Dry-column chromatographic isolation of fatty acid esters of phorbol from croton oil

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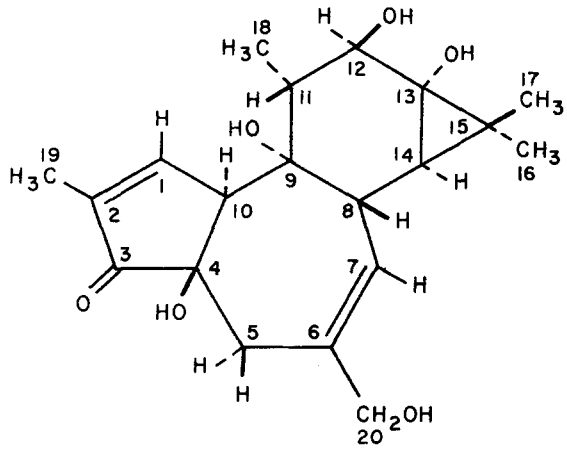
SUMMARY The family of mixed fatty acid esters of 4,9,12,-13,20-pentahydroxy-tiglia-1,6-dien-3-one (phorbol) has been isolated from croton oil by column chromatography on silica gel and preparative dry-column chromatography on silica gel HF/254 packed in a cellophane membrane. The tumor-promoting agents are obtained rapidly and with minimum difficulty.



E. HECKER (1–6) and B. L. Van Duuren (7) have described the preparation of the potent tumor-promoting phorbol esters from croton oil. The methods used for the isolation required extensive countercurrent distribution with apparatus that is not available to many laboratories.

Phorbol esters consist of a mixture of fatty acid esters of 4,9,12,13,20-pentahydroxy-tiglia-1,6-dien-3-one. This communication describes a method for their isolation with readily available equipment.

Each pound of croton oil (N.F. VII, Amend Drug & Chemical Co., New York) was dissolved in 2200 ml of hexane that had been equilibrated with ethanol–water 9:1. The hexane was extracted four times with 750-ml portions of the ethanol–water in a separatory funnel. The alcohol extracts were combined and the solvent was removed in a rotary evaporator at 35°C and 15 mm Hg pressure. Each pound of croton oil yielded 110–120 g or 24% by weight of a yellow oil.

The oil was purified further by column chromatography on silica gel. 560 g of gel (particles of 0.05–0.20 mm diameter, E. Merck, Brinkmann Assoc., New York) was washed with methanol and activated in an oven at 100°C overnight. The column, 4 × 80 cm, was poured as a hexane slurry and washed with 600-ml of

hexane. 50 g of extracted oil in 50 ml of hexane was washed into the gel with 200 ml of hexane and the column was eluted with 2 liters of hexane–diethyl ether 1:1 and 2.5–3 liters of ether (total running time 2.5 hr). Thin-layer chromatography on silica gel in ether–hexane 5:1 showed that the phorbol esters, which run in two groups (R_f 0.24 and 0.47) in this system, were contained in the ether eluate. The ether was removed at 25°C at reduced pressure. Yield, 6.2% by weight from the phorbol ester-enriched fraction and 1.6% over-all from croton oil.

Dry-Column Chromatography. Purification by gradient elution on a standard adsorption column of silica gel leads to modification of the phorbol esters on the gel with loss of biological activity. The speed of dry-column chromatography avoids this difficulty while allowing separation on a larger scale than thick-layer chromatography. The column was prepared according to Dahn and Fuchs (8) but using silica gel HF/254 (Merck). About 135 g of dry gel mixed with 110 g of water was poured into cellophane tubing, 4-cm in diameter, knotted at one end. The gel was allowed to set overnight and activated at 110°C for 1 day; the final activated column measured 4 × 23 cm. 4–5 g of phorbol esters was mixed with an equal weight of dry silica gel and the resultant pellet was packed into the top of the column, which was then turned upside down and inserted into ether–hexane 5:1 for upward adsorption chromatography. A 500 ml graduated cylinder was used as the "tank." The column was supported by means of cord tied to the knot in the cellophane, and the "tank" was covered with tinfoil. When the solvent front had traveled 15 cm (2–2.5 hr) the column was removed and examined under UV radiation (254 nm). Two major bands of phorbol esters, R_f 0.24 (fraction I) and 0.41 (fraction II), were seen, but the column was cut uniformly into 1-cm slices with razor blades or a surgical knife. The gel does not disintegrate if the column is cut within 1 hr after removal from the solvent. The silica gel slices were extracted with ether in a Soxhlet apparatus.

The 6.8 g of phorbol ester-enriched fraction was divided into 5 and 1.8 g fractions and chromatographed on two dry-gel columns (Fig. 1). The yield of phorbol esters were 490 and 180 mg of I and 370 and 130 mg of II. The total yield of phorbol esters from 1 lb. of croton oil was 1.17 g or 0.26% by weight.

Fatty Acid Composition. The esters were saponified with ethanolic KOH in a sealed container at 75°C for 8 hr and then acidified to pH 2.0 with 2.0 N HCl. The methyl esters of the fatty acids were prepared with BF_3–CH_3OH (9) according to Rosenberg and Stern (10) to minimize loss of acetate. GLC was carried out on 10% ethylene glycol adipate polyester and 10% SE-30 (methyl silicone polymer) on 100–120 mesh Chromosorb W, (Applied Science Laboratories, Inc., State College, Pa.). Helium flow rate was 50 ml/min and a temperature program of ambient to 195°C at 2°C/min was used. The fatty acid analysis of fractions I and II (Table 1) demonstrates

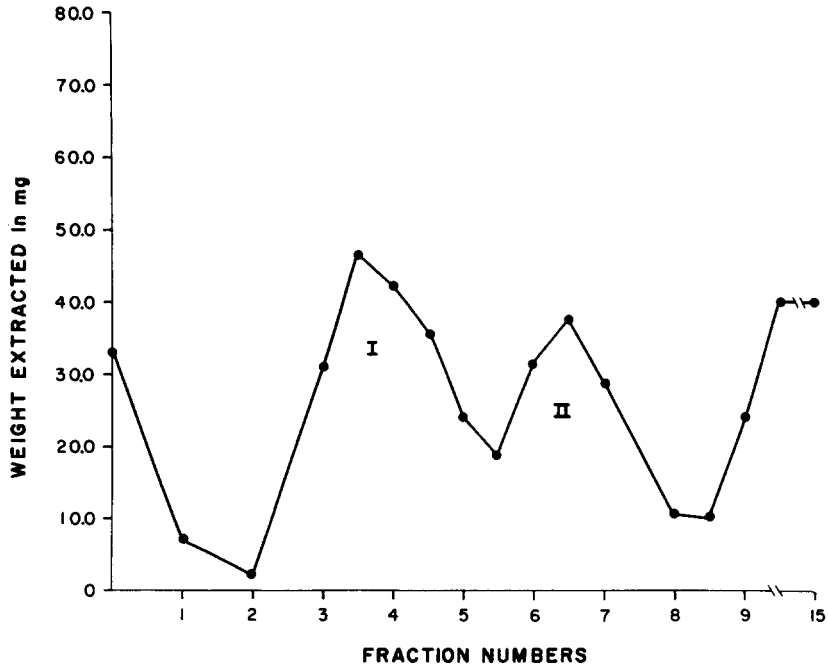


FIG. 1. Elution diagram of dry-column chromatography of phorbol ester-rich fraction on activated silica gel in ether–hexane 5:1. Fraction number coincides with column height in cm, with front at 15 cm. Total charge (two columns), 6.8 g; over-all recovery 6.6 g or 97% by weight.

TABLE 1 FATTY ACID COMPOSITION OF PHORBOL ESTERS FROM DRY-COLUMN CHROMATOGRAPHY

Fatty Acid	Fraction I	Fraction II
Acetic	43.7 ± 2.7	17.2 ± 1.0
Tiglic	—	21.5 ± 1.1
Isobutyric	—	—
Caprylic	—	11.6 ± 0.4
Capric	—	22.7 ± 0.7
Lauric	6.8 ± 0.2	27.0 ± 0.8
Myristic	46.2 ± 1.4	—
Palmitic	0.9 ± 0.1	—

Values represent the mean ± SD of five analyses. Methyl esters were prepared by technique of Rosenberg and Stern (10) to minimize the loss of volatile components. Tiglic acid is $CH_3{=}C(CH_3){-}COOH$; the retention time of its methyl ester relative to methyl acetate is 1.16.

their similarity to phorbol esters obtained by others. Van Duuren (7) reported the chief fatty acids of his group C to be acetic, capric, lauric, and myristic and of his group A to be tiglic, caprylic, capric, and a trace of isobutyric. Hecker (11) found his group A to be phorbol esters of acetic, capric, lauric, myristic, and palmitic and his group B to be esters of isobutyric, caprylic, capric, and lauric acids.

Identification of Phorbol. Fractions I and II were subjected to mild saponification with $Ba(OH)_2$ in methanol (12). The barium soaps were filtered and the solvent was removed by vacuum distillation at 35°C. The residue was taken up in hot ethanol and crystallization was induced by seeding with a crystal of phorbol. The phorbol was recrystallized from 95% ethanol.

The compound showed a single spot, R_f 0.52, on Eastman thin-layer sheets of silica gel in chloroform–methanol 10:1. The phorbol standard had an identical R_f. Other physical characteristics were: mp 238–239°C (lit. 238–240°C); $[\alpha]^{25}D$, 117°C in dioxane (0.4%) (lit. 118°C); λ_{max} 236 and 334 nm in ethanol; IR spectrum, OH peak at 3390, carboxyl at 1720, and C=C at 1625 cm^{-1}. The compound gave a positive color test when heated with HCl, "the phorbol reaction" (12).

Fractions I and II appear, then, to be mixed fatty acid esters of the same alcohol, phorbol, and the grouping of esters appears similar to other preparations (2–5, 7, 11). Although the two fractions I and II are not single components they differ only in fatty acid composition and greater resolution may therefore be unnecessary for some biological work.

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