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VACUUM LIQUID CHROMATOGRAPHY AND QUANTITATIVE ^{1}H NMR SPECTROSCOPY OF TUMOR-PROMOTING DITERPENE ESTERS

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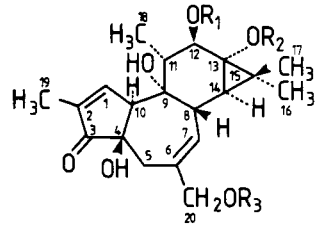
The tumor-promoting activity of diterpene esters of the plant families Euphorbiaceae and Thymelaeaceae is due to the presence of the closely related polycyclic alcohols phorbol, ingenol, and resiniferonol (1). In an earlier publication we described a phytochemical screening method for diterpene esters in biological material using rotation locular countercurrent chromatography (rlcc) and quantitative ^{1}H nmr (2). This method was applied to the quantitative analysis of the phorbol ester constituents of croton oil, the seed oil of *Croton tiglium* L. (Euphorbiaceae). The phorbol 12,13, 20-triesters, which are cryptic tumor-promotors, and the phorbol 12,13-diesters could be determined separately in a quantitative way. It was, however, at that time not possible to differentiate between the tumor-promoting phorbol 12,13-diesters and the short-chain phorbol 12,13-diesters, which are not tumor-promoting (Figure 1). In the present communication the use of vacuum liquid chromatography (vlc) for the separation of the different groups of phorbol diesters is described. Vlc is a relatively new variant of cc, utilizing reduced pressure to increase the flow rate of the mobile phase (3–6).

The combination of vlc and quantitative ^{1}H nmr was also applied to the analysis of the diterpene esters from *Euphorbia ingens* E. Mey (Euphorbiaceae). The latex of *E. ingens* is highly toxic and produces irritation and blistering of the skin in a manner similar to that of croton oil (7,8). Irritant and tumor-promoting activities of an Me_2CO extract were demonstrated earlier (9). The active principles and several nonirritant components of the latex of *E. ingens* have been isolated and identified by Hecker and co-workers (10–14). The biologically active ingenol esters are factors I_1, I_5, and I_6, and the nonirritant diterpene esters are factors I_2 and I_4 (Figures 2,3). Although ingenol 20-esters are inactive, ingenol 3-esters are active as irritants and tumor-promoters.

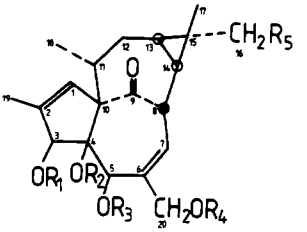
The vlc apparatus used in this work



R_1	R_2	R_3	
H	H	H	phorbol
long[a]	short[b]	H	A-series of phorbol diesters
short	long	H	B-series of phorbol diesters
short	short	H	short-chain phorbol diesters
acyl	acyl	acyl	phorbol triesters

[a]long = carbon chain length 8 to 16
[b]short = carbon chain length 2 to 6

FIGURE 1. Chemical structure of phorbol and its esters.



Ingenol	$R_1 = R_2 = R_3 = R_4 = R_5 = H$
Factor I_1	R_1 = hexadecanoate, $R_2 = R_3 = R_4 = R_5 = H$
Factor I_2	R_4 = hexadecanoate, $R_1 = R_2 = R_3 = R_5 = H$
Factor I_5	R_1 = deca-2,4,6-trienoate, R_5 = O-angelate, $R_2 = R_3 = R_4 = H$
Factor I_6	R_1 = deca-2,4,6-trienoate, $R_2 = R_3 = R_4 = R_5 = H$

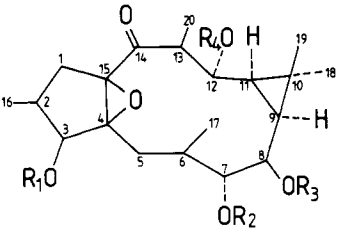
FIGURE 2. Representative structures of ingenol esters.

(Figure 4) is more complicated than some other systems mentioned in the literature (3–6). To maintain a constant solvent flow rate and vacuum, stopcock H should not be opened continuously. Reservoir F can be used as a sluice to avoid direct exposure of the column to the vacuum system, in order to maintain only the minimum vacuum. Column disruption due to bubble formation is avoided by maintenance of only the minimum vacuum required for adequate solvent flow.

Some characteristic ^{1}H-nmr signals of phorbol esters are found at 7.6 ppm for H-1, at 4.5 ppm for H-20 of phorbol 12,13,20-triesters, and at 4.0 ppm for H-20 of phorbol 12,13-diesters. The total amount of phorbol esters in an unknown sample is determined by comparison with an internal standard, namely *p*-dinitrobenzene (*p*-DNB). The total diterpene ester (DE) weight is given by the following equation:

$$\text{weight (DE)} = \text{A(DE)} \times 4 \times \frac{\text{weight } (p\text{-DNB})}{\text{A}(p\text{-DNB})} \times \frac{\text{MW}}{168.1}$$

where A(DE) is the integration value of the signal at 7.6 ppm, weight(*p*-DNB) is the amount of *p*-dinitrobenzene added to the sample, A(*p*-DNB) is the integration value of the corresponding 4-proton singlet at 8.43 ppm, 168.1 is the mol wt of *p*-DNB, and MW is the mol wt of the major phorbol ester present, or a weighted average of the various phorbol esters present in the sample, if known. The proportion of diesters and triesters is de-



Ingol	$R_1 = R_2 = R_3 = R_4 = H$
Factor I_4	$R_1 = R_2 = R_4$ = acetate, R_3 = nicotinate

FIGURE 3. Chemical structure of ingol and factor I_4.

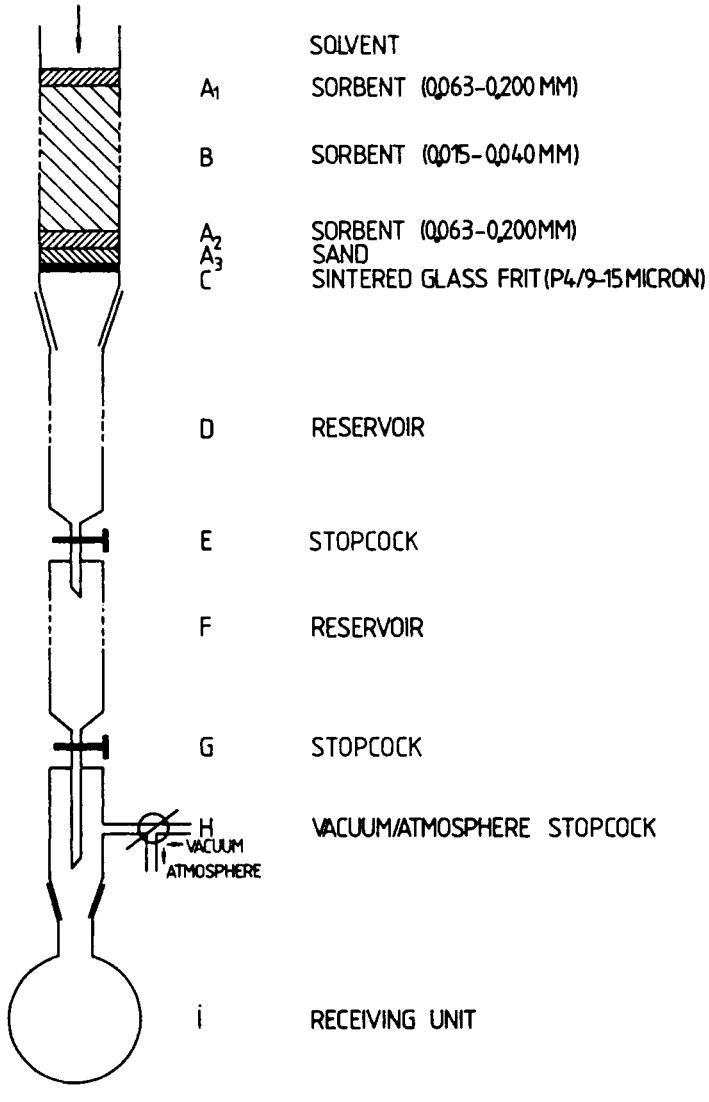


FIGURE 4. Apparatus for vacuum liquid chromatography (vlc).

termined by calculating the ratio of the integration values of the signals at 4.5 ppm and 4.0 ppm. Our sample of croton oil contained $9.1 \pm 0.4\%$ of cryptic tumor-promoting phorbol 12,13,20-triesters, and $2.2 \pm 0.1\%$ of phorbol 12,13-diesters (% of the total oil weight), calculated on the basis of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) having a mol wt of 616.8 (2). The phorbol 12,13-diesters can exist as a series of esters in which a long chain acyl derivate is attached to C-12 of the nucleus and a short-chain acyl derivative to C-13 (the A series), or this may be reversed, as in the B series of diesters. Short-chain phorbol 12,13-diesters, containing two short-chain acyl derivatives, are also present. The diester fraction, isolated from croton oil by rlcc, was separated by vlc. The B series of phorbol diesters was eluted first, then the A series, and finally the short-chain diesters. Pure samples of the different groups of phorbol diesters were obtained and determined quantitatively by ^{1}H nmr. The composition of the phorbol 12,13-diester fraction was as follows (% of the diester fraction): B

series 46.2±1.1%, A series 44.8±1.1%, short-chain diesters 8.9±0.4%. The tumor-promoting A and B series were calculated on the basis of TPA (mol wt = 616.8) and the short-chain diesters on the basis of 12-*O*-tiglylphorbol-13-acetate (mol wt = 488) which is the most abundant short-chain phorbol 12,13-diester (15).

The thin layer chromatogram of the diterpene-ester-containing fraction from *E. ingens* yielded two major spots, with R_f values of 0.46 and 0.54. Additional information was obtained by ^{1}H nmr. The olefinic protons on C-1 and C-7 of ingenol 3,5,20-triacetate resonate at 6.1 and 6.3 ppm, respectively. The geminal protons on C-20 appear as an AB quartet signal at 4.6 and 4.1 ppm in the case of ingenol 20-acylates but as a singlet at about 4.1 ppm in ingenol esters having a free C-20 OH group. In the ^{1}H-nmr spectrum of ingol 3,7,8,12-tetraacetate, the protons geminal to the acyl functions are found between 4.5 and 5.5 ppm, i.e., 5.4 ppm (1H, d, H-3), 5.1 ppm (1H, s, H-7), 4.9 ppm (1H, dd, H-12), and 4.6 ppm (1H, d, H-8) (1,11,12,14).

The diterpene-ester-containing fraction was subjected to a quantitative ^{1}H-nmr experiment. Because all the C-1 and C-7 resonances of the different types of ingenol esters present in *E. ingens* occur between 5.8 and 6.3 ppm, this complex signal was integrated and divided by 2 to measure the total ingenol ester level (ingenol 3-esters and 20-esters), including the 16-OH-ingenol esters. The doublet of doublets at 4.9 ppm, due to H-12 of the ingol nucleus, is characteristic for the ingol esters. The doublet at 4.6 ppm is composed of the overlapping signals of H-8 of the ingol esters and of one half of the AB quartet due to the H-20 protons of the ingenol 20-esters.

The comparative data are as follows: (1/2)A(5.8–6.3 ppm) = ingenol esters, A(4.9 ppm) = ingol esters, A(4.6 ppm)-A(4.9 ppm) = ingenol 20-esters, (1/2)A (5.8–6.3 ppm) − A(4.6 ppm) + A(4.9 ppm) = ingenol 3-esters where A = integration area. Thus, it is possible to measure separately the total ingenol ester level, the amount of tumor-promoting ingenol 3-esters, the amount of biologically inactive ingenol 20-esters, and the total ingol ester level. Formulae similar to those for the phorbol esters from croton oil were used. The results of our sample are given in Table 1. The total ingenol ester level and the amount of ingenol 3-esters were calculated as factor I_5, the principal constituent of *E. ingens*, having a mol wt of 594 (14). The ingenol 20-esters were calculated as factor I_2 (mol wt = 586), and the ingol esters as factor I_4 (mol wt = 597).

EXPERIMENTAL

Croton oil (5.1 g, purchased from Pharmachemic, B-2610 Antwerp, Belgium) was subjected to rlcc, and the phorbol 12,13-diesters were collected (2).

Plant material of *E. ingens* was obtained from the National Botanic Garden, Meise, Belgium, where this plant is kept in continuous growth. Plant material (1.273 kg above-ground parts) was extracted exhaustively with Me_2CO at room temperature. The extract obtained was 7.43% of the dry wt of plant material. A known procedure was used for the isolation of the diterpene esters from this Me_2CO extract (1,15). The final Et_2O fraction was dried over anhydrous Na_2SO_4 and evapo-

TABLE 1. Results of Vlc Isolation (± standard deviation of the mean).

	weight (mg)	% (dry wt)	% of the total ingenol ester weight	number of measurements
Ingol esters	15.1±0.3	0.016		5
Ingenol esters	16.9±0.1	0.018	100	5
Ingenol 3-esters . . .	11.4±0.4		32.0±1.8	5
Ingenol 20-esters . .	5.4±0.3		67.5±2.4	5

rated to dryness under reduced pressure. A residue of 170 mg was obtained, corresponding to 0.18% of the dry wt of the starting material. This residue was subjected to vlc.

DESCRIPTION OF THE VLC APPARATUS.—The apparatus used in this work (Figure 4) was somewhat different from those described in the literature (3–6). A vacuum dry-pack method was used. The sorbent (Si gel 60, Merck, 0.015–0.040 mm) was compressed to a hard layer under vacuum (water aspirator). A pre-adsorbent layer (Si gel 60, Merck, 0.063–0.200 mm) was added. The sample was dissolved in the selected eluent and applied all at once. Elution was performed under vacuum. The eluent was collected in the receiving unit (I), which was brought to normal pressure by opening stopcock H to the atmosphere, while stopcock G was closed (meanwhile the eluent was collected in reservoir F). By closing stopcock E, a second fraction could be collected in reservoir D. After the flask I was changed, stopcock H was opened to the vacuum, which resulted in the evacuation of the receiving unit. Then stopcocks G and E were opened, and the elution was continued.

Separation procedure.—The phorbol 12,13-diester fraction, obtained from *C. tiglium* by a preliminary rlcc experiment, was separated by vlc. The mobile phase was a degassed mixture of CH_2Cl_2-Me_2CO (3:1). To monitor the elution, tlc was used (2).

The diterpene esters from *E. ingens* were separated by vlc using a degassed mixture of *n*-hexane-Et_2O-EtOAc (1:1:1) as the mobile phase. To follow up the elution, tlc on precoated Si gel 60 F_{254} plates, Merck, 0.2 mm, with *n*-hexane-Et_2O-EtOAc (1:1:1) as the mobile phase, was used. For visualization of the spots, two detection modes were used, i.e., uv light (at 254 nm, using plates with fluorescent indicator), and spraying with a reagent consisting of vanillin (3 g) and H_2SO_4 (0.5 ml) in EtOH (100 ml), followed by heating for 20 min at 120° (14, 16–18). A fraction consisting almost entirely of diterpene esters was obtained by vlc.

QUANTITATIVE ^{1}H NMR.—The samples were dissolved in $CDCl_3$ (99.8%), and the nmr spectra were run on a JEOL FX 200 instrument (199.50 MHz). Chemical shift values are reported on the δ-scale, relative to TMS. The recording conditions were selected in order to perform quantitative measurements, and a precisely weighed amount of *p*-dinitrobenzene was added to the samples as an internal standard (19,20).

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