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Note

Isolation of phorbol and 4α-phorbol from croton oil by droplet counter-current chromatography

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Phorbol esters, obtained from the seed oil of *Croton tiglium* L. (croton oil), are the most potent class of tumor-promoters on mouse skin presently known[1]. These compounds have attracted much interest in the scientific community because of their diverse cellular and biochemical effects, which are often apparent at extremely low dose levels[2]. While many phorbol esters are available commercially, they are prepared from their diterpene parent alcohol, phorbol, by semisynthesis[3,4]. Since the published isolation methods for phorbol from croton oil are lengthy and tedious[3,5–7], we wish to report a more reliable method for the isolation of phorbol and 4α-phorbol from this plant source, using droplet counter-current chromatography (DCC). DCC has been applied to a wide range of polar plant constituents[8], and is particularly suitable for labile compounds such as phorbol, since solute autooxidation is limited with this technique[8,9].

EXPERIMENTAL

DCC separations were carried out on a Model-A DCC apparatus (Tokyo Rikakikai, Tokyo, Japan). Low-pressure liquid column chromatography was performed on a LiChroprep RP-8 (40–63 μm), Size A, column (Merck, Darmstadt, G.F.R.). The melting point was determined using a Koffler hot-stage instrument, and is uncorrected. UV measurements were obtained on a Beckman model DB-G grating spectrophotometer. Proton magnetic resonance (PMR) spectra were determined on a Varian T-60A instrument, with a Nicolet TT-7 FT attachment (60 MHz, TMS, internal standard). Mass spectra were obtained on a Varian MAT 112S instrument, operating at 20 and 70 eV.

Materials

Croton Oil NF (Fisher Scientific, New York, NY, U.S.A.) was stored in an amber glass bottle for some years before use in this study. Phorbol was obtained from Chemical Carcinogenesis (Eden Prairie, MN, U.S.A.), and recrystallized from ethyl acetate before use. Reagent-grade solvents were used throughout this work, and were redistilled in glass.

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Hydrolysis and fractionation of croton oil

Croton oil (32.1 g) was mixed with methanol (150 ml), previously saturated with $Ba(OH)_2 \cdot 8H_2O$ (3.5 g), for 14 h. The hydrolysis was carried out under an atmosphere of nitrogen with the reaction vessel protected from light. Methanol was removed under reduced pressure below 40°C. Crude diterpene alcohols were extracted with 8 × 100 ml acetone aliquots, filtered, and, on reduction to dryness, 28.3 g of an acetone extract were obtained. The final acetone wash gave only a faint positive colorimetric test[3] for phorbol. Methanol (150 ml) and water (15 ml) were used to dissolve the acetone extract, and the resultant polar phase was partioned with 3 × 100 ml of hexane. Examination of the hexane layer by thin-layer chromatography (TLC), using ethyl acetate–methanol (10:1) as solvent, on silica gel GF_{254} pre-coated plates (0.25 mm thickness, Merck), showed that no phorbol (R_F 0.28) was present. Solvent was removed from the aqueous methanolic layer to yield 5.4 g of solid residue.

Purification of phorbol and 4α-phorbol

A portion (0.51 g) of the diterpene alcohol-containing fraction (5.4 g) of croton oil was submitted to DCC using hexane–diethyl ether–*n*-propanol–95% ethanol–water (4:8:3:5:4) as solvent. The lower phase was used as the mobile phase, and the sample was injected in a 1:1 mixture of the upper and lower phases, using a 5-ml sample chamber. Fractions (*ca.* 1 ml) were collected at a rate of *ca.* 5 ml/h on a Buchler Fractomette Alpha 200 (Buchler, Fort Lee, NJ, U.S.A.) automatic fraction collector, and were weighed on removal of solvent. DCC, using the above procedure, was repeated for phorbol (23 mg), and the absorbance at 253 nm was measured for each fraction eluted. A void volume, representing the passage of mobile phase from the beginning of the separation until the elution of the first drop of mobile phase, was calculated as 27 ml.

Final purification of phorbol and 4α-phorbol from the DCC of croton oil was achieved by liquid column chromatography of the fractions in tubes 65–125 (60 mg), using octadecylsilyl-bonded silica gel, eluted with methanol–acetonitrile–water (1:1:6), at a flow-rate of 29 ml/min. Phorbol and 4α-phorbol were eluted, respectively, 10–20 ml and 24–27 ml after injection.

Characterization of phorbol and 4α-phorbol

White needles of phorbol–ethyl acetate solvate (35 mg, 0.97% w/w) (m.p. 230°C, lit.[7] 233–234°C) were were obtained after crystallization from ethyl acetate, and exhibited identical TLC and mass spectral characteristics (3,7,10) to an authentic sample of the compound.

4α-Phorbol (5.6 mg, 0.18% w/w, resinous) was characterized by comparison of its PMR spectrum with published data[11]. The mass spectral and PMR data of 4α-phorbol-12,13,20-triacetate and 4α-phorbol-4,12,13,20-tetra-acetate, obtained on the acetylation of 4α-phorbol as previously described[10], were closely comparable with literature data[10,11].

RESULTS AND DISCUSSION

DCC separations of phorbol alone, and phorbol and 4α-phorbol from croton oil extract, are shown in Figs. 1 and 2, respectively. In the design of the solvent system

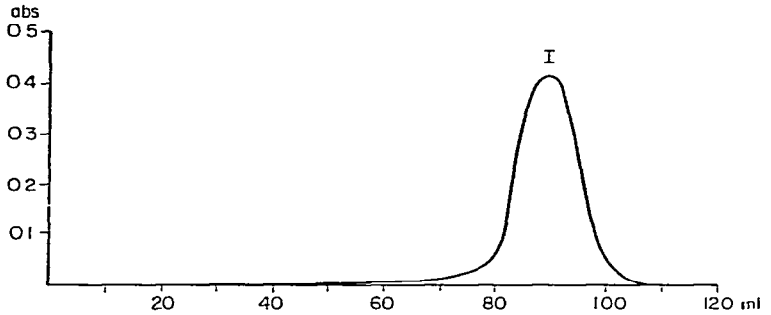

Fig. 1. DCC elution chromatogram of phorbol (I, 23 mg), using hexane–diethyl ether–*n*-propanol–95% ethanol–water (4:8:3:5:4). Mobile phase, lower layer; detection, UV at 253 nm.

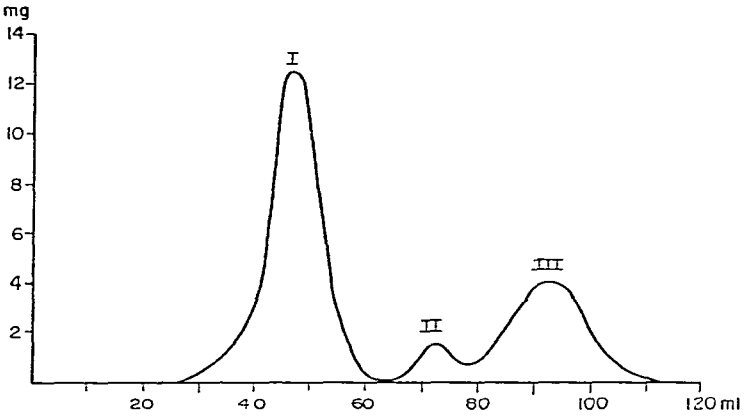

Fig. 2. DCC elution chromatogram of fractionated croton oil (510 mg) I = Solvent front; II = 4α-phorbol; III = phorbol.

it was found that the addition of *n*-propanol increased the solubility of phorbol in the stationary phase and hence the separation of phorbol and 4α-phorbol in croton oil. However, further improvement of the separation, using additional volumes of *n*-propanol, resulted in solvent systems too viscous for DCC. Final purification of the two compounds was achieved by preparative liquid chromatography over C_{18} chemically bonded silica gel. This technique has been used with success for the final purification of other plant constituents following DCC separation[12].

In conclusion, it may be seen that this reproducible DCC system offers considerable advantages over conventional methods for the isolation of phorbol from croton oil. It is less time-consuming, since there is only minimal solvent fractionation after the initial barium hydroxide hydrolysis of the phorbol esters originally present in the oil, and also the need to crystallize phorbol over a period of several weeks[3] is obviated. The concomitant isolation of the extraction artifact 4α-phorbol[3] with phorbol in our method is of potential use, since 4α-phorbol esters are frequently used as negative controls for biologically active phorbol ester homologs in biochemical experiments[4,13]. A similar yield of phorbol (0.97% w/w, expressed as the non-solvated alcohol) was achieved in this DCC procedure to the yields of phorbol in croton oil

obtained by other groups who have used base-catalyzed *trans*-esterification during the isolation of this compound[3,5–7]. The DCC system described in this communication is only the second to date to be designed for weakly polar plant constituents[8,14].

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REFERENCES

1 I. Berenblum, in F. F. Becker (Editor), *Cancer: A Comprehensive Treatise*, Vol. 1, Plenum, New York, 1975, p. 323.
2 A. Sivak, in T. J. Slaga, A. Sivak and R. K. Boutwell (Editors), *Carcinogenesis, Vol. 2, Mechanisms of Tumor Promotion and Cocarcinogenesis*, Raven Press, New York, 1978, p. 553.
3 E. Hecker and R. Schmidt, *Prog. Chem. Org. Nat. Prod.*, 31 (1974) 377.
4 *Technical Bulletin, No. 1002*, Consolidated Midland Corporation, Brewster, NY, 1979.
5 B. Flashenträger and G. Gigner, *Helv. Chim. Acta*, 25 (1942) 569.
6 T. Kauffmann and H. Neumann, *Chem. Ber.*, 92 (1959) 1715.
7 L. Crombie, M. L. Games and D. J. Pointer, *J. Chem. Soc., C*, (1968) 1347.
8 K. Hostettmann, *Planta Med.*, 39 (1980) 1.
9 R. Schmidt and E. Hecker, *Cancer Res.*, 35 (1975) 1375.
10 F. J. Evans and A. D. Kinghorn, *J. Chromatogr.*, 87 (1973) 443.
11 P. Jacobi, E. Härle, H. U. Schairer and E. Hecker, *Justus Liebigs Ann. Chem.*, (1970) 13.
12 K. Hostettmann, M. Hostettmann-Kaldas and O. Sticher, *J. Chromatogr.*, 186 (1979) 529.
13 G. Kreibich, R. Süss and V. Kinzel, *Z. Krebsforsch. Klin. Oncol.*, 81 (1974) 135.
14 Y. Yahasi, T. Matsumoto, Y. Yuki and T. Sakan, *Tetrahedron Lett.*, (1977) 4215.