A Simplified Isolation Procedure of Four Cocarcinogenic Phorbolesters from Croton Oil

When earlier purification experiments succeeded in isolating 2 groups of compounds representing the total toxic, vesicant and cocarcinogenic potency of *Croton tiglium* L., they were classified groups A and B, respectively[1]. Although the earlier separation procedure could be simplified in the meantime[2], so far no detailed information about the preparation of amounts sufficient for analytical and biochemical investigations was available. The following study precisely describes the isolation procedure leading to the pure cocarcinogenic compounds by 3 different steps only.

Methods. Test for cocarcinogenic action. 7-week-old[3] albino mice (SaB) inbred for many generations at the Sandoz laboratories[4] were used in all experiments. The manipulations were performed as described by Boutwell, Bosch and Rusch[5] in a slightly modified manner[1,3,6].

Isolation procedure. Preceding experiment: countercurrent distribution of 3.3 g croton oil DAB 6[7] in a Craig apparatus[8] (49 tubes, upper phase and lower phase 10 ml each of the system[1] *N*-heptane-methanol-water = 1:1:0.1, 48 transfers) achieved separation into the fractions shown in Figure 1.

O'Keeffe countercurrent distribution: croton oil diluted with the upper phase (ratio 1:2) of the above heptane system was fed in 15-ml portions into tube 12 of an automatic 48-tube apparatus[9] with a lower phase volume of 25 ml. About 95% of the croton oil remained in the upper phase, increasing its volume to 40 ml.

Column chromatography: an 8 × 150 cm column was filled with silicagel and eluted with an ethylacetate-chloroform-mixture (1:3). Up to 20 g of the resinous fraction can be applied to such a column.

Craig countercurrent distribution: the automatic apparatus[9] with 200 tubes (both, lower and upper phase 25 ml) was fed once with up to 6 g of the active component A or B eluted from the column. The system carbontetrachloride-methanol-water = 2:1:0.3 was used and 1000 transfers performed.

Results. Cocarcinogenic activity. In all the mice pretreated with dimethylbenzanthracene, the compounds isolated stimulated during their 12 weeks application the appearance of papillomas up to 1.4 cm in diameter. The average values observed were 8.1 papilloma/mouse for A_1, 6.5 for A_3, 6 for B_1, and 6.4 for B_2.

Isolation procedure. Preceding experiment: the fractions shown in Figure 1 contained the following mixtures: the active, resinous fraction with a partition coefficient $p = 0.05$ was found in the tubes 0–6, representing 2.5% of the native croton oil; the tubes 7–21 consisted of a colourless, inactive oil, $p = 0.3$, 3.5% of the oil; the tubes 22–32 contained the free fatty acids, $p = 1.2$, 2%, and finally the neutral lipids were recovered in the tubes 33–48, $p = 15.4$, 92%.

O'Keeffe distribution: dissolved in the upper phase, about 80% of the colourless oil, all the fatty acids and all

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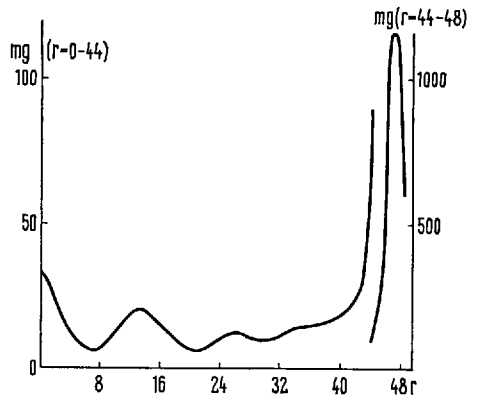

Fig. 1. Craig-countercurrent distribution (3.3 g croton oil, heptane-system). Lower phase = upper phase = 10 ml, 48 transfers. Tubes 0–6 contain the resin, tubes 7–21 a colourless inactive oil, tubes 22–32 free fatty acids, tubes 33–48 neutral lipids. Abszissa: tube No. (fractions), ordinate: weights of the dried fractions in mg.

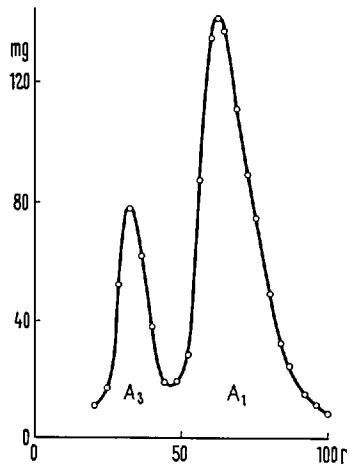

Fig. 2

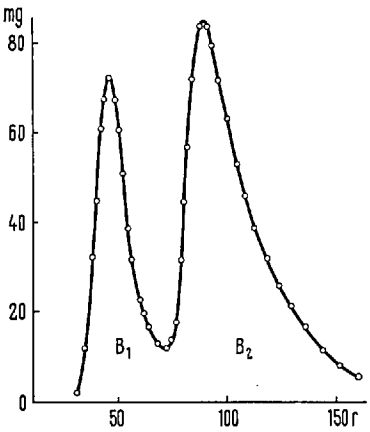

Fig. 3

Figs. 2 and 3. Craig-countercurrent distribution (carbontetrachloride-system) of 4.4 g A (Figure 2) and 4.3 g B (Figure 3), respectively. Lower phase = upper phase = 25 ml, 1000 transfers. Abszissa: tube No. (fractions), ordinate: weights of the dried fractions in mg.

the lipids could be eliminated, i.e. about 97% of the inactive material of the original oil. Thin-layer chromatography (silicagel, ethylacetate-chloroform = 1:2) of the lower phase produced 9 distinct spots, representing the colourless oil (Rf = 0.9) and the active agents A (Rf = 0.25) and B (Rf = 0.35), mainly.

Chromatography: applying 12 g of the active resin obtained by evaporating the lower phase of the O'Keeffe distribution to the column, the active principles were found in fractions (100 ml each) 235–300 (B, 29% of the resin) and 345–405 (A, 16%). These components migrate as single spots in thin-layer chromatography[1].

Craig distribution: group A separated into the pure compounds A_1 (0.45% of the original croton oil) and A_3 (0.15%), group B into B_1 (0.4%) and B_2 (0.8%). The diagrams shown in Figures 2 and 3 are asymmetric, because the partition coefficients are dependent upon the concentration (Figure 4). This behaviour was most pronounced with compound B_2. Consequently, the countercurrent distribution of B_2 resulted in the most asymmetric diagram. The effect decreases from B_2–B_1, from B_1–A_1, and from A_1–A_3, respectively, as may be concluded from Figures 2 and 3 as well as from Figure 4.

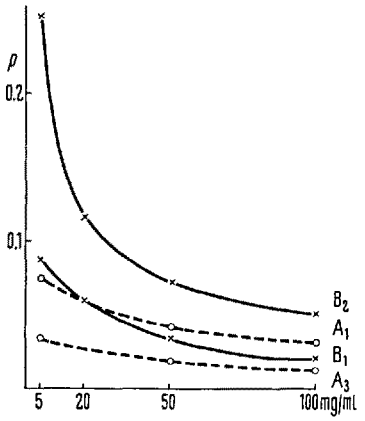

Fig. 4. Partition coefficients (ordinate) of the isolated compounds, determined in the carbontetrachloride-system. They show marked dependence on the concentration (abszissa) of the compounds.

Chemical characterization. IR-absorption spectra of the compounds isolated revealed hydroxylbonds, carbonyl- and esterbonds, and olefinic C=C absorptionbands. Hydrolysis liberated the following fatty acids: from A_1 ($C_{36}H_{56}O_8$) myristic and acetic acid[10], from A_3 ($C_{38}H_{60}O_8$) palmitic and acetic acid[2], from B_1 ($C_{37}H_{58}O_8$) lauric acid and 2-methyl-butanoic acid and from B_2 ($C_{35}H_{54}O_8$) capric and 2-methyl-butanoic acid[11]. All these compounds were shown to be diesters of the polyalcohol phorbol, $C_{20}H_{28}O_6$, analysed in 1934 by FLASCHENTRÄGER[12]. As phorbolesters are known to undergo alkaline hydrolysis easily[12,13] it may be of importance that the simplified procedure no longer uses the elimination of free fatty acids by $2N$ alkalicarbonate, pH = 10.95[1,2,13,14]. It is not yet experimentally excluded that this treatment may cause saponification and re-esterification[12], possibly thus leading to artifacts.

Zusammenfassung. Die schonende Reindarstellung von 4 kokarzinogenen Phorbolestern aus Krotonöl gelingt bereits nach 3 konsekutiven Trennoperationen (O'Keeffe-Gegenstromverteilung, Adsorptionschromatographie, Craig-Verteilung), deren Ausführung präzis beschrieben wird.

J. G. MEYER-BERTENRATH[15]

Medizinische Poliklinik der Universität, 355 Marburg (Germany), 28 August 1968.

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