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THIN-LAYER CHROMATOGRAPHIC BEHAVIOUR OF THE ACETATES OF SOME POLYFUNCTIONAL DITERPENE ALCOHOLS OF TOXICOLOGICAL INTEREST

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SUMMARY

A thin-layer chromatographic method is described for polyhedric diterpene acetates derived from irritant and co-carcinogenic principles from the *Croton* and *Euphorbia* genera of the family Euphorbiaceae. The method consists of an acetylation procedure for the free alcohols followed by thin-layer chromatographic separation and colour reactions on silica gel and alumina with several different solvent mixtures and sprays. Migration is governed by the number and stereochemical configuration of the free hydroxyl groups in rings A and B of the tetracyclic structure and to a lesser degree by the number and configuration of acetate moieties. This provides a rapid micro-screening procedure for these diterpenes, which is readily supported by mass spectra of small quantities recovered from thin-layer plates.

INTRODUCTION

Recent work has demonstrated that the co-carcinogenic and irritant constituents of *Croton tiglum* seed oil and *Euphorbia* latices are esters of a new group of polyhedric diterpene alcohols such as phorbol[1], the deoxy-phorbols[1] and ingenol[1–3]. The parent alcohols are sensitive to light, oxygen, acid and alkaline conditions, and macro-methods for their isolation involved prolonged procedures of counter-current distribution and column chromatography[4]. This communication describes a rapid micro-method for their identification based on thin-layer chromatography (TLC) and colour reactions on the chromatograms. Attempts have been made to correlate TLC migration rates with the structural features of these diterpenes.

EXPERIMENTAL

Preparation of samples for thin-layer chromatography

Phorbol 12,13,20-triacetate. Phorbol was obtained from Schuchardt (Munich, G.F.R.), and produced one spot by TLC[5]. Five milligrams were acetylated for 1 h at 100° in pyridine–acetic anhydride (4:1). A single triacetate was produced as dem-

TABLE I

hR_F VALUES OF DITERPENE ACETATES

Solvent systems: S_1 = Chloroform–ether (95:5).
S_2 = Ether–ethyl acetate–hexane (1:1:1).
S_3 = Hexane–isopropyl alcohol (2:1).
S_4 = Chloroform–ethyl acetate (2:3) (ref. 6).
S_5 = Toluene–ethyl acetate (9:2).
S_6 = Chloroform–acetone–benzene (95:5:50).
S_7 = Chloroform.
S_8 = Hexane–ether–benzene (1:2:1), eluted three times.
S_9 = Benzene–hexane–ether–ethyl acetate (20:40:15:30), eluted three times.
S_{10} = Chloroform–ether–benzene (1:3:3), eluted three times.
S_{11} = Ethyl acetate–benzene (1:3), eluted four times.

	hR_F values							
	Phorbol triacetate	*12-Deoxy-phorbol diacetate*	*4-Deoxy-4α-phorbol triacetate*	*4α-Phorbol triacetate*	*4α-Phorbol tetraacetate*	*Crotophorbolone monoacetate*	*Ingenol triacetate*	*Compound A_1*
Silica gel G								
S_1	16	15	21	7	26	8	44	3
S_2	48	51	52	26	49	34	65	31
S_3	64	68	55	44	57	60	72	71
Silica gel H								
S_4	49	50	53	39	55	41	59	33
Alumina E								
S_5	5	6	12	1	11	2	32	2
S_6	31	33	51	4	54	10	69	7
S_7	53	53	72	10	76	20	80	6
S_8	6	10	11	1	7	2	37	1
S_9	30	38	52	3	45	11	70	6
S_{10}	22	29	36	2	43	7	74	4
S_{11}	35	39	59	5	51	11	86	9

onstrated by TLC and by gas–liquid chromatography (GLC) on SE-30. The mass spectrum had a 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; 91; 93; 95 and 83 (base peak).

12-Deoxy-phorbol 13,20-diacetate. A small sample, received from Professor Hecker, was purified by TLC on silica gel G buffered in phosphate buffer, pH 7.0, and activated at 110° for 30 min. The diacetate produced one spot by TLC and one peak by GLC. The mass spectrum exhibited a molecular ion at *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 12,13,20-triacetate. The parent alcohol (pure by TLC) was acetylated as previously described. The single triacetate gave one spot by TLC and one peak by GLC. The mass spectrum exhibited a small molecular ion at *m/e* 474, a small M—18 ion at *m/e* 456 and significant fragment 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).

4α-Phorbol 12,13,20-triacetate and 4α-phorbol 4,12,13,20-tetraacetate. 4α-Phorbol, obtained from Professor Hecker, produced one spot by TLC. After acetylation three compounds were produced by TLC[6]. The mass spectrum of the bottom spot corresponded to 4α-phorbol 12,13,20-triacetate and had a molecular ion at *m/e* 490 and significant fragment ions at *m/e* 471; 452; 430 (M—60); 412 (M—60 — 18); 388 (M—60 — 42); 370 (M—120); 352 (M—120 — 18); 328; 310 (M—180); 292 (M—180 — 18); 282; 267; 259; 227; 215; 199; 187; 175; 173; 171; 159; 125; 109; 95; 91 and 83 (base peak). The centre spot had a similar R_F value and mass spectrum to phorbol triacetate and the top spot to the 4α-tetraacetate. In the 4α configuration the tertiary hydroxy group is not sterically hindered during acetylation.

Crotophorbolone monoacetate. Crotophorbolone monoacetate was synthesised from phorbol as described by Crombie *et al.*[7]. Crotophorbolone was partitioned from water–methanol into methylene chloride, acetylated and purified by TLC. The monoacetate gave one spot by TLC and one peak by GLC. The mass spectrum had a small molecular ion at *m/e* 388, a small M—18 ion at 370 and 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.

Ingenol 3,5,20-triacetate. A small sample, received from Professor Hecker, was pure by TLC and GLC. The mass spectrum had a small molecular ion at *m/e* 474 and 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).

Compound A_1 (12-O-tetradecanoyl-phorbol 13-acetate). This compound was obtained from Schuchardt (Munich, G.F.R.).

Thin-layer chromatography

Layers. Silica gel G, silica gel H and alumina E layers were used. The layers were activated at 110° for 30 min. The solvent systems applied are listed in Table I.

Reagents. For visualisation of spots the plates were sprayed with one of the following reagents: Reagent A —60% w/w sulphuric acid. The plates were heated for 15 min at 110°. Reagent B —5% w/v vanillin in concentrated sulphuric acid,

TABLE II

COLOURS OF DITERPENE ACETATES WITH ACID SPRAYS ON SILICA GEL AND ALUMINA

Viewed in daylight and under UV light at 366 nm. For reagents A–D, see text.

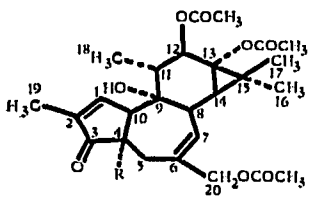
	Phorbol triacetate	*12-Deoxy-phorbol diacetate*	*4-Deoxy-4α-phorbol triacetate*	*4α-Phorbol triacetate*	*4α-Phorbol tetraacetate*	*Crotophor-bolone monoacetate*	*Ingenol triacetate*	*Compound A_1*
Silica gel								
Reagent A								
Daylight	Orange	Red-brown	Yellow-brown	Blue-grey	Grey	Pink	Olive-brown	Brown
UV light	Orange	Pink-brown	Yellow	Yellow-brown	Yellow	Orange	Yellow	Yellow-brown
Reagent B								
Daylight	Purple-brown	Grey-brown	Purple-blue	Blue	Blue	Purple	Grey-brown	Purple-black
UV light	Blue	Dull red	Blue	Dark blue	Dark blue	Blue	Yellow	Dark blue
Reagent D								
Daylight	Red-purple	Orange-brown	Mauve	Blue	Grey-blue	Olive-brown	Yellow-brown	Purple-brown
UV light	Yellow-brown	Pink	Yellow-brown	Pink	Faint pink	Orange	Yellow	Yellow-brown
Alumina								
Reagent A								
Daylight	Orange	Red-brown	Yellow	Yellow-brown	Olive-brown	Orange	Yellow-brown	Brown
UV light	Yellow-brown	Orange	Yellow-brown	Yellow-brown	Yellow-brown	Yellow-brown	Yellow	Yellow-brown
Reagent B								
Daylight	Blue-grey	Grey-green	Blue-purple	Blue-black	Blue-black	Blue-green	Grey-brown	Blue-brown
UV light	Blue-brown	Blue-brown	Blue-brown	Dark blue	Dark blue	Blue	Yellow-brown	Dark blue
Reagent C								
Daylight	Pink	Red-brown	Yellow-brown	Brown	Olive-brown	Pink	Olive-brown	Orange-brown
UV light	Yellow-brown	Dull red	Light yellow	Orange	Yellow-brown	Orange	Light yellow	Yellow-brown

The plates were heated for 5 min at 110°. Reagent C —1 % w/v anisaldehyde–2 % v/v sulphuric acid in glacial acetic acid. The plates were heated for 10 min at 110°. Reagent D —methanol—sulphuric acid (1:1). The plates were heated for 15 min at 110°. All plates were viewed in daylight and under UV light at 366 nm.

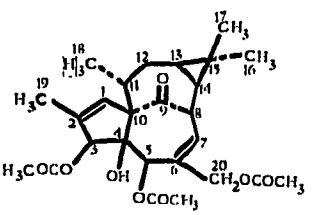
RESULTS AND DISCUSSION

The acetates of the diterpenes (see formulae I–IV) fall into two groups, the phorbol type having lower hR_F values than ingenol. This provides a rapid means of classification of structural types. In the case of silica gel the resolution within the phorbol group is poor, the individual compounds migrating within an hR_F range of only 17–20. This observation is characteristic of non-alkaloidal, polyfunctional compounds on strong adsorbents[8]. An alteration in solvent composition merely increases or decreases the migration distances of the group and has little effect upon group resolution (Table I). Alumina in this case acted as a weaker adsorbent, sensitive to molecular shape as well as to molecular weight. By means of suitably altering the composition of the eluting solvent it was possible not only to separate the ingenol and phorbol types, but also to obtain a three times greater resolution within the phorbol group. If a relatively non-polar solvent mixture is used and the plate repeatedly eluted with adequate drying between runs, then the resolution can be improved still further (Table I).

The phorbol groups of diterpenes have a tetracyclic structure, their migration by TLC being a function of the oxygen-containing moieties at carbons 3, 4, 9, 12, 13 and 20. In phorbol triacetate itself the 1–2 unsaturated tertiary 3,4-ketole group connects the five- and seven-membered rings in *trans* configuration, the tertiary hy-



(I)



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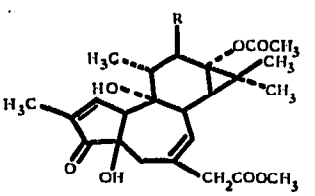
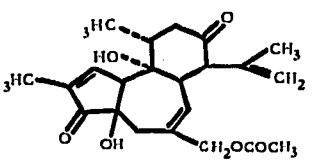


(II)



(IV)

(I) 4-Deoxy-4α-phorbol triacetate, R = H
4α-Phorbol triacetate, R = OH
4α-Phorbol tetraacetate, R = $OCOCH_3$

(II) Phorbol triacetate, R = $OCOCH_3$
12-Deoxy-phorbol diacetate, R = H

(III) Ingenol triacetate

(IV) Crotophorbolone monoacetate

droxyl group at C-9 interlinks the six- and seven-membered rings, the other oxygen moieties being present as acetates at C-12, C-13, and C-20 (ref. 6). hR_F values within this group are influenced by rings A and B, the main functional groups being situated in this part of the molecule. Reference to Dreiding models indicates that adsorption possibly takes place by means of the 9α-tertiary hydroxyl group and also by the tertiary hydroxyl group at C-4. In the case of 4α-phorbol triacetate the 4α-hydroxyl group is on the same side of the molecule as the 9α-hydroxyl group and its influence on migration is considerable, as can be seen from the increase in hR_F values for 4-deoxy-4α-phorbol triacetate, in which the hydroxyl group is replaced with a 4α-hydrogen atom, and also for 4α-phorbol tetraacetate, which is acetylated at the 4α-position. Phorbol triacetate carries a 4β-hydroxyl group orientated on the other side of the molecule and therefore it migrates further up the TLC plate than 4α-phorbol triacetate. The esters of the 12–13 glycol group of ring C also provide alternative sites for adsorbent binding, the α-orientated C-13 ester group being the major contributing site. Hecker's compound A_1 is a long-chain ester at C-12 and an acetate at C-13, compound B_1 being the reversed case[6]. The lower hR_F values of A_1 are explained either by steric hindrance in B_1 of the C-13 ester oxo group, or by the greater electron-withdrawing ability of the long aliphatic chain at this position. The effect of the β-orientated C-12 ester group on adsorption is slight, but when removed as in the case of 12-deoxy-phorbol diacetate, the decrease in polarity is sufficient just to separate it from phorbol triacetate. The C-12 and C-13 ester groups are replaced in croto-phorbolone by a C-13 oxo group and the strained cyclopropane ring D has opened to give a C-14 isopropylene side chain. This results in a stronger C-13 site for adsorption, a lower molecular weight and consequently a low hR_F value. In all systems ingenol triacetate migrates furthest up the plate. The configuration of rings A and B are similar to phorbol but the 3-keto group has been replaced by acetate and no α-orientated tertiary hydroxyl group is present at C-10 on the junctions of rings A and B, thereby explaining its migration characteristics.

These diterpene acetates produce characteristic colours both in daylight and under UV light when sprayed with several acid-based sprays. The type of adsorbent used will affect these colours, as can be seen from Table II. Nevertheless, with the range of solvent systems and several sprays of this type, the possible presence of these compounds may be detected in extracts. On duplicate runs sufficient material (10–15 μg) may be obtained for confirmatory mass spectrometry. This provides a rapid and sensitive technique for screening plants or latex for the presence of co-carcinogenic irritants of the phorbol and ingenol type.

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