

NEW TOXIC, IRRITANT AND COCARCINOGENIC DITERPENE ESTERS FROM EUPHORBIAEAE
AND FROM THYMELAEAEAE*

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Abstract - The biologically active principles from Euphorbiaceae and Thymelaeaceae isolated and structurally elucidated so far are derivatives of three hitherto unknown, chemically related diterpene parent hydrocarbons: the tetracyclics tigliane and ingenane and the tricyclic daphnane. The naturally occurring polyfunctional diterpene parent alcohols phorbol, ingenol and resiniferonol respectively are related structurally to these hydrocarbons. The toxic and irritant principles are esters - in case of the daphnane type orthoesters - of such parent alcohols with a variety of aliphatic, aromatic and aromatic/aliphatic acids. Besides the active principles a number of biologically inactive esters of new macrocyclic diterpenes were isolated and structurally elucidated e.g. lathyrol esters. According to their structure and to the isoprene rule macrocyclics of this type may be considered intermediates in the biogenesis of the diterpene parents of the active principles. Most of the irritant esters also exhibit cocarcinogenic or tumor promoting activity in the skin of mice. Therefore, in the etiology of human tumors such cocarcinogenic diterpene esters may be considered second order carcinogenic risk factors. Recently some of the diterpene esters described have been reported to exhibit antileukemogenic activity.

For centuries, plants and plant materials of species of the family Euphorbiaceae (spurge family) have been known to be poisonous to human beings. Often they are held responsible for the poisoning of live stock and are used as constituents of arrow poisons. Quite a number of species are used in folk medicine, as drugs and raw materials for pharmaceutical preparations, in agriculture, as resources for certain technologies and as ornamental plants in horticulture. In view of their manifold uses in our environment it appears surprising that the chemical nature of the toxic principles of the plants and hence the biological nature of their toxicity and their possible therapeutic value remained relatively undefined until recently.

10 Years ago, in an effort to clarify the intriguing phenomenon of cocarcinogenicity of croton oil we succeeded in the separation, isolation and structural and biological elucidation of its active principles (1). This seed oil from the Euphorbiaceae Croton tiglium was, and still is, used in some parts of the world as a counterirritant and as a purgative drug. The croton oil factors are 12,13-diesters of the tetracyclic diterpene, phorbol. In Fig. 1, as a prototype, the structure of croton oil factor A₁,12-00tetradecanoylphorbol-13-acetate or TPA, is specified. In our standard assay on the mouse ear it exhibits an irritant dose 50 (ID₅₀) - the dose to cause irritation in 50% of the mice treated - with as little as 0,016 nmoles/ear. Also, it is the most active "free irritant and cocarcinogen" known so far, requiring a dose of only p = 10 nmole/application to exhibit cocarcinogenic activity in our standard assay on the skin of mice. The other thirteen phorbol-12,13-diesters isolated from the oil differ in the fatty acid moieties of their ester functions and are more or less active as irritants and as cocarcinogens (1).

The structure and stereochemistry of phorbol was confirmed by X-ray diffraction analysis (1). The corresponding parent hydrocarbon tigliane is a substituted perhydrocyclopropabenzazulene (Fig. 1). The chirality of the asymmetric centres in the tigliane is defined by the chirality of the corresponding centres in the naturally occurring phorbol. In contrast to its esters phorbol is neither irritant nor cocarcinogenic to mouse skin (1).

Besides the phorbol-12,13-diesters, croton oil also contains 12,13,20-triesters of phorbol, representing as much as 50% of the phorbol content of the oil (1). They exhibit relatively little if any irritant and cocarcinogenic activity. Ester functions in the 20-position have not been reported previously but in the prototype triester, Croton Factor S

* Invited half hour lecture

from *Croton sparsiflorus* (Fig. 1), a linolenic acid residue was shown to be present in this position (2).

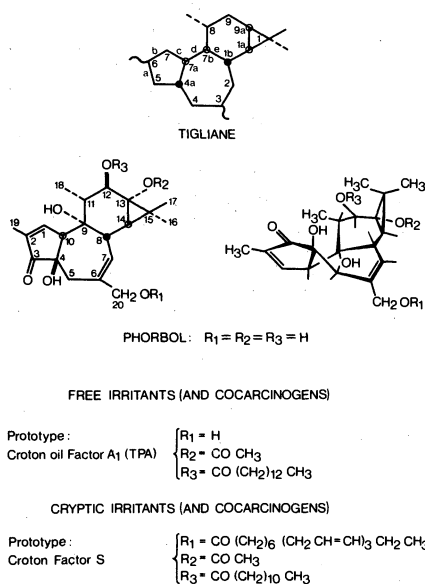


Fig. 1 Structure and stereochemistry of tiglane type diterpenes. Prototypes: phorbol and its 12,13-di- and 12,13,20-triesters, representing free and cryptic irritants and cocarcinogens, respectively.

Interestingly, such 12,13,20- triesters may be converted by acid catalysed transesterification, to the corresponding phorbol-12,13-diester with a concomittant dramatic increase in irritant and cocarcinogenic activity (1,2). In real life such an "activation" may occur under enzymatic or non-enzymatic conditions. Therefore, in addition to the free irritants and cocarcinogens - i.e. phorbol-12,13-diesters - croton oil is shown to contain "cryptic irritants and cocarcinogens" - i.e. phorbol-12,13,20-triesters (1). Croton oil also contains, as minor constituents, biologically inactive esters of 4-deoxy-4 α -phorbol (see also below).

TABLE 1. The Berenblum-experiment of cocarcinogenesis (schematically)

Experiment No.	Exposure of Organism	Tumors
1	■ ■ ■ ■ ■	+
2	■	-
3	□ □ □ □	-
4	■ □ □ □ □	+

■ Solitary carcinogen □ cocarcinogen

The phenomenon of cocarcinogenicity is best demonstrated in the already classical set up of the Berenblum-experiment (Table 1). In Exp. No.1 tumors or cancers are produced by repeated (i.e. chronic) exposure of the host or target tissue to small, identical doses of a solitary carcinogen (3), e.g. a carcinogenic aromatic hydrocarbon. Under otherwise identical experimental conditions exposure of the host or target tissue to just one dose of the solitary carcinogen, as indicated in Exp. No.2, does not elicit tumors or cancers. Hence this single dose is "subcarcinogenic" for the host or target tissue. In Expt. No.3 the host or target tissue is exposed to a number of doses of a cocarcinogen, each representing about 1/100 of the single dose of the solitary carcinogen. This does not cause tumors or cancers even in lifetime exposures of the mice. However, combination of the exposure to the single dose of the solitary carcinogen, followed by the repeated exposure to the cocarcinogen readily causes tumors or cancers, as indicated in Exp. No.4.

In the etiology of human tumors or cancers solitary carcinogens are considered first order carcinogenic risk factors (3). Clinical and epidemiological evidence also suggests that cocarcinogens may play an important role in the etiology of human cancers: exposure of any tissue to even a subcarcinogenic dose of a solitary carcinogen may be causative of cancer if, no matter how much later in life, that same tissue is exposed to a cocarcinogen. Therefore, it is proposed to consider cocarcinogens as second order carcinogenic risk factors (3).

Some years ago, at the German Cancer Research Centre we started a program to evaluate the active principles of further species of the Euphorbiaceae to clarify the occurrence and distribution of cocarcinogens in the human environment. We use systematic isolation procedures monitored by our standardized and quantitative assay for irritant activity on the mouse ear as a rapid biological measure. Recently, other groups joined this effort and cooperated in providing the pure compounds they isolated for quantitative assay of cocarcinogenicity in our laboratory. In their isolation procedures some of them use assays for general toxic (4) or piscicidal activity (5). Thus considerable progress has been achieved in this field in the last few years as summarized below for 1976 and late 1975. For a review with full quotation of all contributions of this and other groups up to late 1975 see reference (6).

Besides esters of phorbol species of the genus *Croton* and species of the genus *Euphorbia* also contain esters of other derivatives of tiglane with the same degree of oxygenation as phorbol but partially different distribution of the hydroxyl functions, such as 4-deoxy-16-hydroxy- and 12-deoxy-16-hydroxyphorbol (O₆, Table 2). The corresponding species (or materials derived from them) are used as folk remedies, as purgative drugs, in homeopathy, for the production of rubber and as honey plants. Tiglane derivatives less oxygenated than phorbol (O₅, Table 2) were isolated from, or demonstrated to be present in a number of *Euphorbia* (7) species, some of which are used in the production of chewing gum, as honey plants, for horticultural purposes and as counterirritant or purgative drugs. Recently (8),

TABLE 2. Tiglane type parent alcohols: occurrence in Euphorbiaceae and Thymelaeaceae species and their use in human environment

Oxygenation of tiglane	Parent alcohol	Isolated from plant species	Use in environment
<u>EUPHORBIACEAE</u>			
O ₅	4-deoxyphorbol	<u><i>Euphorbia tirucalli</i></u> L.	horticulture, live fences
	12-deoxyphorbol	<u><i>E.triangularis</i></u> Desf.	production of chewing gum, honey plant
		<u><i>E.resinifera</i></u> Berg ⁺)	counterirritant, purgative drug

O ₆	phorbol	<u><i>Croton tiglium</i></u> L. <u><i>C. sparsiflorus</i></u> Morong. <u><i>C. oblongifolius</i></u> Roxb.	purgative drug purgative drug folk remedy, homeopathy, purgative drug
	4-deoxy-16-hydroxyphorbol	<u><i>C. flavens</i></u> L.	tea from leaves and root chewing as folk remedy
	12-deoxy-16-hydroxyphorbol	<u><i>E.cooperi</i></u> N.E.Br.	production of rubber, honey plant

O ₇	16-hydroxy-phorbol	<u><i>Aleurites fordii</i></u> Hemsl. <u><i>Croton flavens</i></u> L.	tung oil (drying oil) folk remedy as tea from leaves and root chewing
	12-deoxy-5β-hydroxyphorbol-6α,7α-oxide	<u><i>Hippomane mancinella</i></u> L.	wood for furniture and construction

<u>THYMLAEACEAE</u>			
O ₅	12-deoxyphorbol	<u><i>Pimelea prostrata</i></u> Willd.	cattle poison, horticulture

⁺) and others e.g. *E.fortissima* Leach, *E.coerulescens* Haw., *E.franckiana* Berger, *E.ledienii* Berger, *E.polyacantha* Boiss., *E.unispina* N.E.Br., *E.poissoni* Pax.

the biologically inactive 4 α -sapiine was obtained from the seed oil of *Sapium indicum* Willd. and shown to be 13-O-acetyl-4-deoxy-12-O-(N-methylantranoyl)-4 α -phorbol. It is the first diterpene ester in the tiglane series carrying a nitrogen containing acyl residue and reminiscent of ester alkaloids. In view of the facile and irreversible epimerization of 4-deoxyphorbol (9) to its 4 α -isomer the isolation of 4-deoxy-4 α -phorbol esters or of 4 α -sapiine may well be taken as an indication of the presence of the (postulated) biologically active 4 β -epimers in the corresponding plant materials. 4-Deoxyphorbol derivatives were isolated from *E. tirucalli* (9). Tiglane derivatives more oxygenated than phorbol (07, Table 2) occur as parent alcohols of biologically active esters in the fruit shells of the tropical *Aleurites fordii*, an important source of the drying tung oil as well as in the Caribbean *Croton flavens* (10). The latter species is used in folk medicine and certain uses of it are possibly correlated with the high incidence of esophageal cancer in Curacao (10). *Hippomane mancinella*, the manchineel tree of Central America, the wood of which is used for furniture and construction, contains esters of the 6 α ,7 α -oxide of 12-deoxy-5 β -hydroxyphorbol.

It is of considerable chemotaxonomic as well as environmental interest that a tiglane derivative was found recently in a species of the genus *Pimelea* belonging to the botanically remote family of the Thymelaeaceae (Table 2). Prostratin, isolated from *Pimelea prostrata*, the strathmore weed which frequently causes the intoxication of cattle in New Zealand, was identified as 13-O-acetyl-12-deoxyphorbol (11).

Besides tiglane derivatives, species of the genus *Euphorbia* also may contain irritant and cocarcinogenic esters of the ingenane type (Fig. 2). Compared to tiglane the carbon skeleton of ingenane contains two seven membered rings. This together with the stereochemistry of the asymmetric centers, was established by X-ray diffraction analysis of 3,5,20-tri-O-acetylingenol (6).

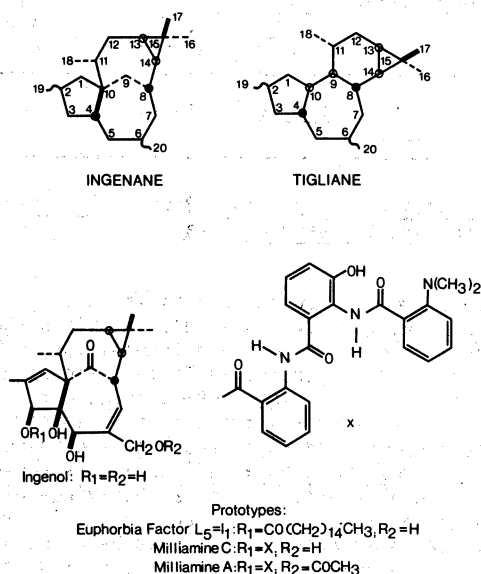


Fig. 2 Structure and stereochemistry of ingenane type diterpenes. Prototype: ingenol and its 3-mono- and 3,20-diesters

The prototype of these irritants and cocarcinogens, Euphorbiafactor L₅ = I₇ i.e. 3-hexadecanoylingenol (Fig. 2), was isolated (6) from both the herbaceous European species, *E. lathyris* (caper spurge, see 05, Table 3) and from the arborescent South and East African species *E. ingens* (cancelaber tree). The seed oil of *E. lathyris* is used as a mild purgative, and in the production of soap and in candles. Also, the plant is grown in gardens as a mule repellent and for ornamental purposes. The latex of *E. ingens* is used as a constituent of arrow poison and at times in the production of rubber. 3-Hexadecanoylingenol exhibits an ID₅₀ of 0.13 nmoles/mouse ear; it is somewhat less irritant and cocarcinogenic than TPA. Most interestingly a 3-ester of ingenol with an oligopeptidyl group was isolated from *Euphorbia milii* (Milliamin C). The tripeptide ester function contains anthranilic, 3-hydroxyanthranilic and dimethylantranilic acid (Fig. 2) and is thus reminiscent of 4 α -sapiine (see above). The related Milliamin A is acetylated in position 20 analogous to the cryptic cocarcinogens derived from tiglane (see Fig. 1).

However, as yet it is impossible to establish this relation by assay because of lack of compound. Very recently, confirmative biological data became available for 3-deca

TABLE 3. Ingenane type parent alcohols: Occurrence in Euphorbiaceae species and their use in human environment

Oxygenation of ingenane	Parent alcohol	Isolation from plant species	Use in environment
0 ₄	5-deoxyingenol	<u>Euphorbia myrsinites</u> L.	horticulture
	20-deoxyingenol	<u>E.kansui</u> Liou.	Chinese drug
0 ₅	ingenol	<u>E.lathyris</u> L.	purgative drug, soap production, horticulture rubber prod., arrow poison
		<u>E.ingens</u> E. Mey.	
		<u>E.milii</u> Ch. des Moulins	
		<u>E.myrsinites</u> L.	
	20-deoxy-16 hydroxyingenol	<u>E.resinifera</u> Berg ⁺) <u>E.marginata</u> Pursh.	horticulture horticulture counterirritant drug horticulture, honey plant
0 ₆	13-hydroxyingenol	<u>E.kansui</u> Liou. <u>E.cyparissias</u> L.	Chinese drug folk medicine, homeopathy
	16-hydroxyingenol	<u>E.lathyris</u> L.	see above
		<u>E.ingens</u> E. Mey	see above
		<u>E.marginata</u> Pursh	honey plant
0 ₇	12-deoxy-16,19-dihydroxyingenol	<u>E.cyparissias</u> L.	folk medicine, homeopathy

⁺) and others e.g. E.lactea L., E.jolkinii Boiss., E.kansui Liou., E.antiquorum L., E.polychroma Kerner, E.desmondii Keay.

2',4'-dienylingenol and the corresponding 20-acetate isolated (12) from the Mediteranian and Near East species, E. myrsinites (see 0₅, Table 3). Besides those Euphorbia species already discussed, many others were shown to contain ingenol (0₅, Table 3), often as the sole diterpene derivative (6,7). Those recorded in Table 3 are used as sources of drugs, in agriculture and in horticulture. It is interesting to note that, as yet, ingenol has been detected only in species of the genus Euphorbia (and Elaeophorbia) but not in those of other genera of Euphorbiaceae nor of the Thymelaeaceae also investigated. 20-Deoxy-16-hydroxyingenol was isolated from E. marginata (13), which is known as a source of toxic honey and used as an ornamental plant. 5- and 20-Deoxyingenol (0₄, Table 3) were detected in E. myrsinites (7) and in E. kansui, respectively. Diterpene parent alcohols more oxygenated than ingenol, i.e. 13-hydroxyingenol have been isolated from E. kansui and E. cyparissias and 16-hydroxyingenol from E. lathyris (6), E. ingens (6) and E. marginata (0₆, Table 3). 12-Deoxy-16,19-dihydroxyingenol was detected in E. cyparissias (0₇, Table 3) used in folk medicine and homeopathy (14).

Recently the chemical relationship between ingenane and tiglliane was demonstrated by chemical means, when we succeeded in reducing the sterically hindered carbonyl group in the 9-position of 3,4,5,20-di-0-isopropylideneingenol (Fig. 3) to yield the corresponding 9(R)- and 9(S)-epimers. The 9(R)-epimer is converted to the corresponding polyfunctional diterpene with the tiglliane skeleton by Wagner-Meerwein rearrangement. Similarly from the 9(S)-epimer the corresponding perhydronaphthalene derivative is obtained (15).

Derivatives of the tricyclic diterpene daphnane occur in Euphorbiaceae and, again, in Thymelaeaceae. They represent a third class of irritant principles. The prototype parent alcohol resiniferonol occurring in E. resinifera, E. unispina (16) and E. poissoni (17) is depicted in Fig. 4. Resiniferonol carries the structural elements of 12-deoxyphorbol, excepting the cyclopropane ring, which is replaced by an isopropenyl group in the 13 β - and a hydroxyl group in the 14 α -position. Typically the irritant esters of this type contain a 9,13,14-ortho-ester function: thus resiniferatoxin (Fig. 4) is an orthoester with phenylacetic acid and, resembling the

structural type of cryptic cocarcinogens, a 20-ester with 5-hydroxy-3-methoxyphenyl-acetic acid. With an ID_{50} of 10^{-4} - 10^{-6} nmole/mouse ear, resiniferatoxin is the most active irritant known so far to occur in Euphorbiaceae and Thymelaeaceae. Investigations into daphnane type esters as to a possible contribution of 20-ester groups to their irritant and cocarcinogenic activity are under way in our laboratory.

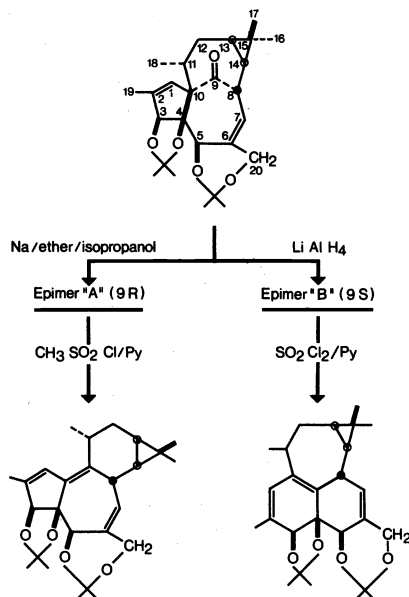


Fig. 3 Preparation and rearrangement of the epimeric 9-deoxo-9-hydroxyingenol

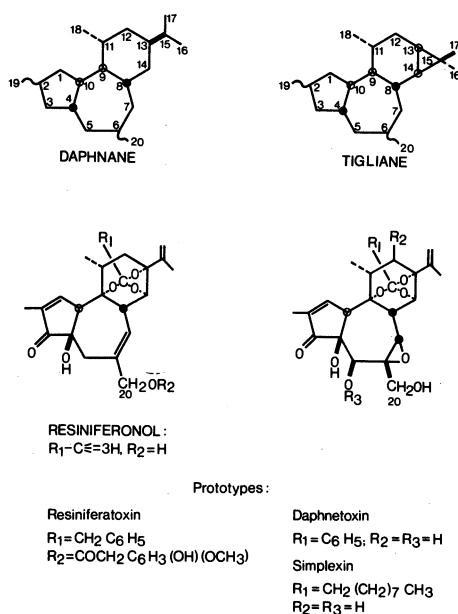


Fig. 4 Structure and stereochemistry of daphnane type diterpenes. Prototypes: resiniferonol and its 9,13,14-ortho- and 9,13,14-ortho-20-esters.

The latices of *E. resinifera*, *E. unispina* and *E. poissoni* are used as sources of counter-irritant drugs and as additives to tobacco snuff and arrow fish poisons, respectively (O_6 , Table 4). Toxins with parent alcohols more oxygenated than resiniferonol (O_8 , Table 4), i.e. orthoesters of 5-hydroxyresiniferonol- $6\alpha,7\alpha$ -oxide have been isolated from the tropical Euphorbiaceae *Hura crepitans* (sandbox tree), *Hippomane mancinella* (manchineel) and *Excoecaria agallocha* (blinding tree).

As yet, with the exception of prostratin (see Table 2), mainly daphnane type diterpene

TABLE 4. Daphnane type parent alcohols: occurrence in Euphorbiaceae species and their use in human environment

Oxygenation of daphnane	Parent alcohol	Isolated from plant species	Use in environment
O_6	Resiniferonol	<i>Euphorbia resinifera</i> Berg	counterirritant drug
		<i>E.unispina</i> N.E.Br.	tobacco snuff, fish
		<i>E.poissoni</i> Pax.	arrow poison, cattle poison
O_8	5 β -hydroxyresiniferonol- $6\alpha,7\alpha$ -oxide	<i>Hura crepitans</i> L.	horticulture
		<i>Hippomane mancinella</i> L.	wood for furniture and construction
		<i>Excoecaria agallocha</i> L.	fish and arrow poison

esters seem to represent the toxic principles in the species of Thymelaeaceae: daphnetoxin and simplexin (Fig. 4) contain as parent alcohol the very same 5 β -hydroxyresiniferonol- $6\alpha,7\alpha$ -oxide as *Hura crepitans*, *Hippomane mancinella* and *Excoecaria agallocha* (O_8 , Tables 4 and 5, respectively). However, the toxins involved differ in their orthoester groups. *Daphne mezereum* (spurge laurel, Table 5) was used as a drug and is of wide use in all temperature climates as an ornamental plant. Simplexin was isolated and characterized from *Pimelea simplex* by the late Prof. Ritchie and coworkers (18) and in our laboratory from both *P. simplex* as well as from *P. prostrata* (19). Both of these species are known in Australia and New Zealand as a source of cattle poisoning. The highest degree of oxygenation (O_9 , Table 5) is exhibited by the parent alcohol of mezerein (20), another toxin from *Daphne mezereum* and of several Gnidia factors: their common parent alcohol is 5 $\beta,12\beta$ -dihydroxyresiniferonol- $6\alpha,7\alpha$ -oxide. The corresponding toxins contain various acyl residues in their 12-ester groups. Of the daphnane derived orthoesters only resiniferatoxin (21),

TABLE 5. Daphnane type parent alcohols: occurrence in Thymelaeaceae and their use in human environment

Oxygenation of daphnane	Parent alcohol	Isolated from plant species	Use in environment
O_8	5 β -hydroxyresiniferonol- $6\alpha,7\alpha$ -oxide	<i>Daphne mezereum</i> L.	horticulture, irritant drugs
		<i>Pimelea simplex</i> F.Muell	cattle poison
		<i>Pimelea prostrata</i> Willd.	cattle poison, horticulture
O_9	5 $\beta,12\beta$ -dihydroxyresiniferonol- $6\alpha,7\alpha$ -oxide	<i>Daphne mezereum</i> L.	horticulture, irritant drug
		<i>Lasiosiphon burchelli</i> Meisn.	cattle poison
		<i>Gnidia lamprantha</i> Gilg.	folk remedy, purgative drug

huratoxin (21), simplexin (21) and mezerein (22) have been assayed so far for cocarcinogenic activity. The latter three were found positive and exhibit moderate to considerable cocarcinogenic activity in mouse skin as compared to TPA.

Most interestingly an antileukemogenic activity was reported recently for a phorbol-12, 13-diester (23), for 3,20-dibenzoylgingenol (23), for mezerein (24), for some Gnidia factors (25) and for extracts of *Pimelea simplex* (26). These findings, indicating an extended

version of "Sir Alexander Haddow's paradox" (see Note a), may open new avenues for the treatment of leukemia patients suffering from this dreadful kind of cancer. In connection with these findings it is interesting to note the reported (28) leukemogenic activity of phorbol.

In addition to the biologically active derivatives of the three new polycyclic diterpenes tigliane, ingenane and daphnane a number of esters of new macrocyclic diterpenes such as lathyrol and duvatriene-diol (Fig. 5) have been isolated from Euphorbiaceae and from tobacco (Solanaceae) as well as from tobacco smoke condensate. As far as assayed,

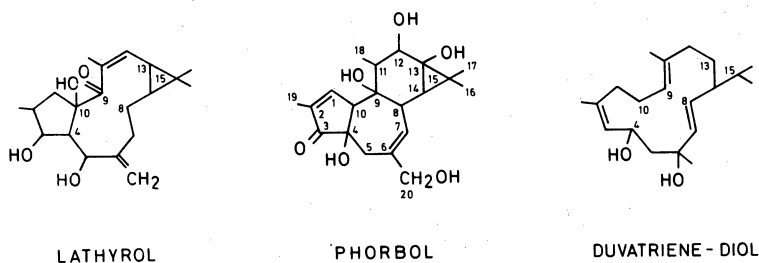


Fig. 5 Comparison of the skeletons of phorbol and lathyrol with duvatriene-diol from tobacco (see also loc.cit. 22).

these esters or their parent alcohols do not exhibit any biological activity. However, their structural relation to tigliane, as exemplified in Fig. 5, together with the classical isoprene rule suggests that lathyrene type macrocyclic diterpenes may be biogenetic precursors of such polycyclic diterpene parents as tigliane, ingenane and daphnane. They may be generated in plants from geranyl geraniol pyrophosphate via the macrocyclic casbene (22) (Fig. 6). Similar routes may lead via duvane type precursors to structures such as rhamnifolane (22), which is isomeric with daphnane (Fig. 6).

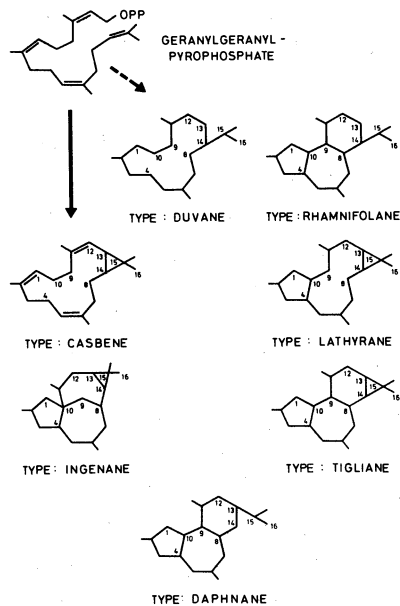


Fig. 6 Isoprene rule in the biogenesis of diterpene parents of cocarcinogens

Note a: This paradox (see 27) was established primarily for certain alkylating agents which often exhibit, besides their desired cytostatic activity, also an undesirable solitarycarcinogenic activity. As yet, the validity of the paradox for cocarcinogens is unknown.

The occurrence of free and cryptic cocarcinogenic diterpenes generated in certain species of Euphorbiaceae and Thymelaeaceae suggests that under certain circumstances indiscriminate use in the human environment of such species, or of plant products and food derived from them may be a potential carcinogenic risk. Consequently, investigations of the distribution of cocarcinogens in the plant kingdom deserve primary interest especially in view of certain new technologies propagated recently (29). Such investigations are challenging exercises in the skills of isolation, purification and structure elucidation of hitherto unknown natural products. The results will help to provide a sound scientific background for environmental hygiene and preventive medicine aimed at the benefit and health of human beings.

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