# Note

## Isolation and purification of phorbol from croton oil by reversed-phase column chromatography

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The diesters of the diterpene alcohol phorbol (Fig. 1A) have generated much interest as pharmacological probes in cancer research and for their diverse biological effects at extremely low concentrations[1-3]. One of these diesters, 12-O-tetradecanoyl-phorbol-13-acetate (TPA, Fig. 1B), is the most potent tumor promoter known in the two stages mouse skin model of carcinogenesis[4,5]. In addition to its tumor promoting activity, TPA also produces a variety of biological effects *in vivo* and *in vitro*[6,7]. Croton oil, the seed oil of *Croton tiglium* L., is a valuable source of the diesters of phorbol and related diterpene alcohols.

Recently, we became interested in preparing some analogues of TPA for use as probes in the study of the mechanism of action of the phorbol esters. These synthetic studies required large quantities of the phorbol alcohol. The relatively high cost of the commercially available product prompted us to explore alternative methods of obtaining phorbol from croton oil which are more efficient than published methods[1,2,8]. In this report we describe a simple, reliable and inexpensive method for the isolation and purification of phorbol from croton oil.

MATERIALS AND METHODS

Melting points were determined in open capillary tubes with a Thomas-Hoover melting point apparatus and are not corrected. Elemental analyses were performed by M-H-W Laboratories (Phoenix, AZ, U.S.A.) and were within ±0.4% of the theoretical values. IR spectra were obtained in KBr pellets using a Perkin-Elmer 281 instrument. UV measurements were obtained on a Beckman Model DU-8 spectrophotometer. Proton nuclear magnetic resonance spectra were recorded at ambient temperature on a Jeol-FX90Q instrument or a Nicollet NT300WB spectrometer with
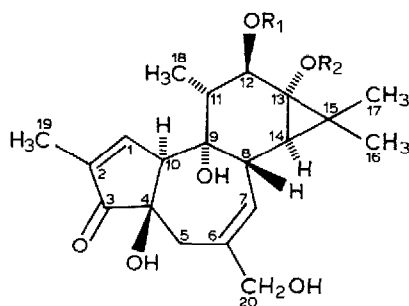
A        $R_1$, $R_2$ = H

B        $R_1$ = $CH_3(CH_2)_{12}CO$— , $R_2$ = $CH_3CO$—

Fig. 1. Structure of (A) phorbol and (B) 12-O-tetradecanoylphorbol-13-acetate.

tetramethylsilane (TMS) as the internal standard. Mass spectra were obtained on a Finnigan 4000 instrument in the chemical ionization (CI) mode using isobutane as the reagent gas.

Croton oil (Fisher Scientific, New York, NY, U.S.A.) was stored in amber glass bottles at 4°C until used. Reference sample of phorbol was obtained from Chemical Carcinogenesis (Eden Prairie, MN, U.S.A.). All other chemicals used throughout the work were of reagent grade. The solvents used in the chromatographic separation were of HPLC grade.

*Preparation of a crude extract of phorbol form croton oil*

Croton oil (200 g) was mixed with a solution of $Ba(OH)_2 \cdot 8H_2O$ (22 g) in methanol (1 l) in a 2-l round bottom flask under an atmosphere of nitrogen. The reaction vessel was wrapped in an aluminum foil to protect the contents from light and was shaken for 10–12 h. The reaction mixture was filtered and the filtrate was concentrated *in vacuo* at a temperature below 40°C. The oily residue was mixed with distilled water (1 l) and extracted with diethyl ether (3 × 200 ml). The aqueous phase was adjusted to pH 5 with 2 N sulfuric acid and treated with a saturated solution of sodium sulfate (16 ml). The mixture was stored at 4°C overnight and filtered. The filtrate was adjusted to pH 7 with 2 N sodium hydroxide and concentrated under reduced pressure. The oily residue was digested with hot absolute ethanol (40–50 ml) and filtered rapidly. The precipitate was washed repeatedly with hot absolute ethanol. The filtrate was concentrated and stored at 4°C for several weeks. Few crystals of phorbol alcohol were added to induce crystallization and the mixture was stored at 4°C for additional several weeks. The mixture was concentrated under reduced pressure to provide crude phorbol fraction as an oily residue. The crude fraction was stored under nitrogen at 4°C until it was subjected to purification by reversed-phase column chromatography.

*Thin-layer chromatography (TLC)*

Normal phase analytical TLC was performed on silica gel GF plates (250 μm, 10 × 20 cm uniscored, Analtech). Reversed-phase analytical TLC was performed on

TABLE I

$R_F$ VALUES OF PHORBOL IN VARIOUS SOLVENT SYSTEMS ON SILICA GEL AND RE-
VERSED-PHASE RPS-F TLC PLATES (ANALTECH)

| Solvent system (v/v) | $R_F$ | TLC plate |
|---|---|---|
| Ethyl acetate–methanol (10:1) | 0.23 | Silica gel |
| Methanol–chloroform (1:9) | 0.70 | Silica gel |
| Acetic acid–chloroform (2:5) | 0.26 | Silica gel |
| Pyridine–chloroform (2:5) | 0.31 | Silica gel |
| Chloroform–acetic acid (1:1) | 0.42 | Silica gel |
| Ethyl acetate–acetic acid (5:2) | 0.80 | Silica gel |
| Ethyl acetate–methanol (1:1) | 0.66 | Silica gel |
| Chloroform–acetonitrile–trifluoroacetic | | |
| acid (7:6:0.05) | 0.12 | Silica gel |
| Acetonitrile–chloroform (95:5) | 0.15 | Silica gel |
| Acetonitrile–methanol (10:1) | 0.36 | Silica gel |
| Methanol–acetonitrile–water (1:1:5) | 0.8 | RPS-F |
| Methanol–acetonitrile–water (1:1:10) | 0.70 | RPS-F |
| Methanol–acetonitrile–water (1:1:20) | 0.56 | RPS-F |
| Methanol–acetonitrile–water (1:1:40) | 0.40 | RPS-F |

reversed-phase RPS-F plates (250 $\mu$m, 10 × 20 cm uniscored, Analtech). A number
of solvent systems were used (Table I). The TLC plates were visualized under short-
wave ultraviolet light followed by spraying with a vanillin–sulfuric acid–absolute
ethanol (3:0.5:100) spray reagent and heating over a hot plate at 120°C.

*Purification of phorbol by preparative reversed-phase column chromatography*

Phorbol was obtained from the crude extract by reversed-phase column chro-
matography using a system composed of metering pump (Model FMI, Fluid Meter-
ing, Oyster Bay, NY, U.S.A.), a PTFE rotary valve (Type 50, Rheodyne, Cotati, CA,
U.S.A.), a multiple wavelength absorbance–fluorescence detector set at 254 nm in
the absorbance mode (Model VA-5, ISCO, Lincoln, NE, U.S.A.) and fraction col-
lector (Retriever II, ISCO). Columns prepacked with LiChroprep RP-8, 40–63 $\mu$m
were used (size A, 240 × 10 mm I.D. and size B, 310 × 25 mm I.D., Merck, Darm-
stadt, F.R.G.). A linear gradient of methanol–acetonitrile–water (1:1:5) and
methanol–acetonitrile–water (1:1:40) was used as the mobile phase at a flow-rate of
10 ml/min.

The crude phorbol fraction was dissolved in distilled water to give a total
volume of 40 ml. When a size A column was used, a 1-ml aliquot of the solution of
the crude fraction was loaded onto the column and the gradient elution was started.
The UV absorbance of the eluate was monitored and 10-ml fractions were collected
(Fig. 2). Phorbol typically eluted within 15 min. After 45 min the column was eluted
with methanol and after 60 min the column was re-equilibrated with methanol–
acetonitrile–water (1:1:40) in preparation for the next separation.

When the size B column was used, a 4-ml aliquot of the solution of the crude
phorbol fraction was loaded on the column and eluted with the same linear gradient
as that used with the size A column at a flow-rate of 10 ml/min. Fractions (10 ml)
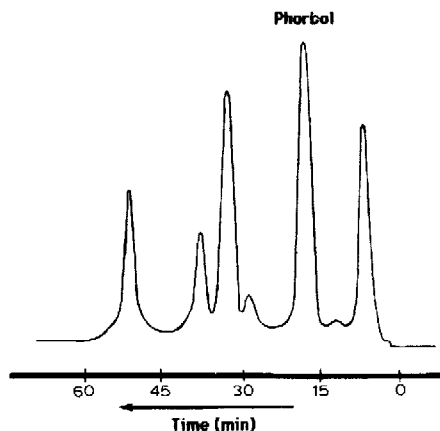were collected and phorbol typically eluted within 1 h.

Phorbol



Fig. 2. Preparative chromatographic separation of phorbol from the crude extract of saponified croton oil. Column: LiChroprep RP-8, 40–63 $\mu$m (size A). Solvent: linear gradient of methanol–acetonitrile–water (1:1:5) and methanol–acetonitrile–water (1:1:40) over 45 min. Flow-rate: 10 ml/min. Detector: UV at 254 nm. Phorbol eluted at 15 min. At 45 min the column was eluted with methanol and at 60 min it was re-equilibrated with methanol–acetonitrile–water (1:1:40).

Fractions containing phorbol were pooled and the solvent was removed under reduced pressure at a temperature below 60°C. Acetone was added to the residue to precipitate the phorbol. The precipitate wass filtered and dried in a vacuum dessicator over phosphorous pentoxide for 24 h. An amount of 3.26 g of pure phorbol was obtained from 200 g of croton oil (yield 1.6%). A sample of the phorbol was re-crystallized from ethyl acetate, m.p. 233–234°C [lit. 234°C (ref. 9)]. IR (KBr) 3400, 2900, 1710 and 1640 cm$^{-1}$. UV (absolute ethanol) $\lambda_{max}$ nm ($\varepsilon$), 210 (6870), 233 (4340). $^1$H NMR (d$_4$-methanol): 0.72 (d, $J = 5$, 1H), 1.1 (d, $J = 6$, 3H), 1.2 (s, 6H), 1.75 (m, 3H), 1.9 (m, 1H), 2.5 (q, AB pattern, 2H), 3.1 (bm, 1H), 3.2 (bm, 1H), 3.9 (s, 2H), 4.1 (d, $J = 11$, 1H), 5.60 (m, 1H), and 7.60 (bs, 1H) ppm. Elemental analysis: calculated (found) for C$_{20}$H$_{28}$O$_6$: C, 65.90 (65.83); H, 7.75 (7.88).

RESULTS AND DISCUSSION

Phorbol and other diterpene alcohols are present in croton oil in the form of esters of fatty acids. These alcohols are sensitive to light and are easily oxidized by atmospheric oxygen. Therefore, croton oil was treated with barium hydroxide under a nitrogen atmosphere and in the absence of light to saponify the esters. This resulted in the formation of the barium salts of the fatty acids, which are insoluble in methanol, and the free diterpene alcohols. The barium salts were removed by filtration and the filtrate was concentrated to remove the solvent. The residue was mixed with water to solubilize the hydrophilic diterpene alcohols and the hydrophobic constituents were removed by ether extraction. The excess barium hydroxide was removed from the aqueous solution by the addition of sulfuric acid to form the insoluble barium sulfate. After the filtration of barium sulfate the filtrate was concentrated to remove water. The crude phorbol alcohol was extracted from inorganic impurities with hot absolute ethanol. Concentration of the ethanol extract is reported to result

in the formation of phorbol crystals[1,2]. However, we were not able to obtain phorbol crystals from the concentrated solution even after the addition of few crystals of phorbol to induce crystallization.

Examination of the alcoholic solution indicated the presence of a major component which had the same chromatographic mobility on TLC as that of the phorbol reference standard (Table I). Therefore, we explored the utility of reversed-phase column chromatography in the isolation and purification of phorbol from the crude extract. Octylsilane bonded to silica gel (40–63 $\mu$m) was used as the stationary phase. A variety of solvents were attempted. A mixture of methanol–acetonitrile–water (1:1:40) in the isocratic mode provided a good separation of phorbol from the other impurities in the extract. The use of a linear gradient of methanol–acetonitrile–water (1:1:5) to methanol–acetonitrile–water (1:1:40) over 45 min resulted in faster elution of phorbol without affecting the chromatographic resolution of the various components in the extract. Also changing the flow-rate between 6 and 10 ml/min did not affect the resolution and maintaining the flow-rate within this range resulted in reproducible results. After the separation with the linear gradient was completed it was essential to wash the column with methanol to remove dark material that was retained at the bottom of the column. Approximately 50 mg of phorbol could be purified in each run using the size A column. A run required approximately 1 h. The crude phorbol fraction obtained from 200 g of croton oil was purified in 30–40 injections.

The phorbol obtained after column purification was of sufficient purity to use for subsequent synthetic work. The purity of the product was ascertained from its homogeneity on examination in a number of TLC systems. In these systems only one product was detected which had the same chromatographic mobility as the reference sample of phorbol. The identity of the product was further confirmed by examining its IR, UV, CI-MS and $^1$H NMR spectra. These spectra were identical to those obtained from the reference standard and were consistent with the structure of phorbol.

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