Diterpene Esters from 'Euphorbium' and their Irritant and Cocarcinogenic Activity[1]

The drug 'Euphorbium' is the air-dried latex of *Euphorbia resinifera* Berg. It has been used as a cathartic and as a constituent of irritant plasters until the early decades of this century[2]. Although much analytical work has been carried out on the resin, resulting in the isolation of a number of biologically inactive triterpenes, the nature of the irritant component(s) remained unknown[3]. In connection with our research program on the distribution and nature of cocarcinogenic principles in the plant kingdom[4], an investigation of the irritant principles of Euphorbium was undertaken[5].

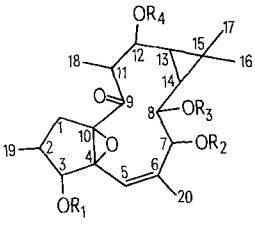
Isolation and identification of compounds. By a combination of multiplicative distribution methods[6] with adsorption chromatography, 3 diterpene fractions R1, R2 and R3 were isolated, which still contained complex mixtures of compounds. In the standard assay on the mouse ear[6], R2 and R3 show irritant activity. R1 does not prove to be irritant.

By preparative TLC (silica gel) of fraction R1, two blackish staining (vanillin/sulfuric acid, V/S)[6], crystalline substances were obtained: **1**: M^+ 610, m.p. 123–124 °C, 0.07% of the resin; **2**: M^+ 640, m.p. 147–148 °C, 0.09% of the resin.

The mass spectrum reveals for **1** the presence of 3 acetate and a phenylacetate group, for **2** three acetate and a methoxyphenylacetate group. This is confirmed by the NMR-spectra (in CCl_4, $\delta_{TMS} = 0.0$ ppm). **1** shows signals for 3 acetyl groups (2.08, 2.05, 2.0 ppm, each 3H, s) and 1 phenylacetyl (7.25 ppm, 5H, s; 3.66 ppm, 2H, s) group. **2** exhibits 3 acetyl groups (2.09, 2.05, 1.95 ppm, each 3H, s), 4 aromatic protons (7.15 ppm, 2H, d; 6.75 ppm, 2H, d), a methoxy group (3.76 ppm, 3H, s) and a methylene group (3.58 ppm, 2H, s), characteristic for a *p*-methoxyphenylacetate. The other signals in the NMR-spectra of the 2 compounds are identical, indicating the presence of the same parent alcohol in both of these esters.

Partial transesterification of **2** with 1.5 equiv. 0.02 *N* KOH/MeOH at −10 °C yields a diacetate-*p*-methoxyphenylacetate (**2c**), a triacetate (**2b**), a diacetate (**2e**) and a monoacetate (**2a**). The MS-, IR-, UV- and NMR-spectra of **2a** are identical with those of ingol-12-acetate as obtained by transesterification of ingol-3, 7,12-triacetate-8-nicotinoate isolated from latex of *E. ingens*[7]. In the NMR-spectrum of the triacetate **2b**, the signal of H-7 is shifted upfield with respect to the corresponding proton of **2** (in CCl_4, $\delta_{TMS} = 0.0$ ppm, 4.08 vs. 4.92 ppm) indicating that the *p*-methoxy phenylacetate moiety of **2** is located at the 7-position of ingol.

Selective transesterification of **1** with 0.1% $HClO_4$/MeOH yields a diacetate-phenylacetate (**2d**). According to the NMR-spectrum of **2d**, the ester groups are located at positions 7, 8 and 12. Subsequent base-catalyzed transesterification of **2d** with 0.05 *N* NaOMe at −10 °C results in the formation of the 8,12-diacetate of ingol (**2e**).



1: $R_2=COCH_2C_6H_5$; $R_1R_3R_4=COCH_3$
2: $R_2=COCH_2C_6H_4$-p-OCH_3 $R_1R_3R_4=COCH_3$
2a: $R_1R_2R_3=H$, $R_4=COCH_3$
2b: $R_1R_3R_4=COCH_3$, $R_2=H$
2c: $R_1=H$, $R_2=COCH_2C_6H_4$-p-OCH_3 $R_3R_4=COCH_3$
2d: $R_1=H$, $R_2=COCH_2C_6H_5$, $R_3R_4=COCH_3$
2e: $R_1R_2=H$, $R_3R_4=COCH_3$

Three resinous, irritant *Euphorbia* factors RR1 (**3**), RR2 (**4**) and RR3 (**5**) staining reddish-brown (V/S), can be isolated from fraction R2 by acid-catalyzed deacetylation, column chromatography, reacetylation and preparative TLC (**3**: M^+ 472, 0.019% of the resin; **4**: M^+ 460, 0.034%; **5**: M^+ 508, 0.014%). Alternatively extensive chromatography of R2 in different solvent systems shows that the compounds occurring naturally are identical with the substances **3–5** obtained as above. However, the isolation procedure with chemical modification provides higher yields of the *Euphorbia* factors than direct isolation.

The mass spectra show for **3** the presence of an acetic acid and a tiglic, dimethyl allylic or angelic acid residue, for **4** an acetic and an isobutyric acid residue and for **5** an acetic and a phenylacetic acid residue. Apart from

[1] Dedicated to Prof. H. HAMPERL, emeritus director of the Institute of Pathology, University of Bonn, Germany, on occasion of his 75th birthday (E.H.).

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Table I. Irritant doses 50 on the mouse ear[6] of the diterpene esters isolated from fractions R2 and R3 of 'Euphorbium'

Croton oil or Euphorbia factor	Structure	ID_{50}^a nMoles/ear
A_1	12-*O*-Tetradecanoyl-phorbol-13-acetate (TPA)	0.016[b]
—	Ingol-7-phenylacetate-3.8.12-triacetate (**1**)	> 460[c]
—	Ingol-7-p-methoxy-phenylacetate-3.8.12-triacetate (**2**)	> 500[c]
RR1	12-Deoxy-phorbol-13-angelate-20-acetate (**3**)	0.18[b]
RR2	12-Deoxy-phorbol-13-isobutyrate-20-acetate (**4**)	0.024[b]
RR3	12-Deoxy-phorbol-13-phenylacetate-20-acetate (**5**)	0.0027[b]
—	Ingenol-3-acylates (mixture)	0.017[c]

Ear reddening was read at 3[b] and 24[c] h after application. Each value is the average of at least 2 independent measurements.
[a] Level of significance $\alpha = 0.05$, standard deviation σ: 1,3.

Table II. Cocarcinogenic effect of diterpene esters from 'Euphorbium' on the back skin of mice[6]

Cocarcinogen	Single dose p [μMoles]	Tumor rate after 12 weeks (% tumor bearers/ survivors)	Tumor yield after 12 weeks (tumors/ survivors)	Tumor rate after 24 weeks	Tumor yield after 24 weeks	Mortality after 24 weeks (%)	No. of experiment
TPA (phorbol-12-tetradecanoate-13-acetate)	0.02	46.5	1.43	66.7	2.09	3	478[a]
12-Deoxy-phorbol-13-angelate-20-acetate (*3*)	0.2	11.1	0.11	11.5	0.12	7	535[b]
12-Deoxy-phorbol-13-isobutyrate-20-acetate (*4*)	0.2	0	0	0	0	28	536[c]
12-Deoxy-phorbol-13-phenylacetate-20-acetate (*5*)	0.1	7.4	0.11	7.15	0.07	50	569[d]
TPA	0.01	59.4	3.21	68	2.52	7	601[e]
Ingenol-3-acylates (mixture)	0.1[g]	42.3	0.89	66.7	1.95	14	541[f]

Numbers of animals per experiment: 28 (females); strain: NMRI. Initiation: 1 application of a single dose i = 0.1 μMole 7.12-dimethylbenz [a] anthracene in 0.1 ml acetone on shaved back skin (~ 6 cm²). Promotion: twice weekly topical applications of single dose p of cocarcinogen in 0.1 ml acetone. Histology of tumors at the end of experiment: [a] No malignant tumor/50 tumors investigated (after 48 weeks of application). [b] 1 malignant/4 tumors investigated (after 48 weeks). [c] No histology. [d] 2 malignant/2 tumors investigated (after 43 weeks). [e] No histology. [f] 2 malignant/30 tumors investigated (after 48 weeks). [g] Based upon an average molecular weight of 516.

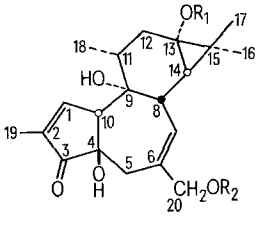
these signals for the fatty acid residues, the NMR-spectra of **3, 4** and **5** are practically identical; these substances are, therefore, diesters of the same parent alcohol.

From its NMR-, IR-, UV- and MS-properties, **4** is identical with 12-deoxy-phorbol-13-isobutyrate-20-acetate, which has previously been isolated from the latex of *E. triangularis*[8].

The NMR-spectrum of **3** (in $CDCl_3$, δ_{TMS} = 0.0) exhibits 2 signals for methyl groups, one at ~2.07 ppm (6H, CH_3C=C (m) and CH_3CO(s), superimposed on a multiplet representing H-11 and H_2-12, and one at ~1.89 ppm (6H, CH_3C=C(m) and CH_3-19 (m)). The olefinic proton centred at 6.19 ppm (1H, qq, $J_{3',5'}$ 2Hz, $J_{3',4'}$, ~7.5 Hz) is characteristic for an angelate[9,10]. Thus, **3** is the 12-deoxy-phobol-13-angelate-20-acetate, the geometrical isomer of 12-deoxy-phorbol-13-tigliate-20-acetate, which was isolated from the latex of *E. triangularis*[8].

The NMR-spectrum of **5** (in $CDCl_3$, δ_{TMS} = 0.0) shows signals attributable to a phenylacetic acid residue (δ = 7.28 ppm, 5H, s; 3.61 ppm, 2H, s) and an acetic acid residue (δ = 2.07 ppm, 3H, s, superimposed on the 3 proton multiplet of H-11 and H_2-12). Thus, **5** is the previously unknown 12-deoxy-phorbol-13-phenylacetate-20-acetate.

The position of the acetate group is confirmed by acid-catalyzed transesterification (0.1% $HClO_4$/MeOH) of **3, 4** and **5** (see above). Thereby, the acetyl group is removed from the allylic oxygen function, as indicated by the shift of the NMR-signal for the vinylic hydroxymethylene group from 4.45 to 4.0 ppm (in $CDCl_3$, δ_{TMS} = 0.0 ppm) and the absence of the 3 proton signal at 2.07 ppm.



3: R_1=$COCH_3H_3$, R_2=COC(=CH_2)CH_3
4: R_1=$COCH(CH_3)_2$, R_2=$COCH_3$
5: R_1=$COCH_2C_6H_5$, R_2=$COCH_3$

Fraction R3 does not show distinct spots on TLC. Further fractionation by column chromatography on silica gel and charcoal columns followed by preparative TLC results in the isolation of a chromatographically relatively uniform resinous mixture of esters of the same parent alcohol (stain: blackish-brown, V/S) representing the entire irritant activity of fraction R3 (0.006%, W) of the resin).

The mass spectrum of the mixture shows M^+ 530, 516, 502. By transesterification with 0.05 *N* NaOMe followed by acetylation, a triacetate is obtained which is identical with ingenol-3, 5, 20-triacetate[11,12]. Treatment of the mixture with acetone/pTsOH produces a corresponding mixture of acetonides (M^+ 570, 556, 542), each containing 1 free hydroxyl. Apart from the signals representing the acyl residues, the NMR-spectrum of this mixture is identical with that of ingenol-3-palmitate-5, 20-acetonide[12]. Therefore, the mixture is composed of esters of ingenol esterified at position 3.

The acyl residues of the mixture of ingenol-3-esters are identified following base-catalyzed hydrolysis, extraction of the acids and methylation with diazomethane. Preparative TLC yields a mixture of 14 methyl esters. By comparison with authentic samples on GC/MS, 6 of them were identified as the methyl esters of 2,6-dimethyl-octanoic acid, 2-methylnonanoic acid, 2,6-dimethyl-nonanoic acid, 2-methyldecanoic acid, 2,6-dimethyl-decanoic acid and 2-methylundecanoic acid. A further peak was tentatively identified by GC/MS as methyl

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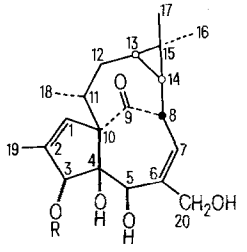
2,6-dimethylundecanoate. 7 other residues were not identified. Thus, the ingenol esters **6** have the structures given in



6: R= $COCH(CH_2)_3CHCH_2CH_3$ (CH_3, CH_3)
$COCH(CH_2)_6CH_3$ (CH_3)
$COCH(CH_2)_3CHCH_2CH_2CH_3$ (CH_3, CH_3)
$COCH(CH_2)_7CH_3$ (CH_3)
$COCH(CH_2)_3CH(CH_2)_3CH_3$ (CH_3, CH_3)
$COCH(CH_2)_8CH_3$ (CH_3)
tentatively
$COCH(CH_2)_3CH(CH_2)_4CH_3$ (CH_3, CH_3)

The occurrence of methyl substituted long chain fatty acids in higher plants is unusual[13]; to our knowledge the only branched long chain fatty acid derivatives isolated from plants are amides of 7-methyl-octanoic and 9-methyl-decanoic acid from Japanese *Capsicum*[14] and methyl-7-methyl-octanoate from hop[15].

Irritant and cocarcinogenic activities. The irritant doses 50 (ID_{50}) of the compounds isolated are given in Table I. For comparison, croton oil factor A_1 (TPA), the main irritant and cocarcinogen from croton oil[6], is used.

As compared to TPA the ingol esters **1** and **2** are not irritant on the mouse ear. The 12-deoxy-phorbol esters are relatively weak irritants and it is interesting to note that, within the variations of the assay, the ID_{50} of **3** is identical with that of the corresponding tigliate[8]. The mixture of ingenol acylates shows considerable irritant activity.

Cocarcinogenic activities were tested in the standard assay[6] on the back skin of mice using TPA for comparison. The results are given in Table II in terms of tumor rates and tumor yields, together with the mortality rates in each experiment. Pronounced necrosis of the skin is observed in the treated area after 2–3 applications of 12-deoxy-phorbol-13-isobutyrate-20-acetate (**4**) and -13-phenylacetate-20-acetate (**5**). Therefore, the mortality of animals in case of **4** and **5** is relatively high. A definite but weak cocarcinogenic effect is exerted by 12-deoxy-phorbol-13-angelate-20-acetate (**3**) (and, perhaps, by **5**). With the mixture of ingenol-3-acylates, the mortality is not exceptionally high. As can be seen from Table II (experiments 541 and 601), $p = 0.1$ μMoles of the mixture appear to be equipotent to $p = 0.01$ μMoles of TPA.

Zusammenfassung. Aus «Euphorbium», dem luftgetrockneten Latex von *Euphorbia resinifera* Berg, wurden 2 neue Ester des tri- und makrozyklischen, polyfunktionellen Diterpens Ingol, 2 neue und ein bereits bekannter Ester des tetrazyklischen polyfunktionellen Diterpens 12-Desoxy-phorbol sowie ein Gemisch von neuen Estern des tetrazyklischen polyfunktionellen Diterpens Ingenol isoliert. Das Gemisch der Ingenolester enthält langkettige, methylverzweigte Fettsäuren. Alle isolierten Ester wurden auf irritierende Wirkung am Mäuseohr und auf cocarcinogene Wirkung an der Rückenhaut der Maus geprüft.

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6 August 1974

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[16] Measurements and stimulating discussions of GC/MS and analytical GC by Dr. Y. NAYA and Dr. Y. HIROSE at the Institute of Food Chemistry, Dojima-naka, kita-ku, Osaka 530, Japan are gratefully acknowledged. Dr. I. KAWASAKI, Osaka University, kindly provided authentic fatty acid esters. Histological investigations and diagnoses of treated skin by Prof. Dr. K. GOERTTLER, Institut für Experimentelle Pathologie, Deutsches Krebsforschungszentrum, are gratefully acknowledged.

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The Analgesic Action of 1-Fluorocodeine

It is well known[1] that introduction of substituents into the aromatic ring of morphine (**1**), codeine (**2**), and their congeners leads to a marked decrease in analgesic effect. For instance, 1-chloro- and 1-bromocodeine show only about $^1/_2$ of the potency of the parent (**2**), and the activity of 1-acetocodeine is likewise markedly smaller than that of (**2**)[1]. A priori, this decrease in pharmacological effect due to the halogen atoms could be caused by their bulk, which might interfere with the proper attachment of the molecule to a binding site on a receptor, or by their electronegativity, which would influence the charge distribution in the aromatic ring, and through this the analgesic action; the diminished effect of 1-acetocodeine could be explained in a similar manner.

To decide between these alternatives, we have prepared the hitherto unknown 1-fluorocodeine (**3**). Since the atom of fluorine is hardly larger than that of hydrogen (van der Waals radii[2]: H, 1.2; F, 1.35 Å), its steric effect should be minimal compared to that of other substituents, which are all significantly larger[2] (e.g. Cl, 1.80; Br, 1.95 Å); on the other hand, this most electronegative of all atoms

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