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Skin irritants of *Euphorbia fortissima*

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By means of a combination of partition and chromatographic methods six irritant constituents were isolated from the fresh latex of *Euphorbia fortissima*. Compounds A-D were di-esters of the common parent diterpene 12-deoxyphorbol, and compounds E and F were mono-esters of the same diterpene. The fresh latex had an irritant dose 50% (ID50) on mice of 0·64 $\mu g\ \mu l^{-1}$. Compounds A-D are short-acting irritants reaching a maximum activity within 4 h of application to the skin, whilst the monoesters maintained potent irritant effects for up to 24 h. Selective hydrolysis of the di-esters at the C–20 primary ester group also produced mono-esters of greater potency after 24 h. An increase in the length of the fatty acid located at C–13 produced greater biological activity in both the mono- and di-ester groups.

In 1947, Berenblum & Shubik were able to show that croton oil, although not a true chemical carcinogen, was able to promote sub-carcinogenic doses of true carcinogens, thereby producing skin sarcomas. Roe & Peirce (1961) later demonstrated that the latex of several species of *Euphorbia* possessed the actions of co-carcinogenicity. On this work was elaborated the controversial "two stage" theory of initiation of skin tumours (Berenblum, 1954). The isolation of diterpene esters of croton oil by Hecker, Bartsch & others (1967) has shed light on the types of chemical structure which possess co-carcinogenic activity. All naturally occurring co-carcinogens of this group are potent skin blistering and reddening agents and if taken internally act as drastic purgatives. Although skin irritancy is a useful guide to co-carcinogenic activity, not all diterpene skin irritants are necessarily co-carcinogenic (Gschwendt & Hecker, 1971).

Euphorbia fortissima is a succulent species indigenous to South and East Africa and the latex is well known in native medicine as a counter irritant. We describe in this communication the isolation of six skin irritants from *E. fortissima* latex.

RESULTS AND DISCUSSION

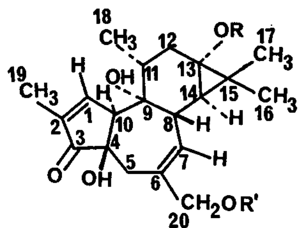
Fresh latex of *E. fortissima* was examined by means of biological analysis using the mice ear irritancy assay as part of a general screening procedure for naturally occurring irritants (Evans & Kinghorn, 1973a). The acetone-soluble dried latex was found to have an irritant dose 50% (ID50) on mice of 0·64 $\mu g\ \mu l^{-1}$ and was of a similar potency to several other *Euphorbia* species (Evans & Kinghorn, 1973a). It differed from other species available to us in that the maximum irritant effects were developed within 4 h of application to the skin and were not maintained for up to 24 h as previously recorded (Hecker, 1968; Evans & Kinghorn, 1973a).

Partition of the polar fraction of the latex against hexane increased the potency by removing steroidal and triterpenoid compounds. All the irritant activity of the polar fraction was ether soluble and free acids and phenols were removed by partition against sodium carbonate solution, leaving an ether soluble residue with an ID50 of 0·2 $\mu g\ \mu l^{-1}$.

Examination of the potent resin by means of t.l.c. indicated that it consisted of several components and these were purified by combinations of column and preparative layer chromatography until the isolated products produced a single spot by analytical t.l.c. and were mass-spectrometrically pure.

Each of the six isolated compounds (Table 1) were biologically active and after strong alkaline hydrolysis produced a common parent alcohol identified as 12-deoxy-4βOH-phorbol. This diterpene was originally isolated from *E. triangularis* (Gschwendt & Hecker, 1969) and has no irritant effects upon mice skin. It is the

Table 1. *The irritants of* Euphorbia fortissima.



Compound	R	R¹	ID50 μg μl⁻¹ 4 h	24 h
A	Dodecenoate	Acetate	0·04	0·4
B	Tigliate	,,	0·44	1·2
C	iso-Butyrate	,,	0·1	1·3
D	α-Methyl-butyrate	,,	0·14	0·6
E	Dodecenoate	H	0·006	0·02
F	Tigliate	H	0·06	0·1

esterified form which gives rise to irritancy (Table 1) and the isolated factors were divided into two groups. The first group, compounds A-D were di-esters of 12-deoxyphorbol and formed the bulk of the active fraction. These compounds all produce a rapid reddening of mice ears within 4 h of application. The second group (Table 1) E and F were mono-esters of the same diterpene and exhibited a greater irritant effect 24 h after application than did compounds A–D. Structurally the esterifying acids of 12-deoxy-phorbol are located at C-13 and C–20, the tertiary hydroxy groups at C–4 and C–10 being sterically hindered. The primary ester moiety at C–20 is readily cleaved by dilute alkali or acid (Gschwendt & Hecker, 1971) and therefore the higher molecular weight fatty acids could in each case be assigned to C–13 by means of spectral data. The di-esters contained an extra acetate moeity located at C–20. Of the four di-esters isolated two occurred naturally in the latex as the mono-ester equivalents (compounds E and F) with a free primary hydroxy group at C–20. Trace components of the mono-ester fraction were also present which possibly correspond to the mono-ester homologues of the remaining di-esters, but were present in insufficient quantities for confirmation.

From Table 1 comparisons of irritant activity for two mono-ester/di-ester pairs is possible. Compound A which is a novel dodecenoate-acetate of 12-deoxy-phorbol has an ID50 of 0·04 $\mu g\ \mu l^{-1}$ after 4 h and is comparatively weak 24 h after application, with an ID50 of 0·4 $\mu g\ \mu l^{-1}$. The novel dodecenoate mono-ester, compound E, on the other hand has a lower ID50 after 4 h, but with an ID50 of 0·02 $\mu g\ \mu l^{-1}$ after 24 h is twenty times as potent as its di-ester homologue. A similar comparison is possible for compounds B and F. It would appear therefore that, although for biological activity esterification of the parent alcohol is necessary, a free C–20 alcoholic group confers longer lasting irritant effects upon the molecule, possibly by binding more strongly to the site of action in the skin. If the di-esters are selectively hydrolysed at C–20 to give mono-esters the same effect is evident as with the naturally occurring mono-ester forms. It is also of interest to note that within both groups of irritants an increase in the molecular weight of the C–13 esterifying group from C–5

as in compounds B and F to C–12 as in compounds A and E also increases the potency of the molecule possibly due to increased lipid solubility.

EXPERIMENTAL CHEMISTRY

Extraction

Euphorbia fortissima latex was collected directly into methanol and dried below 40°. The dried latex was exhaustively extracted with acetone until the marc showed no irritant activity on the skin of mice. The residue from acetone was dissolved in methanol–water and the triterpenoids removed with hexane. This non-polar fraction had an ID50 in excess of 8 $\mu g\ \mu l^{-1}$ and was rejected as an inactive fraction. The polar fraction was re-extracted with ether and the whole of the irritant activity was found to be ether soluble. Phenolic and acid impurities were removed from the ether phase by partition against 1% sodium carbonate solution leaving an irritant, neutral, ether soluble resin with an ID50 of 0·2 $\mu g\ \mu l^{-1}$ 4 h after application to the skin. Thin-layer chromatography of the resin (200 mg) indicated several major components and these were obtained as entities by column chromatography using Florisil as adsorbent and eluting in a gradient: hexane–benzene–ethyl acetate–acetone –methanol. The eluted bands were finally purified by repeated preparative layer chromatography (p.l.c.) on buffered silica gel using chloroform–benzene–ether (1 : 1 : 3) as solvent. Each of the isolated zones had similar infrared spectra exhibiting absorbences at 3385, 1725, 1705, 1695, 1630 cm^{-1} (KBr microcells in $CHCl_3$) and similar C.D. spectra with negative Cotton effects at 272 and 360 nm. They also produced single spots by means of t.l.c. in several solvent systems (Evans & Kinghorn, 1973b).

Identification of parent alcohol

By means of hydrolysis with saturated barium hydroxide in methanol compounds A–E (Table 1) produced a common parent alcohol ($M^+C_{20}H_{28}O_5$). After acetylation (Kinghorn & Evans, 1974) the product was recrystallized from acetone (m.p. 138°). The infrared spectrum was similar to before and the mass spectrum exhibited a parent ion at *m/e* 432 (M^+ $C_{24}H_{32}O_7$) with 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). The nmr (60 MHz) ($CDCl_3$ $\delta = 0{\cdot}00$ ppm) exhibited signals at δ 0·89d ($J = 4$ Hz) (3H–18; H–14); δ 1·16d ($J = 8$ Hz) (6H–16, 17); δ 1·80d ($J =$ 2·1 Hz) (3H–19); δ 2·05 (6H–CH_3CO–); δ 2·18s (2H–12); δ 2·44s (2H–5); δ 3·02m (H–8); δ 3·29m (H–10); δ 4·47s (2H–20); δ 5·75d ($J = 4{\cdot}3$ Hz) (H–7); δ 7·64s (H–1); δ 2·47 and δ 5·58 (2OH–deuterium exchange) ppm. The circular dichroism spectra had Cotton effects at 206 nm $\Delta E = 11{\cdot}33$; 230 nm $\Delta E = +\ 10{\cdot}86$; 260 nm $\Delta E =$ 0·00; 270 nm $\Delta E = -0{\cdot}66$; 360 nm $\Delta E = -0{\cdot}71$ (solvent methanol). The parent alcohol isolated as its diacetate was pure by t.l.c. (Evans & Kinghorn, 1973b) and by g.l.c. (Kinghorn & Evans, 1974), and was identified as 12-deoxy-4βOH-phorbol.

Identification of di-esters

Compound A. (5 mg) was eluted first from the column and was a resin which would not re-crystallize from several solvents. It produced a single orange spot by t.l.c. (R_F 0·8) when sprayed with 60% H_2SO_4 and heated at 110°. The mass-spectrum produced a molecular ion at *m/e* 570 (M^+ $C_{34}H_{50}O_7$), and fragment ions at *m/e* 552

(M–18); 534 (M–36); 510 (M–60); 492 (M–60 + 18); 372 (M–198); 354 (M–198 + 18); 312 (M–198 + 60); 294 (M–198 + 60 + 18) and then as for 12-deoxy-phorbol Trans-esterification in 0·5 M KOH and MeOH produced a mono-ester (M+ *m/e* 528 $C_{32}H_{48}O_6$) which by means of mass spectrometry lost 198 units from the parent ion. Complete hydrolysis followed by methylation of the free fatty acid identified the acid as a C_{12} mono unsaturated compound using g.l.c. on a Carbowax column (Evans, 1973). Acetylation of the mono-ester produced a compound identical in the mass spectrum to compound A. Compound A was therefore assigned as 12-deoxy-4βOH-phorbol-13-dodecenoate-20-acetate. Because of an insufficiency of material, no attempt was made to assign position or stereochemistry of the double bond of the side chain.

Compounds B and C. The second resin eluted from the column (50 mg) although a single spot by t.l.c. (R_F 0·75) produced two peaks by g.l.c. and was shown by mass spectrometry to consist of two di-esters (Compound B M+ *m/e* 472 $C_{27}H_{36}O_7$, Compound C M+ *m/e* 460 $C_{26}H_{36}O_7$). The two were separated by column chromatography on charcoal eluting with a gradient: water–alcohol–acetone–ethyl acetate and finally purified by p.l.c. Compound B (20 mg) by mass spectrometry was shown to have one acetate moiety (M–60 *m/e* 412) and a second acid of molecular weight 100 (M–100 *m/e* 372). The nmr (Fig. 1) was identical to 12-deoxyphorbol diacetate with the exception that only one acetate was present at δ 2·05 and in addition the two methyl groups of tiglic acid were present at δ 1·80 and the vinyl proton as a multiplet at δ 6·8. Tiglic acid was identified as its methyl ester after complete hydrolysis by g.l.c. as before. Trans-esterification of B produced a mono-ester (M+ *m/e* 430 $C_{25}H_{34}O_6$) which lost 100 mass units in the mass spectrum. Compound B was re-formed by acetylation of the mono-ester and was identified as 12-deoxy-4βOH-phorbol-13-tigliate-20-acetate.

Compound C (10 mg) had a molecular ion by ms at *m/e* 460 and fragment ions at *m/e* 442 (M–18); 400 (M–60); 382 (M–60 + 18); 372 (M–88); 312 (M–60 + 88); 294 (M–60 + 88 + 18); and then as before. The nmr (60 MHz. $CDCl_3$ δ = 0·00 ppm) had signals at δ 0·89d, (3H–18, H–14); δ 1·16d (12H); δ 1·80d (3H–19); δ 2·05s (MeCO–); δ 2·18s (2H–12); δ 2·44s (2H–5); δ 2·50m (IH-isobutyric acid); δ 3·02m (H–8); δ 3·29 (H–10); δ 4·47s (2H–20); δ 5·75d (H–7); δ 7·64s (H–1); δ 2·47 and δ 5·58 (2OH-deuterium exchange). Trans-esterification produced a mono-ester (M+ 418

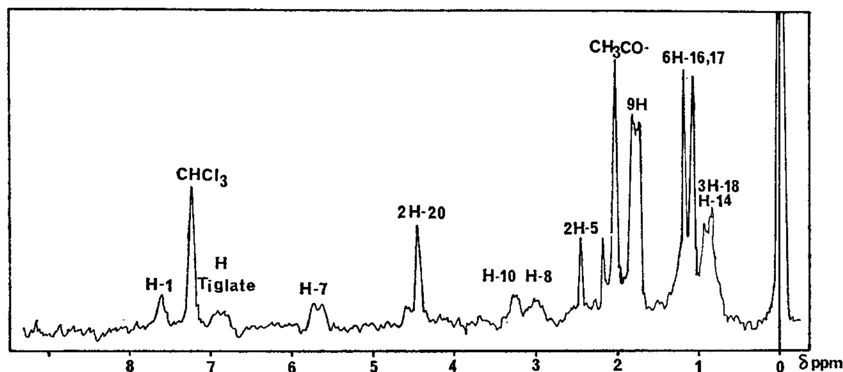


FIG. 1. 60 MHz nmr spectrum of 12-deoxy-phorbol-13-tigliate-20-acetate (deuterium exchange).

$C_{24}H_{34}O_6$) which lost 88 units in the mass spectrum. Complete hydrolysis produced isobutryric acid identified as its methyl-ester by g.l.c. As before, acetylation of the trans-esterification product produced a di-ester identical to Compound C which was identified as 12-deoxy-4βOH-phorbol-13-isobutyrate-20-acetate.

Compound D. On elution from the Florisil column this fraction was contaminated with Compounds B and C. Impurities were removed by repeated elution p.l.c. producing 10 mg of t.l.c.-(R_F 0·6) and mass spectrometrically pure-resin. The mass spectrum exhibited a parent ion at *m/e* 474 ($C_{27}H_{38}O_7$) and fragment ions at *m/e* 456 (M–18); 414 (M–60); 396 (M–60 + 18); 372 (M–102); 354 (M–102 + 18); 312 (M–60 + 102); 294 (M–60 + 102 + 18); and then as before. The nmr which had a methyl doublet at δ 1·14 and a methyl triplet at δ 0·93 ppm suggested the presence of α-methylbutyric acid as one of the esterifying acids. Trans-esterification produced a mono-ester (M^+ *m/e* 432 $C_{25}H_{36}O_6$) which lost 102 mass units in the mass spectrum. By g.l.c. after complete hydrolysis α-methylbutyric acid was identified as its methyl ester. Compound D was regenerated by acetylation of the transesterification product and was therefore identified as 12-deoxy-4βOH-phorbol-13-α-methyl butyrate-20-acetate.

Identification of naturally occurring mono-esters

Compound E, (3 mg) was a resin giving a single orange-brown spot by t.l.c. (R_F 0·3). C.D. and infrared specta suggested an ester of 12-deoxy-4βOH-phorbol. This was confirmed by hydrolysis and methylation of the free fatty acid when dodecenoic acid was identified by g.l.c. Compound E in mass spectrum had a parent ion at *m/e* 528 (C_{32} $H_{48}O_6$) and significant fragment ions at *m/e* 510 (M–18); 492 (M–36); 330 (M–198); 312 (M–198 + 18); 294 (M–198 + 36). The ester was resistant to trans-esterification but acetylation produced Compound A. Substance E was therefore assigned as 12-deoxy-4βOH-phorbol-13-dodecenoate.

Compound F. (1·0 mg) was pure by t.l.c. (R_F 0·15). In the mass spectrum the resin had a molecular ion at *m/e* 430 ($C_{25}H_{34}O_6$) and fragment ions identical to the trans-esterification product of Compound B. Although E was resistant to trans-esterification Compound B was produced by acetylation. The infrared and C.D. were identical to the mono-ester produced degradatively from B and Compound F was therefore assigned as 12-deoxy-4βOH-phorbol-13-tigliate.

Biological analysis

Both the fresh latex and the isolated compounds were used for ID50 determinations on LACA mice using the method previously described (Kinghorn & Evans, 1974).

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