1	48	1/28	4/28			100		26/8	0

[a]Twenty-eight female NMRI-mice/experiment; as initiator a single dose i = 100 nmole of 7,12-dimethylbenz[*a*]anthracene (DMBA) was administered.
[b]See Table 3.
[c]PEC: squamous cell carcinoma.

Each of the mixtures MF_3-MF_6 was dissolved in small volumes of Me_2CO and treated with catalytic amounts of *p*-toluenesulfonic acid hydrate for 1 h. The reaction was stopped by adding phosphate buffer pH=6.7. Each of the reaction products was extracted with EtOAc. Tlc of each extract yielded a single product: Rf=0.7 (Et_2O-petroleum ether, 4:1). (b) Transesterification of the acetonides obtained from mixtures MF_3-MF_6 affording ingenol-5,20-acetonide (**14**).

TABLE 3. Overview of the Structures of Diterpene Constituents Isolated and Identified from Latex of *Euphorbia tirucalli* Originating from Madagascar. The Long-Chain Acyl Moieties Are of the General Structure CH_3-$(CH_2)_m$-$(CH=CH)_n$-COOH with an Overall Chain Length of N=2n+m+2

Euphorbia factors and mixtures	Parent alcohol	Acid moieties in ester groups		Structure of the long chain acy moiety[a]		
		C-12	C-13	n	m	N
MF_1	phorbol	CH_3CO	CH_3-$(CH_2)_m$-$(CH=CH)_n$CO	2,3	2	8,10
Ti_9				5	2	14
MF_2	phorbol	CH_3CO	CH_3-$(CH_2)_m$-$(CH=CH)_n$CO	3,4	4	12,14
Ti_5				2	2	8
MF_7	phorbol	CH_3-$(CH_2)_m$-$(CH=CH)_n$CO	CH_3CO	2,3	2	8,10
Ti_6				3	2	10
TI_7				5	2	14
MF_8	phorbol	CH_3-$(CH_2)_m$-$(CH=CH)_n$CO	CH_3CO	2,3,4	4	10,12,14
Ti_8				4	4	14
		C-3				
MF_3	ingenol	CH_3-$(CH_2)_m$-$(CH=CH)_n$CO[a]		3,4,5	2	10,12,14
MF_4						
MF_5	ingenol	CH_3-$(CH_2)_m$-$(CH=CH)_n$CO[a]		2,3,4	4	10,12,14
MF_6						

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

Each of the acetonides obtained from mixtures MF_3-MF_6 was transesterified under alkaline conditions ($5 \cdot 10^{-2}$M sodium methoxide in MeOH) for 12 h. Subsequent extraction (EtOAc) of the neutralized reaction mixture afforded, besides unsaturated carboxylic acid, methyl esters (see above), the acetonide **14**, Rf=0.4 (Et_2O-petroleum ether, 4:1). The spectroscopic data of **14** were identical with those of an authentic sample (12).

Gas chromatography of carboxylic acid methyl esters.—*Euphorbia* factors and mixtures MF_1-MF_8 of *Euphorbia* factors were each transesterified (10^{-2}M sodium methoxide in MeOH). The mixtures of carboxylic methyl esters, obtained from the reaction mixture by tlc, were hydrogenated (EtOH/Pd/charcoal, 6 h). The hydrogenation was stopped by filtration of the catalyst. The mixtures of hydrogenated carboxylic acid methyl esters were resolved by glc and identified by co-chromatography of authentic samples (Table 3).

RESULTS

ISOLATION OF *EUPHORBIA* FACTORS AND THEIR MIXTURES.—For the isolation of *Euphorbia* factors and mixtures thereof, a separation procedure was developed similar to that used in fractionation of latex of *E. tirucalli* from South Africa (1). The steps of the fractionation were monitored quantitatively for irritant activity on the mouse ear (9).

Extraction of the methanolic latex preparation with Me_2CO yielded the acetone extract, which was separated by means of O'Keeffe distribution in the hydrophilic fraction I containing essentially all irritant activity and the weakly irritant hydrophobic fraction (see Experimental). The hydrophilic fraction I was subjected to a second O'Keeffe distribution yielding the highly irritant hydrophilic fraction II, in addition to non- or weakly irritant side fractions. By a third O'Keeffe distribution, the hydrophilic faction II was separated into the irritant fractions ET-1 and ET-2.

Both fractions were subjected to multistage Craig distributions yielding various irritant sections from which the irritant *Euphorbia* factors Ti_5-Ti_9 (see Experimental) and

mixtures of *Euphorbia* factors MF_1-MF_8 (Table 1) were isolated by column chromatography.

CHEMICAL CHARACTERIZATION OF *EUPHORBIA* FACTORS AND REMAINING MIXTURES OF *EUPHORBIA* FACTORS.—The elucidation of the chemical structures of the *Euphorbia* factors Ti_5-Ti_9 has been reported briefly (10). Together with other compounds, they are compiled in Figure 1. *Euphorbia* factor Ti_5 is 12-*O*-[(2*Z*,4*E*)-2,4-oc-

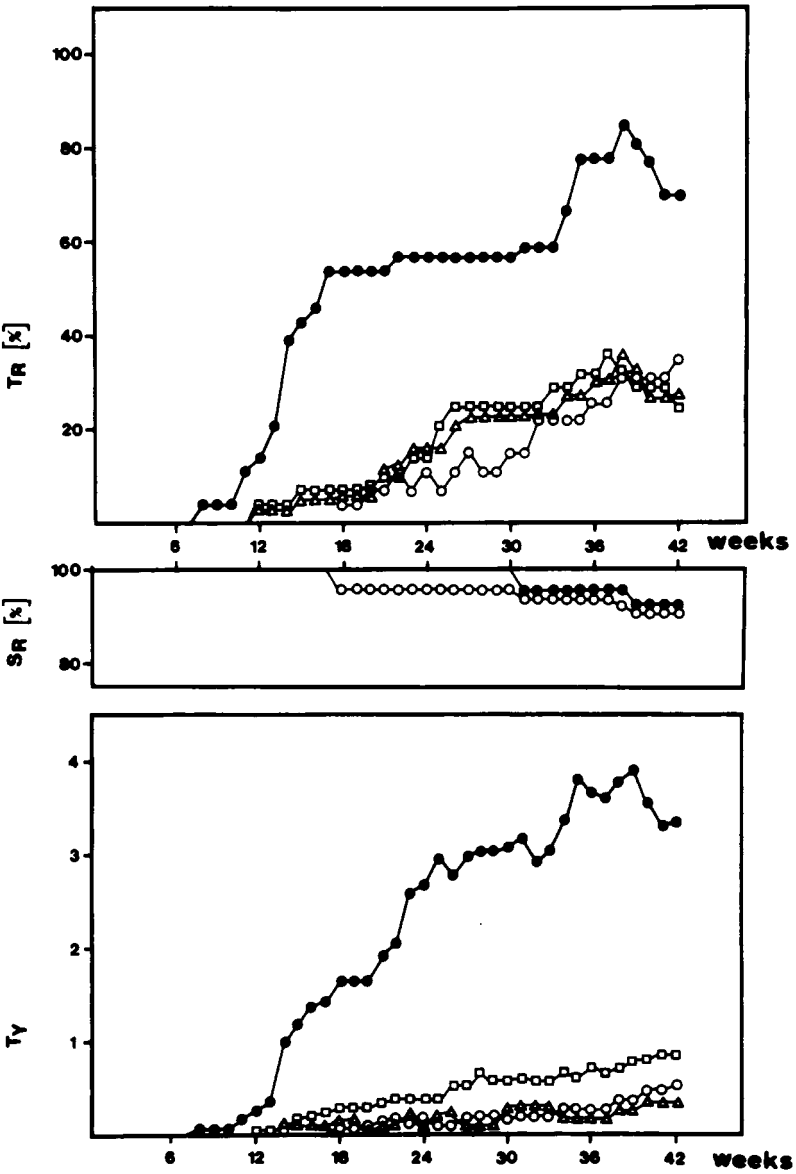

FIGURE 2. Time course of the tumor-promoting activities in the standard assay for tumor promoting activity on the back skin of 28 ♀ NMRI mice up to 42 weeks; Initiation: one single dose i=100 nmole of DMBA; promotion twice a week a single dose of the compound to be tested TPA, p=2.5 nmole (●, Exp. No 719); Ti_5, p=55 nmole (○, Exp. No 746); Ti_7, p=15 nmole (□, Exp. No 745), Ti_8, p=8 nmole (Δ, Exp. No 744). The promoting activity is expressed as tumor rate T_R (number of tumor-bearing animals/survivors in percent) and as tumor yield (number of tumors/survivors).

tadienoyl]phorbol-13-acetate (**2**), Ti_6 is 12-*O*-[(2*Z*,4*E*)-2,4,6-decatrienoyl]phorbol-13-acetate (**3**). Ti_7 represents 12-*O*-(2,4,6,8,10-tetradecapentaenoyl)phorbol-13-acetate (**4**) and Ti_8 12-*O*-(2,4,6,8-tetradecatetraenoyl)phorbol-13-acetate (**5**). *Euphorbia* factor Ti_9 is an isomer of Ti_7 (**4**) with respect to the ester positions representing 12-*O*-acetylphorbol-13-(2,4,6,8,10-tetradecapentaenoate) (**6**).

The mixtures MF_1 and MF_2 as well as MF_7 and MF_8 (Table 1) represent 12,13-diesters of the diterpene parent phorbol (**1**). In their ^{1}H-nmr spectra, the signals at 5.42-5.55 ppm indicate that both the secondary hydroxyl function at C-12 and the vicinal tertiary hydroxyl function at C-13 are esterified (10) by HOAc, as revealed by the nmr signal at 2.0-2.2 ppm and by long chain carboxylic acids carrying carbonyl conjugated double bonds. Thus, uv spectra of the mixtures show maxima between 267 and 356 nm (Table 1). Gc analysis of the carboxylic acid esters obtained upon base-catalyzed transesterification and subsequent hydrogenation yielded mixtures of octanoic, decanoic, dodecanoic, and tetradecanoic acid methyl esters (Table 1). The signals between 4.00 and 4.04 ppm (2H, 20-H_2) in the nmr spectra of mixtures MF_1, MF_2, MF_7, and MF_8 indicate a free hydroxyl function at C-20 of phorbol (**1**).

Selective transesterification with sodium methoxide/MeOH of mixtures MF_1 and MF_2 yielded 12-*O*-acetylphorbol (**7**), demonstrating that the long-chain acyloxy residue is located at C-13. Base-catalyzed transesterification of MF_7 and MF_8 afforded 12-*O*-acylates of phorbol (**8**), as indicated by the chemical shift of the vicinal 12-H to higher field (11) in the nmr spectra of both mixtures. Thus, mixtures of factors MF_1 and MF_2 comprise 12-*O*-acetylphorbol-13-acylates and mixtures MF_7 and MF_8, isomeric 12-*O*-acylphorbol-13-acetates (Tables 1 and 3).

The mixtures of factors MF_3-MF_6 are characterized as monoacylates of the diterpene parent ingenol (**11**) carrying carbonyl conjugated double bonds in the carboxylic acid, as revealed by absorption maxima between 310 and 352 nm in the uv spectra of the mixtures (Table 3). Upon treatment of MF_3-MF_6 with Me_2CO/*p*-tolunesulfonic acid hydrate 5,20-isopropylidene derivatives are formed. Base-catalyzed transesterification of these derivatives yields 5,20-*O*-isopropylideneingenol [**14**, (12)] and unsaturated carboxylic acid methyl esters. After catalytic hydrogenation, the latter were identified by glc analysis as mixtures of methyl-octanoate, -decanoate, -dodecanoate, and -tetradecanoate (Table 1). The sharp singlet at 5.60-5.62 ppm in the nmr spectra of the mixtures MF_3-MF_6 is consistent with the localization of the acyloxy residues at C-3 of ingenol [**11**, (13)]. An additional support for this localization is given by the formation of the 5,20-*O*-isopropylidene derivatives of mixtures MF_3-MF_6. Thus, the mixtures MF_3-MF_6 represent 3-acylates of ingenol (**11**) (Tables 1 and 3).

DITERPENE PROFILES OF LATICES OF *E. TIRUCALLI* FROM DIFFERENT ORIGIN.—The analysis of diterpenoid compounds from latices of *E. tirucalli* revealed that esters of 4-deoxyphorbol which represent the main constituents of latex of South African origin (1) are not present in that originating from Madagascar. Instead, phorbol (**1**) was found to be the predominant diterpene parent. Both latices were shown to contain different amounts of ingenol (**11**). Moreover, latex collected from *E. tirucalli* plants grown in green-houses in Heidelberg exhibited practically no irritant activity (ID_{50}: >300 μg/ear). Under the analytical conditions used, no diterpene parent alcohols could be detected.

BIOLOGICAL ACTIVITIES.—*Irritant activity on the mouse ear.*—With forthcoming purification of the active principles in active fractions, the irritant activities increase (see Experimental section). The *Euphorbia* factors Ti_5-Ti_9 represent highly active irritants (see Experimental section and Table 4) as compared to the standard irritant tumor promoter TPA (ID_{50}: 0.016 nmole/ear). The mixtures of factors MF_1-MF_8 exhibit a similar degree of irritant activity (Table 1).

Tumor promoting activity.—Since the purification of the *Euphorbia* factors followed an established separation procedure (1), individual fractions were not tested for tumor-promoting activity. Also, due to the lack of material, *Euphorbia* factors Ti_6 and Ti_9 were not tested for promoting activity. The *Euphorbia* factors Ti_5, Ti_7, and Ti_8 all exhibit very weak tumor-promoting activity, even when applied ion high doses (Figure 2, Table 2); their biological response is lower than that of TPA even at single doses of p of 10 to 220 times the single dose of TPA. The promoting activity of pure ingenolesters of the type found in mixtures MF_3-MF_6 is described by Opferkuch and Hecker (14).

DISCUSSION

EUPHORBIA FACTORS IN THE LATEX OF *E. TIRUCALLI* ORIGINATING FROM MADAGASCAR.—Isolation and chemical and biological characterization of the irritant principles from latex of *E. tirucalli* originating from Madagascar revealed as main irritant constituents diterpene esters of the tigliane [phorbol (**1**)] and of the ingenane types [ingenol (**11**)]. Quantitatively, phorbol (**1**) esters predominate and were investigated in detail. Biologically active diterpene constituents were identified as molecularly uniform *Euphorbia* factors Ti_5-Ti_9 and, in addition, in the nonseparated mixtures MF_1-MF_8 (Table 1).

Chemically, the *Euphorbia* factors Ti_5-Ti_9 and the mixtures of *Euphorbia* factors MF_1, MF_2, MF_7, and MF_8 represent a new structural type of 12,13-diesters of phorbol carrying, in addition to acetoxy residues, long-chain carboxy groups of the general formula CH_3-$(CH_2)_m$-$(CH{=}CH)_n$-COOH (m=2,4; n=2,3,4,5; N=2n+m+2) (Table 3). Saturated analogues thereof have been isolated from *Croton tiglium* and from other Euphorbiaceae (9,15). Moreover, 12-*O*-(2,4,6)-decatrienoylphorbol-13-acetate, i.e., *Euphorbia* factor Ti_6, was also isolated from *Sapium japonicum* (16) and from *Aquilaria malaccensis* (17).

The same structural type of long chain carboxylic acids CH_3-$(CH_2)_m$-$(CH{=}CH)_n$-COOH (m=2,4; n=2,3,4,5; N=2n+m+2) is present in the ingenol-3-monoesters from the mixtures of *Euphorbia* factors MF_3-MF_6 (Tables 1 and 3). Ingenol-3-(2,4,6-decatrienoate) (m=2, n=3, N=10) was found in latex of *Euphorbia ingens* (13,14) and together with ingenol-3-(2,4,5,8-dodecatetraenoate) (m=2, n=4, N=12) in latex of *Euphorbia helioscopia* (18). Ingenol-3-(2,4,6,8,10-tetradecapentaenoate) (m=2, n=5, N=14) was identified from latex of *Euphorbia lathyris* (19) and from roots of *Euphorbia jolkinii* Boiss. (20). Ingenol-3-(2,4-decadienoate) (m=4, n=2, N=10) was characterized from latices of *Euphorbia kansui* (21), *Euphorbia helioscopia* (18) and together with its 20-acetate from *Euphorbia myrisinites* (22).

STRUCTURE/ACTIVITY RELATIONSHIPS.—*Irritant activity.*—12-*O*-acylphorbol-13-acetates carrying long chain carboxylic acids of the general structure CH_3-$(CH_2)_m$-$(CH{=}CH)_n$-COOH display rising irritant activity with an increasing number of double bonds in carboxylic acids of increasing chain length (factors Ti_5-Ti_7; m=2; n=2,3,5; N=8,10,14; see Table 4). A similar relation was also observed for the irritant activity of saturated 12-*O*-acylphorbol-13-acetates (23). The *Euphorbia* factors Ti_5-Ti_9 are almost as irritant as the corresponding saturated esters [Table 4; see also (25)]. The practically identical irritant activities of Ti_7 and Ti_9 indicate that there is no influence of positional isomerism of the long chain unsaturated carboxylic acid (Table 4), thus supporting results obtained previously with saturated analogs (23).

Tumor promoting activity.—On the other hand, the higher the degree of unsaturation in the ester moiety is, the lower the promoting activity of corresponding 12-*O*-acylphorbol-13-acetates (Table 4) is. This supports results obtained with factors from *E. tirucalli* originating from South Africa (1) and unsaturated phorbol 12,13-diacylates obtained by partial synthesis (25). From these data, it may be concluded that generally

TABLE 4. Irritant and Tumor-promoting Activity of Selected Unsaturated Phorbol-12,13-diesters as Compared with Those of Structurally Analogous Saturated Phorbol-12,13-esters

Factor[a]	Irritation ID_{50} [nmole/ear]	Tumor response after 24 weeks[b]			Structure of the long chain carboxylic acid[c]		
		single dose p [mole]	tumor rate [%]	tumor yield (pap./surv.)	N	m	n
Ti_5 (2)	0.10	55	11	0.19	8	2	2
OPA	0.06	50[d]	30	0.82	8	6	0
Ti_6 (3)	0.07		n.d.		10	2	3
DPA	0.10	20	25	0.35	10	8	0
Ti_7 (4)	0.02	15	14	0.4	14	2	5
Ti_8 (5)	0.03	8	7	0.15	14	4	4
		2	0	0			
TPA	0.016	0.25	0	0	14	12	0
		2.5	57	2.7			
		10	75	4.3			
Ti_9 (6)	0.03		n.d.		14	2	5
APT	0.02		n.d.		14	12	0

[a]12-*O*-octanoylphorbol-13-acetate (OPA); 12-*O*-decanoylphorbol-13-acetate (DPA); 12-*O*-tetradecanoylphorbol-13-acetate (TPA); 12-*O*-acetylphorbol-13-decanoate (APT).

[b]Twenty-eight female NMRI mice were used; as initiator a single dose i = 100 nmole of 7,12-dimethylbenz[*a*]anthracene (DMBA) was administered.

[c]The long chain acyl moieties are of the general structure CH_3-$(CH_2)_m$-$(CH{=}CH)_n$-COOH with an overall chain length of N = 2n + m + 2.

[d]H.J. Opferkuch and E. Hecker, unpublished results.

irritant activity is a necessary but not a sufficient requirement for promoting activity (23-26). In this respect, *Euphorbia* factor Ti_8 (**5**) was considered a model compound of an irritant and hyperplasiogenic phorbol ester exhibiting practically no promoting activity (23,27-29). On the other hand, Ti_8 (**5**) has been found to be a potent second-stage promoter, allowing the subdivision of the process of promotion into two stages, the first being characterized by a single exposure to the promoter TPA and the second by reported exposures to Ti_8 (**5**) (28,29).

TOXICOLOGIC ASPECTS.—The structural elucidation and biological characterization of the irritant plant constituents represent important information for the handling of *E. tirucalli* as an ornamental plant, for utilization in folk medicine, or for large-scale cultivation (1). The weak tumor-promoting activities of the irritant factors identified and their chemical instability may be used to cope with the toxicological problems involved in handling of the plant.

CHEMOTAXONOMIC ASPECTS.—Determination of the irritant constituents in latices collected either in Madagascar or in South Africa (1) established qualitatively and quantitatively different diterpene pattern. Latex obtained from *E. tirucalli* plants grown in greenhouses in Heidelberg contains neither tigliane- nor ingenane-type diterpene esters and does not exhibit irritant activity (6), thereby supporting similar data reported previously in the literature (30). Obviously, the biosynthetic pathways generating irritant diterpene esters depend on the soil and/or the climate in the habitats in which the plants grow. These findings indicate a possible existence of chemical races and may be relevant for chemotaxonomic considerations (31).

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