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A quantitative gas-liquid chromatographic method for phorbol and related diterpenes as their acetates

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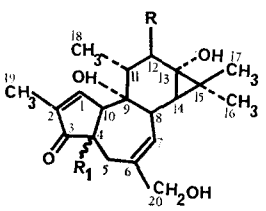
A qualitative and quantitative gas-liquid chromatographic technique has been described for the acetates of the parent alcohols of several irritant and co-carcinogenic factors of the genera *Croton* and *Euphorbia*. Four columns have been found to be useful for the identification of the acetates of phorbol, 4-deoxy-4α-phorbol, 12-deoxyphorbol, ingenol and crotophorbolone. A mixed SE-30, E.G.S. column was used for quantitative analysis using codeine as internal standard. Percentage standard errors of the estimated relative weight ratios of diterpenes to codeine varied from 0·9 to 1·27% for between 21 and 42 estimations. With plant latex samples the total recoveries of added ingenol were 98·3 ± 3%. The method has been used to evaluate the latex of *E. lathyris*, *E. millii*, *E. desmondi*, *E. triangularis* and *E. tirucalli*. The same samples were also assayed by an irritancy biological assay developed by Hecker.

Two genera of the family Euphorbiaceae, *Croton* and *Euphorbia*, are the sources of one or more of a class of diterpenes which have co-carcinogenic effects upon the skin of mice (Hecker, 1971a). This action is due to the esters of phorbol (Hecker, 1971b), the deoxyphorbols (Hecker, 1968) and ingenol (Evans & Kinghorn, 1973a; Uemura & Hirata, 1973). These compounds have been estimated biologically by the Berenblum test on the backs of mice (Hecker, 1971b), an assay which takes up to 30 weeks to complete, or more rapidly in crude extracts by the irritancy assay developed by Hecker (1968). The speed and accuracy of chemical evaluation provides a more convenient approach to this problem and we describe here a qualitative and quantitative technique for several of these compounds as their acetates.

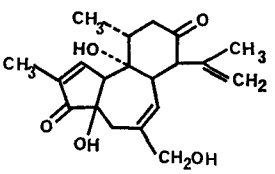
RESULTS AND DISCUSSION

The *Croton* and *Euphorbia* irritant factors are unstable, and macro-methods for their isolation have involved procedures of counter-current distribution and column chromatography (Hecker, 1971a). Because they have a high potency and occur in small yields, quantitative assessments have involved biological techniques (Hecker, 1971b). It has been shown that the acetates of the parent alcohols are sufficiently stable for a microanalysis based upon combined t.l.c.-mass spectrometry, and the acetates are also suitable for gas-liquid chromatography, as little as 10 μg being sufficient for a determination (Evans & Kinghorn, 1973a). Four columns of different polarities were found useful for qualitative evaluation, but in each case the order of separation was the same, the retention time being a function of molecular weight and configuration. Phorbol triacetate (Ia) had the longest retention time (Table 1); removal of the free 4β-hydroxy moiety as in 4-deoxy-4α-phorbol triacetate (Ib)

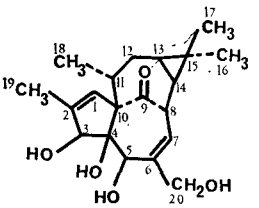
reduced the retention time on all columns. If the molecular weight of phorbol triacetate was reduced by an acetate unit, for example in 12-deoxyphorbol diacetate (Ic), the retention time was shorter still. Ingenol triacetate (III) had the shortest retention time of all four compounds, but differs from 4-deoxy-4α-phorbol triacetate (Ib) in the configuration of rings B and C and the keto-group, which is present at carbon 9 in ring B, and not carbon 3 or ring A as in the phorbols and deoxyphorbols. Crotophorbolone monoacetate (II) is not a natural product but is the compound obtained on hydrolysis and acetylation of 12-deoxy-16-hydroxyphorbol esters extracted from *E. cooperi*. It had poor gas-chromatographic properties and was not estimated quantitatively.



I



II
Crotophorbolone



III
Ingenol

a. R=OH; R_1=4β-OH phorbol.
b. R=OH; R_1=4α-H 4 deoxy-4α-phorbol.
c. R=H; R_1=4β-OH 12-deoxyphorbol.

Determinations of the relative weight ratios of compounds I-IV were made using codeine as internal standard, between 21 and 42 determinations being carried out for each compound. Percentage standard errors were between 0·9 and 1·27% (Table 1, Fig. 1). Replicate analysis of latex samples was used to assess the reproducibility of the assay for all four compounds and, in the case of *E. lathyris*, experiments involving addition of ingenol to latex indicated 98·3 ± 3% recovery of added material.

Table 1. *Gas chromatography of diterpene acetates.*

Compound	*Relative retention column 1	2	3	4	Column 1 relative weight ratio	n	% Standard error
Ingenol triacetate	2·78	2·52	5·58	2·52	1·11	42	1·09
Phorbol triacetate	6·16	5·47	24·4	7·33	0·72	30	1·22
12-Deoxyphorbol diacetate	3·95	3·59	12·6	4·17	1·21	21	0·91
4-Deoxy-4α-phorbol triacetate	4·65	4·16	17·1	5·25	0·85	30	1·27
Crotophorbolone monoacetate	3·23	2·95	13·9	4·24	—	—	—

*Relative to codeine Column 1. 10% SE-30 and 0·05% EGS. Oven temperature 213° nitrogen flow rate 60 ml min^{-1}. Codeine retention time 17·0 min.
Column 2. 10% SE-52. Oven temperature 228° nitrogen flow rate 60 ml min^{-1}. Codeine retention time 29·1 min.
Column 3. 2% QF-1. Oven temperature 186° nitrogen flow rate 60 ml min^{-1}. Codeine retention time 3·65 min.
Column 4. 2% OV-17. Oven temperature 220°, codeine retention time 12·0 min.

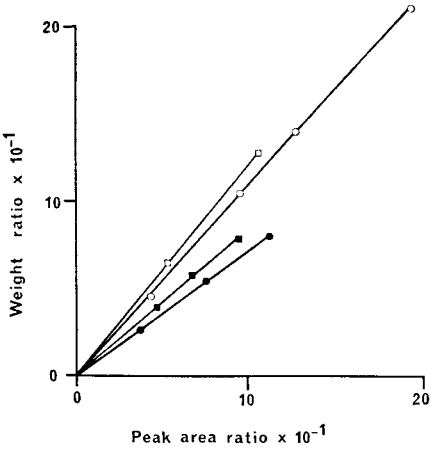


FIG. 1. Determination of relative weight ratio of diterpene to codeine. ○ Ingenol. ■ 4-Deoxy-4α-phorbol. □ 12-Deoxyphorbol. ● Phorbol.

In the plant latex the alcohols are esterified with long and short chain fatty acids, and it is the esterified form which gives rise to toxicological reactions (Hecker, 1971a). Each parent alcohol can give rise to several irritants of varying potency depending upon the nature of the esterifying acids. Nevertheless from Table 2 it is evident that there is some correlation between content of ingenol in *E. lathyris* latex and irritant dose 50% on mice. Samples collected from the same biennial *E. lathyris* plants in successive growing seasons exhibited a two-fold difference in ID50, and in the comparative content of ingenol.

Table 2. *Diterpene content of several irritant* Euphorbia *species.*

	Acetone extract of plant latex	*Diterpene present	ID50 μg per 5 μl	% w/v* diterpene
E. lathyris	1.	Ingenol	10·4	1·67
	2.	Ingenol	5·2	3·24
E. millii		Ingenol	<50	0·09
E. desmondi		Ingenol	8·0	1·05
E. triangularis		12-Deoxyphorbol	<50	0·16
E. tirucalli		4-Deoxy-4α-phorbol	12·5	0·58
E. cooperi		12-Deoxy-16-hydroxyphorbol	11·0	—

*Mean of four determinations per sample. Two separate samples were analysed in each case.

Ingenol was shown to be present for the first time in *E. desmondi*, a cactus-like plant indigenous to Ghana and Nigeria. The latex was more irritant than latex from first year *E. lathyris*, but the content of ingenol was less, suggesting that the ingenol esters of *E. desmondi* are more irritant than those of *E. lathyris*. Three diterpenes have been reported from the latex of *E. tirucalli* (Fürstenberger & Hecker, 1972), however, in the sample available to us, only 4-deoxy-4α-phorbol was isolated. The biological assay of irritancy would not detect differences in parent alcohol content and this underlines the necessity for a chemical evaluation which involves qualitative as well as quantitative assessments.

EXPERIMENTAL CHEMISTRY

Preparation of samples for gas-liquid chromatography

Ingenol triacetate. Ingenol was isolated from *Euphorbia lathyris* as described by Hecker (1968). The triacetate was produced and the ether-soluble oil purified by t.l.c. on silica gel eluting with benzene–hexane–ether–ethyl acetate (20:40:15:30). The recovered material was subjected to a second t.l.c. purification eluting with benzene–hexane–ether (1:1:2). The purified solid recrystallized from methanol, m.p. 195–7°, was found to be chromatographically pure by several solvent systems (Evans & Kinghorn, 1973b). Mass spectrum parent ion at *m/e* 474, ($C_{26}H_{34}O_8$), significant fragment ions at *m/e* 414 (M−60); 401; 372 (M−60+42); 354 (M−120); 341; 336 (M−120+18); 326; 312 (M−120+42); 294 (M−180); 284; 251; 223; 135; 122; 121; 97 and 83 (base peak). Infrared KBr discs, 3430, 1740, 1705, 1640 cm^{-1}. Nmr $CDCl_3$ (tetramethylsilane $\delta = 0{\cdot}00$ ppm) 6·28 (H-7d); 6·09 (H-1S); 5·36 (H-5S); 4·98 (H-3S), 4·51 and 4·08 (H_2-20); 4·27 (H-8d); 3·22 (1 OH deuterium exchange); 2·21, 2·12, 2·0 ($3CH_3CO$); 1·78 (H_3-19); 1·05-1·10 ($3CH_3$-) ppm. C.D. 202 nm $[\theta] = +21780$; 210 nm $[\theta] = +5148$; 224 nm $[\theta] = -20064$; 300 nm $[\theta] = +3003$: solvent methanol.

Phorbol triacetate. Phorbol, obtained from Schuchardt (Munich), m.p. 249-50°, gave one spot by t.l.c. (Crombie, Games & Pointer, 1968), mass spectrum parent ion *m/e* 364, $C_{20}H_{28}O_6$; C.D. 204 nm $[\theta] = -27291$; 229 nm $[\theta] = +53295$; 270 nm $[\theta] = -5181$; 340 nm $[\theta] = -3894$; solvent methanol. The triacetate was produced and purified as before, m.p. 120-1°; it gave one spot by t.l.c. (Evans & Kinghorn, 1973b). Mass spectrum, molecular ion at *m/e* 490 and fragment ions at *m/e* 430 (M−60); 388 (M−60+42); 387; 370 (M−120); 352 (M−120+18); 328; 310 (M-180); 292 (M−180+18); 282; 267; 227; 215; 199; 173; 159; 145; 133; 125; 123; 121; 109; 95; 91; 83 (base peak).

12-Deoxyphorbol diacetate. A small sample was received from Professor Hecker and purified by t.l.c. as before, m.p. 138°. The diacetate produced one spot by t.l.c. (Evans & Kinghorn, 1973b), mass spectrum, small parent ion *m/e* 432 and significant fragment ions at *m/e* 414 (M−18); 401; 372 (M−60); 354 (M−60+18); 336 (M−60+36); 312 (M−120); 294 (M−120+18); 284; 266; 253; 251; 241; 233; 223; 190; 177; 161; 151; 135; 122; 121; 107; 93 and 83 (base peak).

4-Deoxy-4α-phorbol triacetate. The parent alcohol was obtained from Professor Hecker (pure by t.l.c.) and acetylated and purified as before. The triacetate gave one spot by t.l.c. (Evans & Kinghorn, 1973b). Mass spectrum, a small molecular ion at *m/e* 474, a small M^+-18 ion at *m/e* 456 and significant fragmentation ions at *m/e* 414 (M−60); 396 (M−60+18); 372 (M−60+42); 354 (M−120); 312 (M−120+42); 294 (M−180); 279; 199; 125; 97 and 83 (base peak).

Crotophorbolone monoacetate. This was synthesized from phorbol as described by Crombie & others (1968). It had m.p. 227-9°, and was pure by t.l.c. (Gschwendt & Hecker, 1970); it was acetylated and purified as described previously. The monoacetate, m.p. 104-5°, was pure by t.l.c (Evans & Kinghorn, 1973b). Mass spectrum small molecular ion at *m/e* 388 and a small M^+-18 ion at *m/e* 370; significant fragment ions at *m/e* 328 (M−60); 310 (M−60+18); 292 (M−60+36); 267; 251; 241; 223; 208; 207; 179; 137; 122; 121 (base peak); 109; 91 and 83.

Gas-liquid chromatography

Four columns were used for qualitative analysis (Table 1). Column 1 was used for all quantitative work at 213° and a nitrogen flow rate of 60 ml min^{-1}. Standard solutions in several concentrations of compounds I to IV were prepared in ethanol containing codeine as internal standard. The relative weight ratio of diterpene acetate/codeine was determined with several solutions of different weight ratios. (Table 1. Fig. 1).

Extraction and determination of error of g.l.c. analysis of diterpenes

Samples of *E. lathyris* latex were divided into two equal portions by weight and to each was added a known weight of ingenol. The portions were extracted separately with acetone until the residue showed no irritant activity on the ears of mice. About 50 mg of the acetone-dried extract was accurately weighed and dissolved in 20 ml of methanol–water (17:3) and partitioned with 2 × 5 ml of hexane to remove lipids. The residue from the alcoholic phase was hydrolysed in dry methanol at ambient temperature for 30 min with 0·5 M KOH. Water (4 ml) and methanol (5 ml) were added and ingenol extracted with 30 ml of methylene dichloride in aliquots. On removal of the solvent the residue was acetylated at 100° for 1 h with 400 μl of pyridine and 100 μl of acetic anhydride. The reagents were removed in a nitrogen stream and the triacetate partitioned between water–methanol (2:1) and ether. The ether layer was reduced to dryness and the residue dissolved in a 1 ml ethanolic solution of codeine (0·07% *w/v*) for g.l.c. using column 1 (Table 1). Peak areas were estimated by the product of the height × width at half height. The diterpene content of the irritant fractions of *E. millii*, *E. desmondi*, *E. triangularis* and *E. tirucalli* was similarly estimated.

Biological analysis

Acetone dried latex samples were estimated by means of the irritancy test of Hecker (1968).

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