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150 Years of Croton Oil Research

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It was at a session of the Société Philomatique, Paris, in 1818, that PELLETIER read a paper concerning attempts to separate the active substances from croton oil[1] which he had performed together with CAVENTOU[2]. The investigators, of course, at that time still believed in examining the oil from *Jatropha curcas* L. (Figure 1), but they recognized the error a few years later[3].

These early experiments were carried out by simple extractions and distillations, by saponifications and precipitations. Although with regard to the situation in natural sciences 150 years ago one cannot expect precise elaboration of analytical data, 2 findings should be taken into consideration: (1) Neutralization of the acid croton oil by the hydroxides of barium or magnesium, and subsequent separation of the soaps formed, yielded an oil with raised efficiency. This effect, of course, was estimated rather subjectively, the pungency tasted serving as a criterion. Today it is to be explained by the concentration of the active agents after elimination of free fatty acids from the native oil. (2) The pharmacological potencies of the croton oil could be nullified by intensive saponification, e.g. by boiling with sodium hydroxide. It will be seen later that this loss of activity is the inevitable consequence of the alkaline hydrolysis of the phorbolesters accounting for the biological activities of the untreated oil.

Several findings made by PELLETIER and CAVENTOU have been confirmed by BUCHNER[4], BRANDES[5], and NIMMO[6], who, moreover, subdivided the croton oil into a liquid fraction called fatty oil and the pungent resin with high activity, prepared also by SOUBEIRAN[7].

The croton resin, known nowadays as a strong concentrate of phorbolesters, was separated by consecutive alcoholic extractions[6]. This original method was also employed by BUCHHEIM[8,9], who stated that the oily residue still had the laxative effect of the original oil, which was lacking, however, in the vesicant alcoholic extract. Therefore, these most pronounced pharmacological qualities known to the first investigators were explained rather early as being due to different compounds.

In 1858 SCHLIPPE[10] published elementary analyses for the first time. He had compared the efficiencies of croton oil squeezed out of the seeds, on the one hand, with an alcoholic extract of ground seeds on the other hand, with regard to the vesicant potencies, which were rather exactly determined by the degree of inflammation caused after application to the skin. The vesicant activity of the alcoholic extract was found to be drastically higher than that of the squeezed oil. SCHLIPPE analyzed the extract and stated its contents to consist of C, H, and O only. Moreover, he made the remarkable suggestion that the resinous active agent obviously showed the qualities of an alcohol with more than 1 hydroxyl group.

Our knowledge of the chemistry of the croton oil agents was enlarged by DUNSTAN and BOOLE[11]. They saponified the resinous fraction under analytical conditions, leading to the knowledge that hydrolysis liberated of course fatty acids, but no glycerine. The acids were later identified by BOEHM[12] as a mixture of acetic, iso-butylic, tiglic, and higher homologues with straight chains from caprylic to myristic acid. The alcoholic component was described as biologically inactive and the suggestion of SCHLIPPE confirmed that this alcohol possessed several hydroxyl functions. The activity of croton resin, therefore, was due to its esters consisting of that alcoholic component and fatty acids already mentioned. Moreover, BOEHM found these esters to possess optical activity. He therefore used this criterion, as well as the acidity of the oil, for estimation of the success of each separation step. Decreasing acidity and increasing optical rotation indicated rising

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[2] J. PELLETIER and J.-B. CAVENTOU, Journal de Pharmacie *4*, 289 (1818).
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concentration of the active agent. This excellent method was also used later[13–15].

When PAAL and ROTH[16] nullified the potencies of croton oil by catalytical hydration, BOEHM proved that this effect was caused by the reduction of the agents concentrated in the resinous fraction thus indicating their unsaturated quality – a new, interesting hint concerning the chemical structure.

Moreover, from the pharmacological point of view, the work of BOEHM made remarkable contributions, especially with regard to the toxicology of the croton oil, which he examined quantitatively. The resinous fraction in aqueous dilutions in the ratio of $1:10^8$ killed all the tadpoles put into that medium, and a single dose of 0.02 mg proved to be lethal after s.c. injection into frogs. The toxic potency was also examined with rabbits.

After the first World War, BOEHM continued his work together with KÜLZ and succeeded in crystallizing the alcoholic component of the active agent[17], which was later called phorbol. Thereupon the analytical efforts were forced, and, with the aid of FLASCHENTRÄGER, BISCHOFF, BENEDIKT, and VON WOLFFERSDORFF, the inactive phorbol was converted into the highly active acetyl phorbol[18,19]. This was the first partial synthesis of a vesicant and toxic phorbol ester, confirming the knowledge about the interrelation of structure and qualities of the active agents from *Croton tiglium*.

Nevertheless, these investigators also published inconsistent interpretations about the origin of the croton resin, which was temporarily supposed to be an artifact[19–22], while the unaltered concentrate of the active agents was named the 'honeylike natural substance'. This confusion, however, was eliminated by the proof of CHERBULIEZ and coworkers[23] that the vesicant compounds are of resinous consistence. These experiments took advantage of the data on the solubilities of the phorbol esters elaborated by BOEHM[12,19], and this led to the physical concentration of these compounds by consecutive separations using the 2-phase-system benzene/aqueous methanol. This procedure resembled on principle an O'KEEFFE-countercurrent distribution[24].

The results in croton oil research up to 1934[25,26] are summarized in a lecture read by FLASCHENTRÄGER[21]. As shown in Figure 2, the formula of phorbol, $C_{20}H_{28}O_6$, was already known, as well as the most important fact that this compound had a more-cyclic, unsaturated structure with 5 hydroxyl groups, 2 of which were tertiarily and 2 primarily, the fifth one secondarily linked. Esterifying of the inactive phorbol with fatty acids led to biologically highly active agents. With regard to the limited analytical possibilities of that time, these remarkable findings merit more attention than which is often given in later publications in this field.

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ESSAI ANALYTIQUE

Sur la graine du médicinier cathartique (jatropha curcas).

Par MM. PELLETIER et CAVENTOU.

Lu à la Société Philomatique, le 27 juin 1818.

LA graine du médicinier cathartique, vulgairement appelé *pignon d'Inde*, est un des plus violens purgatifs connus. L'énergie de son action sur l'économie animale, doit même la faire ranger dans la classe des substances vénéneuses. Sous ces deux points de vue, son examen chimique nous a paru mériter d'être entrepris.

Cent grammes de pignon d'Inde mondés de leur partie corticale ont été broyés, réduits en pâte grossière, et traités à plusieurs reprises par l'éther sulfurique rectifié. Lorsque l'éther a paru être sans action sur le résidu, les teintures ont été distillées au bain-marie. l'éther a passé pur, et il est resté dans la cornue une huile épaisse, jaune, et d'une âcreté extrême. Nous reviendrons plus bas sur cette huile particulière.

IV^me^. *Année.* — *Juillet* 1818. 19

Fig. 1. Title-page of the first publication[2] that describes an attempt to separate the active material from croton oil by chemical procedures.

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[14] J. G. MEYER, Dissertation, Universität München (1962).
[15] H. BRESCH, Dissertation, Universität München (1964).
[16] C. PAAL and K. ROTH, Ber. dt. chem. Ges. *42*, 158 (1909).
[17] R. BOEHM, daybook p. 309 of 8.12.1923, in R. v. WOLFFERSDORFF, Dissertation, Leipzig (1929).
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[20] K. WAGNER, Dissertation, Leipzig (1929).
[21] B. FLASCHENTRÄGER, *Zangger Festschrift* (Rascher u. Cie. AG, Verlag Leipzig, Zürich und Stuttgart 1934), vol. 2, p. 857.
[22] R. BOEHM, B. FLASCHENTRÄGER and L. LENDLE, Arch. exp. Path. Pharmak. *177*, 212 (1935).
[23] E. CHERBULIEZ, E. EHNINGER and K. BERNHARD, Helv. chim. Acta *15*, 658 (1932).
[24] E. HECKER, *Verteilungsverfahren im Laboratorium* (Verlag Chemie, Weinheim 1955).
[25] B. FLASCHENTRÄGER and R. v. WOLFFERSDORFF, Helv. chim. Acta *17*, 1444 (1934).
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When BERENBLUM[27,28] detected the cocarcinogenic potencies of croton oil, the research was expanded. However, the scope of this review does not cover the manifold work done from the pathologist's point of view[29–32] but is limited to the problem of isolation and chemical characterization of the active agents. Therefore, the investigations undertaken by GWYNN[33] are mentioned next, who succeeded in separating the croton resin, prepared by liquid-liquid extraction[23], into several fractions by adsorption chromatography using alumina oxid columns. This technique yielded a thirtyfold concentration of the active agents, which were found to be colourless resins with both highly cocarcinogenic and vesicant activities.

When the attempt is made to separate the active agents from the biologically indifferent materials of the original croton oil, it is considered essential to possess methods which allow the impartial estimation of the above-mentioned potencies. Most useful prescriptions were first elaborated by BOUTWELL, BOSCH, and RUSCH, who determined the inflammatory activity by counting the number of mitoses in the epidermis of the treated mouse ear, the other one serving as control[34], while the cocarcinogenic potency was estimated quantitatively by counting the papillomas induced during a 12-week period of croton oil application after pretreatment with a single dose[35,36] of a carcinogenic hydrocarbon[37]. It may be of interest that 5 years later a number of details of that prescription were independently[38] discerned to be most favourable in this topic[39].

Using the techniques of countercurrent distribution[24] and basing his work upon that of former investigators[12,13,23], MEYER published in 1962 the successful purification of 2 highly active components of croton oil, which in thin-layer chromatography migrated as single spots and were named A and B, respectively[14]. These components represent all the toxic, vesicant, and cocarcinogenic potencies of the original oil: Using a method for estimation of the toxic activity of BOEHM and coworkers[22], it was found that 50% of the frogs treated were killed within 12 h by 10 μg A or B/50 g body weight, i.e., $LD_{50} = 10\ \mu g/50$ g. Furthermore, 0.01 μg A or B caused inflammation of a mouse's ear, and 10 μg A or B per mouse and week induced on the average the appearance of about 10 papillomas per mouse[14]. These biological activities correspond with the data elaborated for each of the phorbolesters of group A or B, respectively.

In common successful separation of mixed compounds by countercurrent – distribution depends on the properties of both, the compounds as well as the 2-phase-system of the solvents: Firstly the partition coefficients of the compounds have to possess the greatest differences possible; secondly, the upper phase of the system has to separate as soon as possible from the lower phase, i.e., they must not emulsify mutually or with the compounds to be separated. Using the systems of the above-mentioned procedure[14] BRESCH[15] succeeded in the further separation of group A into the pure compound A_1 and other components representing still a mixture, and of group B into B_1, B_2, and B_3. Although BRESCH did not consider these

Auf Grund der bisher vorliegenden experimentellen Ergebnisse kann man die Formel für Phorbol: $C_{20}H_{28}O_6$ vorläufig folgendermassen auflösen:

$C_{20}H_{23}$ { —OH, —OH tertiär; —OH sekundär; —OH; —OH; —O—; 3 Doppelbindungen; 3 Ringe.

Für Krotophorbolon ergibt sich: $C_{20}H_{26}O_5$:

$C_{20}H_{23}$ { =O; —OH sekundär; —OH; —OH; —O—; 3 Doppelbindungen; 3 Ringe.

Das Phorbol könnte u. a. durch Pinakolinumlagerung in Krotophorbolon übergehen. Der Grundkohlenwasserstoff $C_{20}H_{42}$ und das Krotophorbolon stimmt mit diesen Überlegungen gut überein. Der ätherartig gebundene Sauerstoff kann natürlich auch ringförmig angeordnet sein. Die Natur des tertiären und der sekundären Hydroxyle ist noch weiter sicherzustellen.

Zum Schluß sei das Gesamtergebnis kurz zusammengefaßt: Der Giftstoff im Krotonöl enthält als ungiftige Grundsubstanz einen komplizierten, hochmolekularen Polyalkohol. Durch Veresterung mit Fettsäuren, entstehen giftige Ester, die, je nachdem eine oder mehrere Fettsäuren eingebaut sind, einmal das amorphe, spröde Krotonharz und dann den honigähnlichen Naturstoff liefern können. Durch die Ergebnisse der vorsichtigen Spaltung des Giftstoffes ist diese Vorstellung wohl begründet. Auf Grund dieser Versuche ist es wohl möglich, daß bei der Denaturierung von Alkohol mit Krotonöl,

Fig. 2. Page 827 of the 'ZANGGER-Festschrift' (Zürich 1934) showing the formula of phorbol and several properties of its chemical structure[21].

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[28] I. BERENBLUM, Cancer Res. *1*, 807 (1941).
[29] I. BERENBLUM, Archs Path. *38*, 233 (1944).
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compounds to be pure because of their asymmetric partition diagrams, it can now be stated that compounds B_1 and B_2 have been gained absolutely pure, since the asymmetric diagrams could be shown[40] to be due to the non-linear partition isotherms.

A remarkable merit of BRESCH's work consists in the proof that the active agent A_1 is a diester of phorbol with acetic and myristic acid. These acids had already been liberated from group A in earlier experiments[14].

In the course of the next few years, the purification and identification of further compounds was published. Independently of each other, CLARKE et al.[41] described the compounds B_3–B_7, and, on the other hand, VAN DUUREN and coworker isolated compound B_4[44] as well as A_1 and B_2, which however were already known at that time. Finally, A_3 was described[42], and A_4 as also the active compound A_2[43], which so far had been considered to be inactive[45].

These 11 phorbolesters and their acid residues are shown in the Table. It may be worthy of note that

Compound	Formula	Acid residues
A_1	$C_{36}H_{56}O_8$	Acetic and myristic
A_2	$C_{32}H_{48}O_8$	Acetic and decanoic
A_3	$C_{38}H_{60}O_8$	Acetic and palmitic
A_4	$C_{34}H_{52}O_8$	Acetic and dodecanoic
B_1	$C_{37}H_{58}O_8$	Methylbutanoic and dodecanoic
B_2	$C_{35}H_{54}O_8$	Methylbutanoic and decanoic
B_3	$C_{35}H_{52}O_8$	Tiglic and decanoic
B_4	$C_{34}H_{52}O_8$	Acetic and dodecanoic
B_5	$C_{33}H_{50}O_8$	Methylbutanoic and octanoic
B_6	$C_{33}H_{48}O_8$	Tiglic and octanoic
B_7	$C_{32}H_{48}O_8$	Acetic and decanoic

The Table shows the formulae and acid residues of the phorbolesters isolated so far. Isomeric formulae are explained in the text.

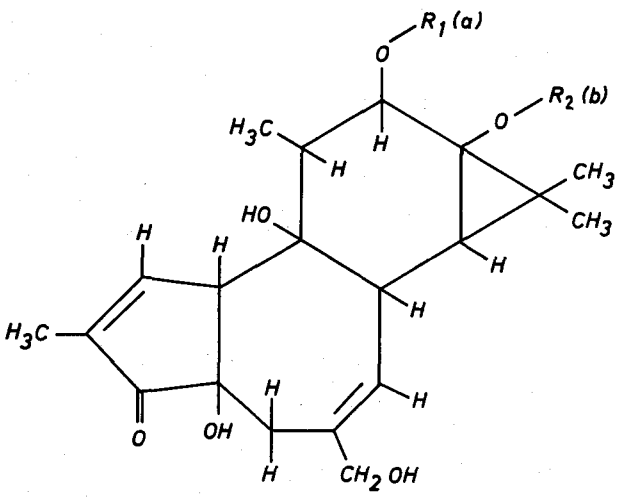

Fig. 3. The proposed[43] structure of phorbol ($R_1 = R_2 = H$). Substitution of R_1 and R_2 by fatty acid residues leads to the biologically active esters.

in contrary to the separation procedures used for the isolation of the greater part of those compounds, VAN DUUREN[44] first of all esterified the biologically active mixture with *p*-nitrophenylazobenzoic acid and separated the compounds A_1, B_2, and B_4 as their azoderivatives. This technique had been employed in experiments with group B, too, leading to 4 distinct monoazoderivatives which were found to be no longer of vesicant or of cocarcinogenic activity[14].

The recent development of sensitive physical methods, such as nuclear magnetic resonance and mass spectrometries, has provided ideas for the chemical structure of phorbol[43]. After the formula $C_{20}H_{28}O_6$ could be confirmed by splitting off 1 molecule of water from the crotophorbolon, $C_{20}H_{26}O_5$ about 40 years ago[21], the early knowledge of several structural properties of phorbol already mentioned (Figure 2) was enlarged by KAUFFMANN and coworkers[46,47], who found the α,β-unsaturated keto- and the α-glycol group and synthetized, moreover, several active derivatives. The further hydroxyl group of the phorboldiesters that could be esterified was shown to be a primary allylic one, and phorbol was proved to possess a carbon skeleton of 4 cycles, one of which being cyclopropane[15]. The analytical studies, supported by the above-mentioned physical methods, explain the existence of groups A and B: esterification of the secondary hydroxyl function (a) (Figure 3) with a long chained acid and of the tertiary one (b) with acetic acid characterizes the A-compounds. The diesters of the B-group, vice versa, are derivatives with long chained fatty acid residues in position (b), while in (a) the smaller residues of acetic, methylbutanoic, or tiglic acid are linked to the alcoholic groups of the phorbol[48].

This knowledge at present terminates 150 years of experimental effort in croton oil research, as far as the chemistry of the active agents is concerned. Unfortunately, their biochemical mode of action in carcinogenesis is not yet understood, as experimental data in this field are almost completely lacking. Discussions of the hypothesis of a 2-stagemechanism in carcinogenesis, therefore, allow the implication of phorbolesters simply and solely as a speculative contribution[49], and it is much to be desired that this

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problem should induce qualified experimental studies to elucidate the cocarcinogenic mechanism at the molecular level.

Zusammenfassung. Die Übersicht befasst sich mit der Entdeckungsgeschichte der entzündlich, toxisch und kokarzinogen hochwirksamen Phorbolester aus *Croton tiglium* L., wobei insbesondere die Anreicherungs- und Isolierungsversuche sowie die Beiträge zur Chemie der Wirkstoffe berücksichtigt werden. Nach ersten Untersuchungen durch PELLETIER und CAVENTOU im Jahre 1818 erbrachten vor allem die Arbeiten der Arbeitskreise um BOEHM sowie FLASCHENTRÄGER in den zwanziger Jahren dieses Jahrhunderts entscheidende Fortschritte in der chemischen Charakterisierung der Giftstoffe, die als Fettsäureester des Phorbols, $C_{20}H_{28}O_6$, erkannt wurden. Nach der Entdeckung der kokarzinogenen Aktivität hielt das Crotonöl Einzug in die experimentelle Krebsforschung und gab Anlass zu intensiviertem Bemühen um die Isolierung der Wirkstoffe, die durch Anwendung von Gegenstromverteilungen schliesslich 1962 erstmals frei von inaktiven Begleitstoffen dargestellt werden konnten. Inzwischen ist die Chemie der Phorbolester weitgehend geklärt und ein Strukturvorschlag für das Phorbol erarbeitet worden. Die Biochemie dieser Substanzen im Hinblick auf ihre Tumorpromotion bei der Karzinogenese ist jedoch bislang völlig ungeklärt.

SPECIALIA

Two Types of Carbonate Substitution in the Apatite Structure

The apatite structure (e.g. $Ca_{10}(PO_4)_6(OH)_2$) allows varied substitutions to take place (e.g. Sr^{2+} or Ba^{2+} for Ca^{2+}; $(SiO_4)^{4-}$ for $(PO_4)^{3-}$; Cl^- or F^- for $(OH)^-$) without a significant alteration in its basic structure. These substitutions in the structure are manifested by changes in the lattice parameters (*a*- and *c*-axes) of the apatite depending on the size of the substituting cation or anion. The incorporation of carbonate into the apatite structure, however, has been the subject of study and speculations for more than 3 decades. On the basis of chemical analysis, CO_3-for-OH substitution was proposed by EITEL[1]. Carbonate-containing mineral apatites demonstrate a contraction of the unit cell[2–4] but biological apatite (e.g. human enamel) demonstrate an expanded *a*-axis, 9.44 Å. Since all naturally occurring apatites have other constituents besides those of Ca, CO_3 and PO_4, it is necessary to study synthetic systems in which the incorporation of only the carbonate could be directly correlated with the physical properties of the apatite. 2 types of carbonate-containing synthetic apatites have been reported in literature: (a) prepared at high temperatures, 900–1000 °C, with exclusion of water[5,6]; and (b) prepared from aqueous systems by direct precipitation[7,8] and by conversion of monetite, $CaHPO_4$, in carbonate solutions at 100 °C[8]. The results from X-ray diffraction and IR-absorption studies are briefly reported in this paper. A more extensive report will be published elsewhere.

X-ray diffraction analysis. Figure 1 shows diffraction patterns of the 2 types of carbonate-containing synthetic apatites: (A) and (B) are apatites prepared at 1000 °C before and after passing dry CO_2 over them; (C) and (D) are patterns of apatites precipitated from phosphate solutions at 100 °C without and with added carbonate, respectively. The expansion of the *a*-axis in the former case is determined from the shifting of the (300) reflection to lower angles (2θ), and the slight contraction of the *c*-axis from the shifting of the (002) reflection to higher angles. In the precipitated apatites, carbonate incorporation shifts the (300) reflection to higher angles, indicating a contraction of the *a*-axis, and the (002) reflection to lower angles, indicating a slight expansion of the *c*-axis. Since the planar CO_3 group is larger than the (OH) group, an expansion of the unit cell would be expected to accompany a CO_3-for-OH substitution in the apatite (Table I). On the other hand, the planar carbonate is smaller than the tetrahedral PO_4 group, and a contraction of the unit-cell would be expected to accompany a CO_3-for-PO_4 substitution (Table II). It is also of interest to note that the *a*-axes of the precipitated carbonate-free apatites as precipitated, are slightly longer (i.e. by 0.014–0.022 Å) than those of apatites which have been heated at high temperature or those of the mineral OH-apatite from Holly Springs, Ga. (i.e. 9.42_2 Å). This slight expansion of the *a*-axis observed in the precipitated apatites could be due to the presence of some HPO_4^- or entrapped water[4].

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