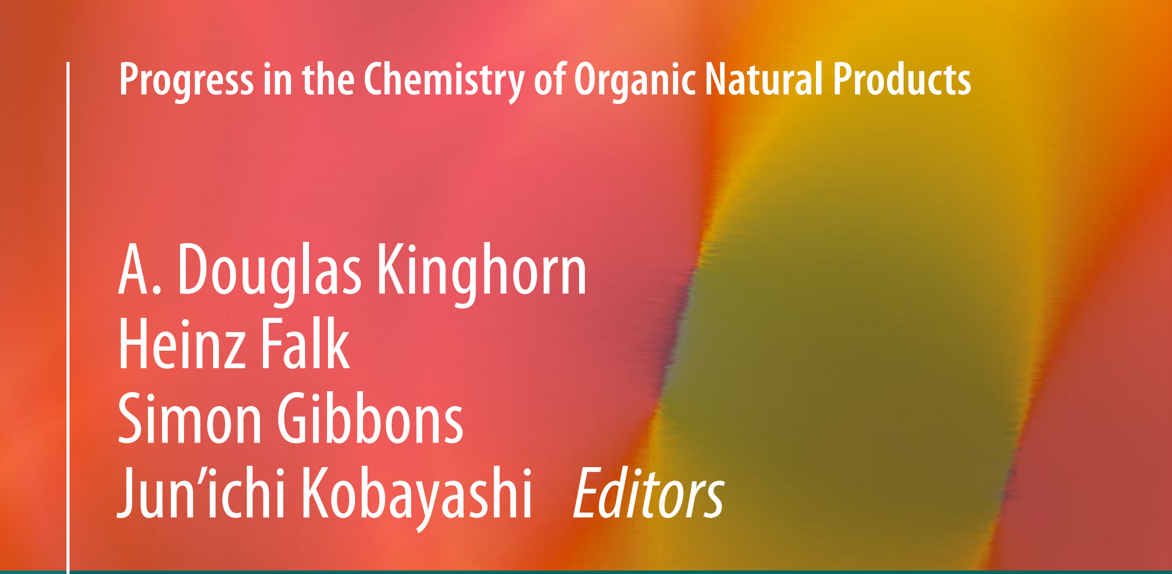
Progress in the Chemistry of Organic Natural Products



A. Douglas Kinghorn
Heinz Falk
Simon Gibbons
Jun'ichi Kobayashi *Editors*

102

Progress in the Chemistry of Organic Natural Products

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A. Douglas Kinghorn • Heinz Falk •
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Editors

Progress in the Chemistry of Organic Natural Products

Volume 102

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Springer

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Contents

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Ingenane Diterpenoids

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Contents

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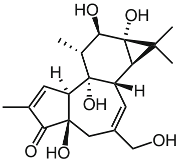
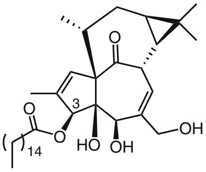
1 Introduction

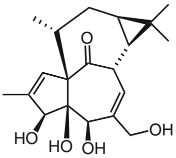
Ingenane diterpenoids are a biogenetically advanced group of phorboids, a family of polycyclic diterpenoids structurally related to the tigliane skeleton of phorbol (**1**) that also includes the tiglianes and daphnanes. A combination of limited distribution, very low concentration in plant tissues, chemical instability, and difficulty of detection delayed the discovery of ingenane derivatives (ingenoids) compared to the tiglianes, the major class of phorboids. Thus, while phorbol (**1**), the archetypical tigliane, was first obtained in pure form in 1934 [1], the first ingenol derivative (ingenol-3-hexadecanoate = ingenol 3-palmitate, **2**) was only reported in 1968 [2, 3]. On the other hand, while phorbol went into a long series of constitutional and configurational revisions before its structure was clarified unambiguously by X-ray crystallography in 1967 [4], the basic ingenane polyol ingenol (**3**), just like the archetypical daphnane daphnetoxin (**4**) [5], benefited from an early (two years after the isolation) crystallographic determination of its relative and absolute configuration [6]. In retrospect, the early structure elucidation of ingenol, along with the lack of an abundant plant source, is responsible for the limited information still available on its chemistry compared to phorbol, for which the reactivity was thoroughly investigated in attempts to elucidate its structure by classic studies of chemical degradation [7].



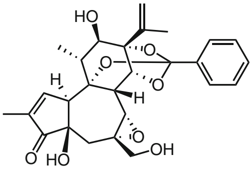
1 (phorbol)



2 (ingenol-3-palmitate)



3 (ingenol)



4 (daphnetoxin)

To detect the presence, often in trace amounts, of ingenol derivatives in crude extracts and chromatographic fractions, the early studies relied extensively on non-selective bioassays common also to other types of phorboids (e.g. cytotoxicity, fish toxicity, skin irritancy). The improvements in analytical techniques and the introduction of hyphenated chromatographic techniques like HPLC-MS have now simplified the detection and the isolation of ingenol esters. Despite these advancements and the growth of the natural products chemistry community, the diversity of ingenol derivatives has not expanded significantly in terms of chemotypes or euphorbiaceous sources since the pioneering work of the groups of Erich Hecker (Plate 1) in Heidelberg and Fred J. Evans (Plate 2) in London during the seventies and the eighties of the past century [8], suggesting that

Plate 1 Erich Hecker (taken from the brochure “Cancer Research and Cooperation, German-Israeli Cooperation in Cancer Research – The first 20 years”. Deutsches Krebsforschungszentrum (1999)

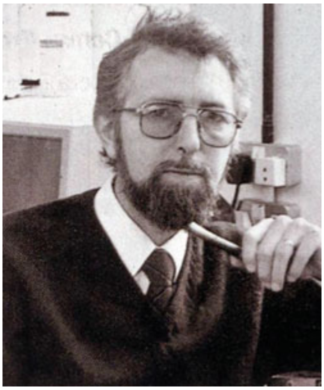
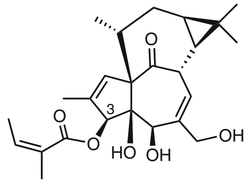


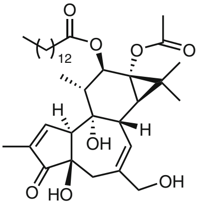
Plate 2 Fred J. Evans (1943–2007) (photograph provided courtesy of Prof. Elizabeth M. Williamson)

this class of compounds has a very narrow distribution in Nature and a limited structural diversity.

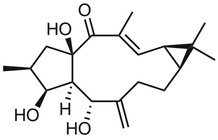
After an initial interest for the oxymoronic tumor-promoting and anticancer properties of ingenol derivatives, activities remained for a long time limited to the chemical synthesis community and the total synthesis of the parent alcohol. On the other hand, the successful and paradoxical development of the (weak) rodent tumor promoter ingenol mebutate (= ingenol-3-angelate, I3A, **5**) into a topical human chemopreventive drug (Picato®) (see Sect. 4.3), has rekindled a multidisciplinary interest for this compound and its biological potential.



5 (ingenol-3-angelate, I3A)



6 (phorbol-12-myristate-13-acetae, PMA)



7 (lathyrol)

Just like phorbol, ingenol does not occur in Nature as a free polyol, but only in esterified form, and its direct isolation from plant extracts is associated with the facile hydrolysis of the 3- and 20-monoesters. The first member of the class was reported in 1968 as part of a screening for co-carcinogenic diterpenoids in euphorbiaceous plants and the products obtained thereof [2, 3]. This systematic search for the distribution of co-carcinogenic compounds was undertaken by the German Cancer Research Centre in Heidelberg in the wake of the identification of phorbol 12-myristate-13-acetate (PMA, **6**) (= tetradecanoylphorbol acetate, TPA) as the major tumor-promoting principle of croton oil (*Croton tiglium* L.) by Hecker [7]. The purification of the compound later structurally elucidated as ingenol 3-hexadecanoate (**2**) was reported in 1968 from the seeds of the caper spurge (*Euphorbia lathyris* L.) [2] and from the latex of the African candelabra tree (*E. ingens* E. Mey.) [3]. Just like croton oil, a major source of phorbol esters, also the sap of the candelabra tree and the oil from caper spurge seeds have long been known for their high irritancy and skin-blistering properties, and the sap of the candelabra tree was also known to display co-carcinogenic properties [9]. Unexpectedly, both sources lacked phorbol esters, containing rather a complex mixture of ingenol esters and macrocyclic diterpenoids. Hecker, who pioneered studies in this field, assigned codes to diterpenoid constituents obtained in his laboratory, possibly to cope with the delay before isolation and structure elucidation that used to plague phytochemistry in the decades before pre-high-field NMR spectroscopic techniques became available. These codes were related to the plant source and to the chromatographic elution order, and the major irritant and co-cocarcinogenic constituents of *E. ingens* and *E. lathyris* respectively, dubbed Factors I_5 and L_1, turned out to be the same compound, namely, ingenol-3-hexadecanoate (**2**), with the structure of the parent polyol being eventually clarified by X-ray analysis of its crystalline triacetate [6]. Ingenol was named after *E. ingens*, possibly because laboratory work on this plant actually predated that on *E. lathyris*, or, alternatively, because the name lathyrol (**7**) was already being used, as epoxylathyrol [10], for the major macrocyclic diterpenoid polyol of caper spurge oil, a compound previously known as *Euphorbia*-steroid and first reported in 1937 [11].

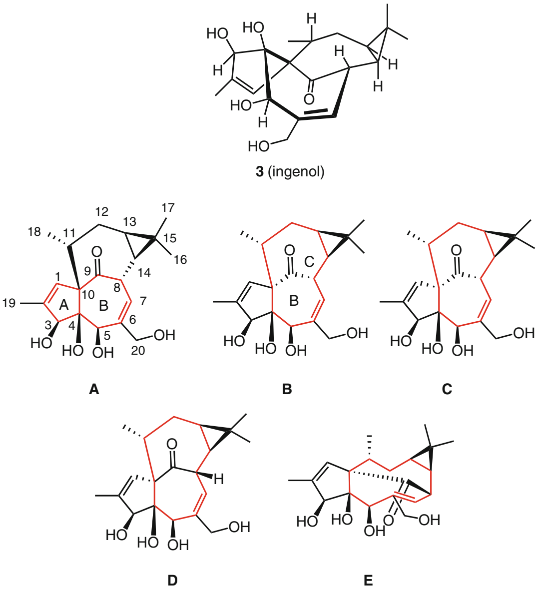
The total synthesis of ingenol is well covered in the secondary literature (see Sect. 3.2), while the other aspects of its properties have not been comprehensively reviewed since the chapter on ingenoids by Evans and Taylor in Volume of 42 of this Series [8] and the monographic book on phorboids edited by Evans [12]. This work aims at filling this gap, providing a platform for further investigation and the full exploitation of the pharmacological potential of this remarkable and fascinating parent diterpene natural product.

2 Phytochemistry

2.1 Structure and Spectroscopic Properties

The ingenane skeleton is based on a 5/7/7/3 tetracyclic system featuring a *trans*-bridgeheaded bicyclo[4.4.1] undecane core. The two-dimensional structural representation of ingenol is not straightforward, and several formulas have been used in the literature. The formula A (Fig. 1) is based on the azulenoid A,B ring system, and avoids the ambiguities associated with the two-dimensional representation of the *trans*-bridge, even though it depicts with a wedge/broken line endocyclic bonds

Fig. 1 Three-dimensional and two-dimensional representations of ingenol (**3**)



centered on stereogenic carbons. Thus, C-11 and C-14 of the cycloheptane C-ring bear an endocyclic wedge/broken line descriptor in addition to the one of their exocyclic substituent. The cyclodecane-based formulas B-D are also documented in the literature, but are ambiguous in terms of *(R),(S)* descriptors for the stereogenic centers at the bridge. As a consequence of this, *Chemical Abstracts Service* (Columbus, Ohio, USA) uses a different cyclodecane-based representation (*E*) for ingenol, unambiguous from the standpoint of translation in *R,S* configurational descriptors, but rather unfamiliar in the chemical literature, ambiguous in terms of configuration of the ring B double bond, and reminiscent of an Escher painting in the way the bridge is drawn. In conformational terms, the cyclodecane rim of the B, C-ring system approaches a sofa conformation, with C-10 upwardly oriented outside the mean plane through the other atoms, while the C-9 bridge and ring A are oriented toward the opposite (α-) face of the molecule.

The *trans*-bridge between C-8 and C-10 is an example of a conformationally locked *in,out*-bridge junction [13], a very rare structural element within the pool of natural products of which the major examples among the isoprenoids belong to the *seco*-trinervitane class of insect diterpenoids [14, 15]. The configuration is *in* at C-8 (angle C-10/C-8/H-8 < 90°, Fig. 1) and *out* at C-10 (angle C-8/C-10/C-11 > 90°, Fig. 1), and the bridge results, when referred to the de-apicalized cyclodecane system, from the merging on the C-9 methylene of a β-bond at C-8 and of an α-bond at C-10 (Fig. 2). The homeomorphic interconversion [13] of the *in*/*out* and the *out*/*in* forms is impossible in ingenol because of the small size of the ring system and because of the presence of the junction with the cyclopentane A-ring and the cyclopropane D-ring. Models show angular strain at several carbon atoms, with flattening at C-5, C-8 and C-11. Although the original X-ray data on ingenol triacetate [6] were not made available, the X-ray structural analysis of 20-deoxyingenol (**3**) found indeed that the bond angles of these carbons are significantly larger than the tetrahedral value (125.0°, 126.4° and 119.5°, respectively) [16].

In general, the relative stabilities of isomeric bicyclic [x.1.y] ring systems depend on their size. Isomers of the *out,out*-type are only possible in rings up to cyclononane, and they are also the most stable form up to cycloundecane, with the *in,out* becoming the preferred form for cyclotridecane and larger rings [13]. Unsurprisingly, the C-8 epimer of ingenol, the *out,out*- stereoisomer **8**, is significantly (24.7 kJ/mol) more stable than the natural polyol, and is characterized by a reduced angular strain [17]. The esters of this isomer, at least in the tris-*nor* version lacking the D ring (**9**), are devoid of tumor-promoting properties, and have been assumed to

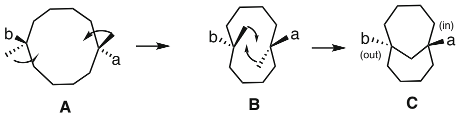
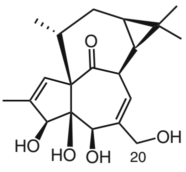
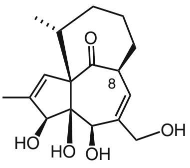


Fig. 2 Effect of de-apicalization on the configurational descriptors of the bridgehead carbons in a cyclodecane bearing a 1,6-*trans* bridge (bold and hashed wedges are used to indicate the orientation of the substituents a and b compared to the mean plane through the cyclodecane ring)

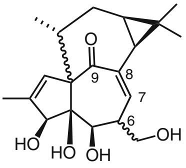
be biologically inactive [18]. The parent tris-*nor* polyol was synthesized by Paquette in 1988 [18], 20 years after the isolation of ingenol and 14 years before the first total synthesis of the natural product by Winkler [19]. In the light of the articulated and multifaceted nature of the biological profile of ingenol esters and of the lack of ring D, this assumption should be better evaluated before generalization.



8 (8-epi-ingenol)



9 (8-epi-tris-*nor*ingenol)



10 (isoingenol)

The isomerization of ingenol does not lead to 8-epi-ingenol (**8**), but to iso-ingenol (**10**), a compound where the double bond has shifted to the bridgehead carbon and is formally conjugated with the C-9 carbonyl, although the two π-systems cannot achieve planarity [20]. There is, unfortunately, a certain confusion in the literature between epi-ingenol (**8**) and isoingenol (**10**), and the situation is further compounded by confusion between epi-ingenol (**8**), a compound so far unknown, and its synthetic tris-*nor* derivative **9**. Isomerization of ingenol to isoingenol (**10**) leads to total loss of tumor-promoting properties of its 3-esters, and iso-ingenol 3-esters have been used as negative controls in biological assays just like 4-epi-phorbol (4α-phorbol) esters have been used for the activity of tumor-promoting phorbol esters [20].

The ^{1}H- and ^{13}C-NMR features of ingenol are summarized in Fig. 3 [21]. The most remarkable features of the NMR spectra are the downfield resonance of the non-oxygenated carbon C-10 (δ 72.6) and of the H-8 methine (δ 4.13), and the upfield resonance (δ 3.80) of the 5-proton, presumably because of its location in the anisotropy cone of the C-9 carbonyl. Although the ^{1}H NMR spectrum of the *out,out* isomer **9** was not assigned, all oxymethines resonated at $\delta > 4$ ppm [18], as expected from a different orientation of the C-9 carbonyl and H-5. Esterification causes predictable α,γ-downfield and β-upfield shifts in the proton and carbon resonances of the parent polyol. Remarkable differences were noticed between

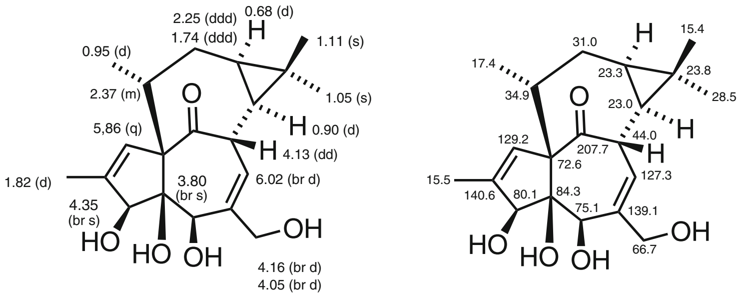


Fig. 3 ^{1}H- and ^{13}C NMR data for ingenol (**3**) ($CDCl_3$, 400 and 100 MHz, respectively). *J* values: 1,19 = 1.5 Hz; 7,8 = 4.5 Hz; 8,14 = 12.5 Hz; 11,12 = 3.0 Hz; 11,12′ = 8.5 Hz, 12,12′ = 15; 12,13 = 6.0 Hz; 12′,13 = 8.5 Hz; 13,14 = 8.5 Hz, 20,20′ = ca. 7 Hz [21]

the IR carbonyl frequencies of ingenol and its *out,out* model analog **9** ($\nu = 1705$ and 1675 cm^{-1}), presumably because of different degrees of deviation from planarity, of ground-state strain energy, or of a combination of both [18]. An extensive compilation of ^{13}C NMR data for ingenol esters has been published, facilitating the characterization of the acyl decoration of its esters [22].

The MS fragmentation of ingenol is significantly different from those of phorbol and deoxyphorbol, and this makes it possible to selectively detect its derivatives in complex extracts using MS-MS techniques [23]. The mass spectra of ingenol esters are characterized by the stepwise loss of the ester groups, and their fragmentation profile has been investigated in the positive-ion mode, which readily ionizes them [23]. While the nature of the acyl moiety can be inferred from the pattern of mass fragmentation loss, the elimination order is of limited general value for the establishment of the esterification profile, since it was found to be dependent on the experimental conditions and the nature of the ester group [23].

Ingenol triacetate and ingenol-3-angelate (I3A, **5**) [24] have been investigated by X-ray diffraction, and such data on several semi-synthetic analogs obtained during various total synthesis campaigns are also available [16]. Crystalline forms of I3A were obtained from ethanol by addition of water, and from acetonitrile by cooling. Apparently, the crystalline forms are easier to formulate than the amorphous form, and their preparation has been patented [24]. In accordance with observations made on 20-deoxyingenol, angular strain was particularly evident at the angles C4–C5–C6 (123.0°) and C10–C13–C14 (119.3°). Angular strain was also present at the carbonyl bridge, with the angle C8–C9–C10 being only 111.2°. Linear strain was especially marked at the bond C4–C10, which is considerably longer than normal [1.602 (5) Å] [24].

Ingenol was originally reported as dextrorotatory ($[\alpha]_D^{25} = +41° cm^2 g^{-1}$, methanol) [21], and further studies confirmed a positive optical rotation also for the synthetic product. Nevertheless, a high-purity sample prepared in the course of a large-scale isolation of the product at LEO Pharma, the company that developed Picato®, showed a negative optical rotation ($[\alpha]_D^{25} = -18° cm^2 g^{-1}$, methanol), confirmed with an ad hoc prepared synthetic sample ($[\alpha]_D^{25} = -13° cm^2 g^{-1}$, methanol), with the absolute configuration of the levorotatory sample supported by X-ray analysis of a heavy-atom containing derivative (3-(4-bromobenzoate) of the 5,20-acetonide) [16]. More than the presence of impurities, for which the profiles should be different between samples of natural and of synthetic origin, it is possible that the reversal of the optical rotation might be related to degradation, since ingenol is not stable (see Sect. 2.3). Ingenol esters are mostly dextrorotatory, while those of 13-hydroxyingenol show an opposite optical rotation [8]. Some exceptions are known, but, since the purification of ingenol esters is difficult and the observed optical rotations are sometimes small, this could be related to the presence of impurities. Similar considerations hold also for the CD spectra. Ingenol esters show a positive Cotton effect at 300 nm, while 13-hydroxyingenol esters show a negative Cotton effect at 290 nm [8]. No systematic study has been done so far on the chiroptical properties of ingenoids.

2.2 Biogenesis

Euphorbiaceous plants are prolific producers of diterpenoid skeletal types, many of which, like the ingenanes, are unique to this large plant family [25]. According to their derivation from a linear tetraprenyl phosphate, *Euphorbia* diterpenoids can be broadly divided into compounds derived from the cascade cyclization and those derived from the head-to-tail cyclization of a tetraprenyl phosphate precursor through the effects of type II and type I cyclases, respectively (Fig. 4).

The first cyclization mode is at the origin of the so-called polycyclic diterpenoids, represented in euphorbiaceous plants mainly by abietanes, pimaranes, kauranes, atisanes, and trachylobanes, while the second one generates, in a stepwise fashion and with the cation processes typical of the isoprenoid cyclizations, macrocyclic diterpenoids, namely, the monocyclic cembranes, the bicyclic casbanes, and the tricyclic lathyrane derivatives (Fig. 5). Alternatively, cyclopropane ring opening generates jatrophanes from lathyranes (Fig. 5).

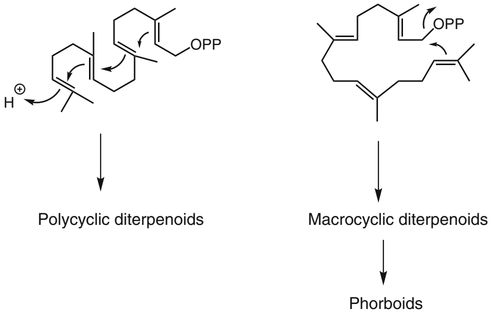


Fig. 4 Cascade and head-to-tail cyclization mode of a tetraprenyl phosphate

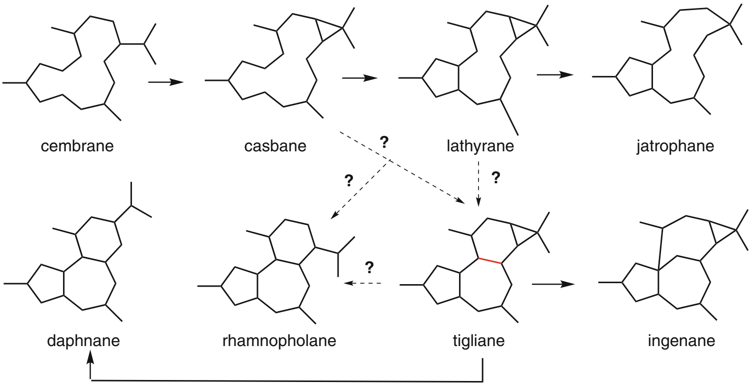


Fig. 5 Topological relationship between the macrocylic diterpenoid skeleta (upper line) and those of phorboids (lower line), and their possible biogenetic relationship via lathyrane derivatives (topological proposal) or via casbane derivatives (Jakupovic proposal)

From a structural standpoint, phorboids are polycyclic diterpenoids, but are biogenetically derived by macrocyclic diterpene precursors. Therefore, they are considered products of a "head-to-tail" cyclization mode of a tetraprenyl phosphate, in one of the many oxymoronic relationships between biogenesis and structure that plague natural products chemistry, as exemplified by the isoquinoline alkaloid quinine, an indole alkaloid from a biogenetic standpoint. The relationship between macrocyclic diterpenoids and phorboids has long remained a mechanistic black box, despite obvious structural similarities between the lathyrane and tigliane skeleta. Tiglianes can next, at least formally, be converted to the other structural types of phorboids by cationic rearrangements typical of isoprenoid chemistry, and the present mechanistic uncertainties on the biogenesis of phorboids center, therefore, on the relationship between lathyranes and tiglianes and, in particular, on the formation of the single bond that differentiates compounds representative of these two classes.

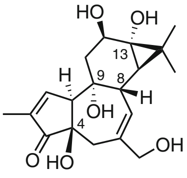
Despite the structural similarity between the carbon-carbon connectivity of the lathyrane and tigliane skeleta, several issues make questionable a direct relationship between compounds of the two classes:

(a) While tiglianes have a broad distribution, occurring in several species of Euphorbiaceous plants, lathyranes have a much more limited distribution, exclusive to only a few species of the genus *Euphorbia*. If compounds from the two types were biogenetically related, one would expect a better overlapping occurrence, and not a "traceless" biogenetic relationship.

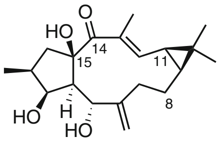
(b) As exemplified by phorbol (**1**) and lathyrol (**7**), the cyclopropyl carbon C-13 (tigliane numbering, C-11 in terms of lathyrane numbering) is hydroxylated in all natural tiglianes, as well as in many ingenanes, while no lathyrane discovered so far in Nature is hydroxylated at the cyclopropane carbons. In biological systems, C-H functionalization is basically an iron-mediated radical process [26], and the direct hydroxylation of a cyclopropane ring would therefore be biased toward the formation of a cyclopropylalkyl radical rather than a cyclopropyl radical [26]. In turn, the cyclopropylalkyl radical would rapidly generate the 3-butenyl radical, with eventual opening of the cyclopropane ring [27]. These considerations suggest distinct mechanisms of formation for the cyclopropane ring in lathyranes and tiglianes.

(c) In topological terms, the conversion of lathyranes to tiglianes involves the formation of a bond between the carbons 8 and 14 (corresponding to the carbons 8 and 9 of the tiglianes, cf. **1** and **7**), but no lathyrane derivative isolated so far bears the correct functionalization for this cyclization, which would require complementarity in terms of nucleophilic and electrophilic properties between these two carbons. Thus, C-14 of the lathyranes is generally a carbonyl, while C-8 is either hydroxylated, and therefore electrophilic, or is part of an unfunctionalized sequence of methylenes, as exemplified by lathyrol (**7**). Furthermore, most lathyranes contain at least one endocyclic (*E*)-double bond [25], which, apart from preventing a spatial interaction between the functionalities at C-8 and C-14, makes the cyclization to tiglianes impossible, since (*E*)-double bonds can only exist in eight-membered or higher ring systems. Unsurprisingly, the cationic cyclization of lathyranes has provided a wealth of rearranged diterpenoid skeleta, with none of these, however, bearing any resemblance with the tigliane skeleton [28].

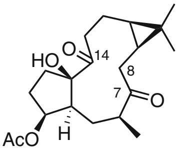
A high-yield lathyrane-to-tigliane conversion was reported via an anionic process that involved the intramolecular aldol reaction of an ad hoc prepared semi-synthetic lathyrane 7,14-diketone devoid of unsaturations on the eleven-membered ring (**11**) [29]. The resulting tigliane (**12**) was assumed tentatively to have the same configuration as natural phorbol esters at the stereogenic carbons at the B,C ring-junction, but the functionalization of these two rings was completely different from that of the naturally occurring compounds of this class. Furthermore, aldol-type anionic cyclizations, although well documented in the chemistry of isoprenoids [30], are rarely involved in their biogenesis.



1 (phorbol)



7 (lathyrol)



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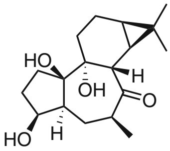

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(d) Naturally occurring lathyranes and tiglianes show an opposite hydroxylation at the A,B ring junction, as exemplified by comparison of phorbol (**1**) and lathyrol (**7**).

Owing to these differences, and the implausibility of the hydroxylation of a cyclopropane carbon under biomimetic conditions, the biogenetic relationship between lathyranes and tiglianes has been questioned, without, however, proposing a possible biogenetic alternative [31]. As a result, tiglianes and ingenanes may be added to the thapsane family of sesquiterpenoids, the ryanodane family of diterpenoids, and the *Iboga* alkaloids as veritable biogenetic enigmas.

The late Jasmin Jakupovic (Plate 3) (1949–2005) of the Technische Universität Berlin and later Analyticon elaborated an elegant biogenetic proposal to solve this

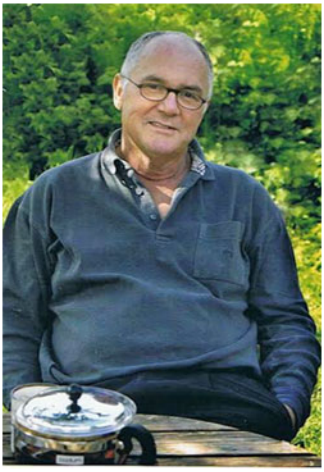


Plate 3 Jasmin Jakupovic (1949–2005) (photograph provided courtesy of Fotini Tsichrtzis)

riddle, providing a unifying mechanistic setting for the generation of lathyranes and tiglianes from casbanes that explains the differences in the functionalization pattern of these two class of compounds. This proposal has important implications for the biogenesis of ingenol, and is also capable of shedding light on the biogenesis of rhamnofolanes, another biogenetically enigmatic class of phorboids. The premature death of Jasmin Jakupovic has precluded the dissemination of his ideas within the natural products community, and his views remained confined to laboratory meetings, private discussions, and a Ph.D. thesis [32]. Thanks to the help of the Jakupovic family and his collaborator Frank Jeske, as well as to ideas and comments exchanged during personal discussions before Jakupovic's death, sufficient material has been retrieved to present in an organic fashion what will be referred to as the "Jakupovic biogenetic proposal" for phorboids. Despite radically deviating from some current ideas in the area, this proposal is the only one capable of rationalizing the biogenetic derivation and the functionalization pattern of phorboids in light of the isoprenoid rule.

The starting point for the elaboration of this proposal is a retrosynthetic analysis of the tigliane skeleton according to the isoprenoid rule. Thus, the reversal of the cyclopropane ring closure by protonation of the C-13 α-carbon unambiguously connects the tigliane skeleton (**13**) to the cembranyl cation **14** (Fig. 6). The alternative opening of the cyclopropane by protonation at C-14 is, in fact, incompatible with the isoprenoid rule, which sets the relative location of the alkyl substituents on isoprenoid chains. Conversely, the topologically symmetric cembranyl cation **14** could be generated by two distinct foldings of the acyclic tetraprenyl precursor. The folding **15** of pathway A seems more obvious, since it leads to tiglianes with the correct location of the cyclopropane on ring C. Conversely, the folding **17** of pathway B is apparently counterintuitive, since, by (formal) insertion

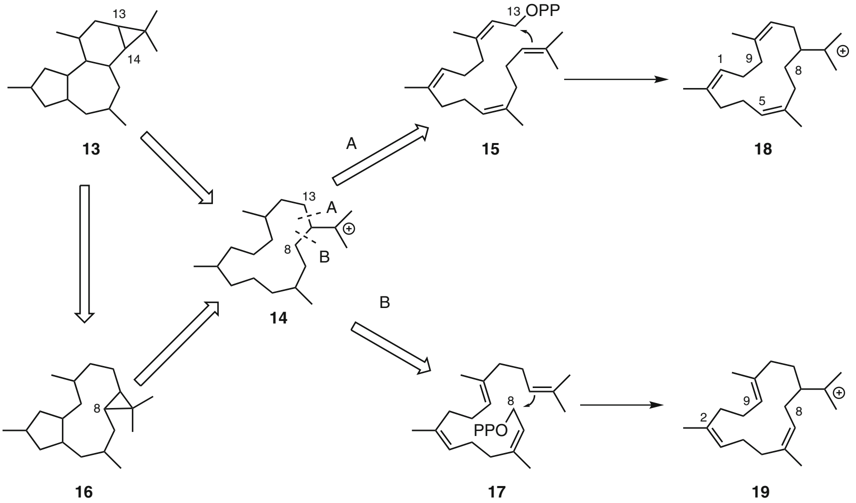


Fig. 6 Retro-biogenetic analysis of the tigliane skeleton

of the side-chain cation center into a C(8)-H sigma bond, it would generate compounds with the unnatural location of the other cyclopropane bond at C-8 (represented in **16** in its lathyrane version), and a rearrangement would be needed to bring it to its natural location at C-13. This rearrangement is, indeed, at the core of the Jakupovic biogenetic proposal, and provides a mechanistic explanation for all the "unknowns" of the biogenesis of phorboids. Thus, pathway A requires the triply-isomerized all-(*Z*) tetraprenyl precursor **18**, and will lead to the "functionalization" "cul-de-sac" discussed before, since neither C-8 nor C-9 are properly functionalized to form the critical B, C ring junction. Furthermore, it leaves unresolved the issue of the hydroxylation of the cyclopropane carbon.

The configuration issue for the double bond located in what will become the cyclopentane ring A (Δ^1 in **18** and Δ^2 in **19**) is irrelevant, since the isomerization can be associated with functionalization and the generation of the five-membered A-ring, with a similar argument holding also for the allylic C-9 methylene of **18**. However, this issue is difficult to resolve by simply advocating functionalization for the Δ^5 double bond of **18**, since this occurs as an unfunctionalized methylene in most tiglianes. Conversely, in **19** the configuration of the endocyclic double bond is not an issue, since the Δ^9 double bond can be accommodated in an (*E*)-configuration, and the Δ^6 double bond is allylic to the phosphate group, and therefore can isomerize via the classic rearrangement of primary γ,γ-dimethylallyl phosphates to tertiary α-dimethylvinyl phosphates [33]. In general, the isomerization of the pyrophosphate starter from a terpenyl-geranyl to the terpenyl-linalyl type (Fig. 7,

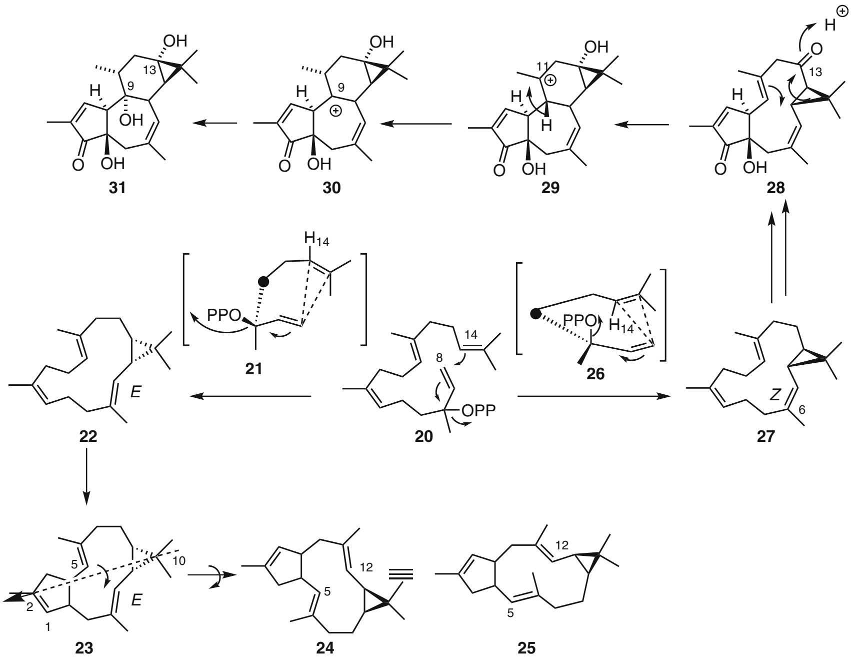


Fig. 7 The Jakupovic proposal for the biogenesis of phorboids and lathyranes

20) is associated with an increase of the possibility of cyclization modes, and this conformational diversity (Fig. 7, **21** and **26**) might ultimately be responsible for the generation of both lathyranes and tigliane from the same precursor. Thus, electrophilic attack on the *re*-face (H-14 “up”) of the terminal double bond from the vinyl antiperiplanar to the phosphate leaving group, would translate into an *E*-configuration of the newly generated trisubstituted double bond, and, when the resulting casbene derivative is viewed in a tigliane orientation, an α-orientation of the cyclopropane ring (Fig. 7, **22**). Conversely, attack on the *si*-face of the terminal double bond (H-14 “down”) from the vinyl group *synclinal* to the phosphate leaving group will generate an opposite configuration of the double bond and the cyclopropane ring [27]. After formation of the cyclopentane ring A, rotation around the C-2–C-10 axis (lathyrane numbering) of the casbene **23** generates a lathyrane with the double bond locations (Δ^5 and Δ^{12}) and configuration typical of this class of products. Conversely, the casbene **27** has the correct double bond configuration and location to generate, after closure of the A-ring, the phorboid skeletal of tiglianes, ingenanes and rhamnofolanes. Thus, introduction of a carbonyl function at C-13 (tigliane numbering) triggers the formation of a stable non-classic cyclopropylmethyl cation, which is then quenched by the C-9 (tigliane numbering) endocyclic double bond and closure of the cyclopropane ring at C-13 (see **28**), with formation of the critical C-8—C-9 bond and of the hydroxylated version of the cyclopropane D ring. Hydride migration and quenching by water will eventually provide a tigliane (**31**) with hydroxy groups at C-9 and C-13, the hallmark of all natural compounds of this class (Fig. 7).

For ingenanes that lack a 13-hydroxy group, like the parent polyol ingenol, the electrophilic trigger for the migration of the cyclopropane could be provided by the generation of an oxonium ion at C-13 (Fig. 8), while their rearranged skeleton might, in principle, derive from an alternative quenching of the C-9 cation **34**.

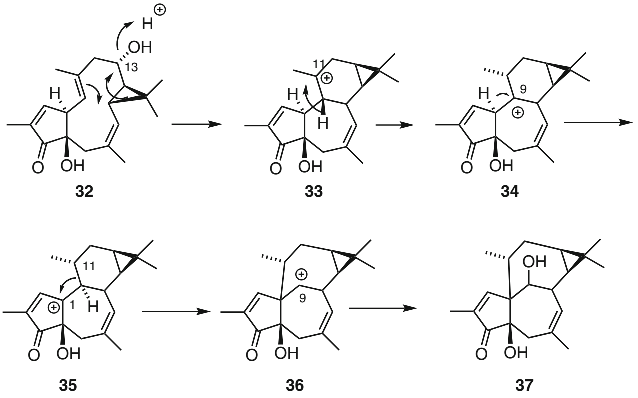
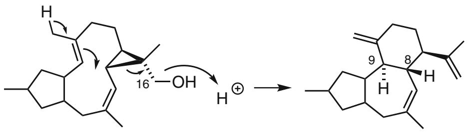


Fig. 8 Possible direct biogenetic derivation of the ingenane skeleton from a C-13 hydroxylated (tigliane numbering) bicyclic precursor

Rather than directly undergoing water trapping like the cation **30**, quenching could occur after a 1,2 hydride shift to generate a C-1 cation that next undergoes a 1,2-alkyl shift, eventually rearranging to the ingenyl cation **36**. A detailed study on the tigliane-to-ingenane rearrangement during the Baran synthesis of ingenol [16] has highlighted the role of the presence of a hydroxy substituent at C-9 to overcome the thermodynamic barrier involved in the reaction, suggesting that a direct derivation of the ingenane skeleton from a lathyrane-type bicyclic precursor seems unlikely.

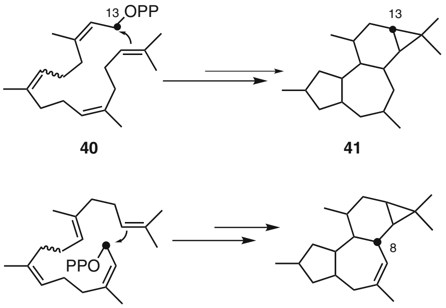
Finally, oxidation of the casbene **27** at C-16 (tigliane numbering) and the generation of lathyranes like **38** (Fig. 9) may trigger a different mode of transannular opening of the cyclopropane ring, generating the typical ring C functionalization of rhamnofolanes (**25**) via the backside attack of the double bond to the cyclopropane C-8 carbon.

The two different retro-biogenetic disconnections of the tigliane skeleton according to the isoprenoid rule (Pathways A and B in Fig. 6) could be distinguished easily by labeling experiments. Thus, labeling C-1 of geranyl-geraniol will lead, via the phosphate **40**, to a tigliane labeled at C-13 according to pathway A (**41**), but, via the phosphate **42**, to a tigliane labeled at C-8 (**43**), if pathway B is indeed the one occurring in Nature (Fig. 10).



38 **39**

Fig. 9 Generation of the rhamnofolane skeleton by rearrangement of a C-16 hydroxylated lathyrane precursor (tigliane numbering throughout)



42 **43**

Fig. 10 Location of the labeling of the C-1 carbon of a tetraprenyl phosphate precursor according the two possible retro-biogenetic disconnections of the tigliane skeleton

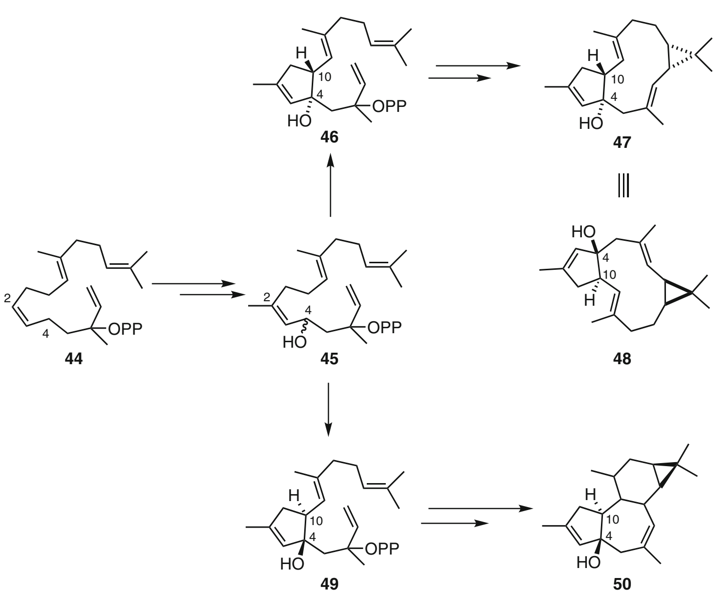


Fig. 11 Possible rationalization of the opposite hydroxylation of the A,B-ring junction in lathyranes and tiglianes (tigliane numbering throughout)

According to the Jakupovic biogenetic proposal, lathyranes and tiglianes have, in biogenetic terms, a different location of the cyclopropane ring. The conventional representation of this class of compounds hides this, and generates inconsistencies between the functionalization pattern of these compounds and phorboids. Regarding the opposite functionalization of the cyclopentane A-ring in lathyranes and tiglianes, it is tempting to imagine that the allylic oxidation of the Δ^2 double bond (tigliane folding) of a tetraprenyl precursor like **44** could trigger formation of the cyclopentane A ring, and that the different configuration of this hydroxy group, which generates diastereoisomers in a tetraprenyl starter of the geranyl-linalyl type, might be involved in the pre-organization of the cyclization step involving the pyrophosphate group (Fig. 11), thus explaining the apparently different hydroxylation of the A, B ring junction in lathyranes and tiglianes.

The Jakupovic proposal provides a mechanistic framework to relate biogenetically the functionalization pattern of lathyranes and phorboids, elegantly by-passing the “cul-de-sac” represented by the apparently unrelated configuration and location of the double bonds within these compounds and providing a mechanistic rationale for the oxygenation of the cyclopropane ring in tiglianes and ingenanes. Since it rationalizes all the many “unknowns” of the biogenesis of phorboids, its wider appreciation will, hopefully, trigger studies aimed at its confirmation or invalidation.

2.3 Isolation

Many ingenol esters are potent irritant and vesicant agents as well as tumor promoters, and during their extraction and purification great care has to be taken to avoid contact with the skin, mucous membranes, and the eyes. Accidental eye contact is characterized by massive lacrimation and acute pain, and may require hospital treatment to be managed correctly. In turn, skin contact induces within 2–3 h the formation of an erythema that can persist for well over 24 h, but which if the exposed area is limited, can be soothed by topical treatment with local anesthetics and ice blocks [34], or with a solution of a polyamphoteric detergent [35]. Hospitalization might, however, be necessary in case of the accidental contamination of a large skin area [34]. Based on observations done with I3A, the inflammatory response to ingenoids is transient and resolves spontaneously without scarring within 2–4 weeks, during which topical treatment with corticosteroids may be recommended to control pain and inflammation [34]. Severe allergic reactions, including throat tightness, difficulty in breathing, feeling faint, or swelling of the lips or tongue, have been reported with Picato® [36], and they might well result also from contact with other ingenoids. Facial, especially the eyes, and skin protection is therefore mandatory during the laboratory manipulation of ingenol esters, as well as of plant material, extracts, and chromatographic fractions containing compounds of this type. Glassware should be treated with bases before washing, and contaminated laboratory spaces should be treated in the same way. It is known that 5% KOH in methanol quickly hydrolyzes ingenol esters, and is preferred to washing with aqueous solutions that can not quickly penetrate fatty matrices [37].

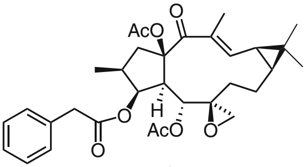
The literature is rife with exaggerated statements on the difficulties involved in the isolation of ingenol esters, and, in general, of phorboids. Thus, the claim that "*the isolation (of phorboids) must be conducted in oxygen-free conditions; solvents must be degassed and extraction should be conducted under continuous flow of nitrogen or argon*" [38] is exaggerated, even though ingenol derivatives are unstable toward hydrolysis and transesterification, and are easily oxidized by atmospheric oxygen. Undoubtedly, their isolation in the native form requires care and the need to avoid acidic and basic conditions, which even if mild, can promote degradation and/or isomerization reactions, but these features are not exclusive to this class of natural products, and do not preclude working under normal laboratory conditions. Although no systematic study has yet been done, ingenoids are probably also easily degraded by heating, explaining the edible uses of the seeds of the caper spurge (*E. lathyris*), which after roasting, were used as a coffee replacement during the shortages associated with World War I [39]. Heating is also use to detoxify this oil from this species before its technical uses may be applied [39].

Ingenane polyols do not occur as free alcohols, but only in the esterified form. Ingenol esters are lipophilic and insoluble in water. They are extracted from plant material with organic solvents, and, depending upon their degree of acylation and the nature of the ester groups, they show different polarities and therefore partition differently between hydrocarbon and more polar solvent phases. Extraction from a plant biomass typically involves the use of solvents like ethanol, acetone or chloroform, evaporation, and next partition between hexane and water-methanol, with ingenol esters ending up in the more polar and hydrophilic phase. Conversely, when the primary extract is partitioned between water and chloroform, or between water and ethyl acetate, ingenol esters end up in the organic phase. Partition is especially critical to recover long-chain ingenol esters from fatty matrices like seed oils. In this case, the system acetonitrile-cyclohexane was found to work much better than other partition systems [21]. Crude ingenoid mixtures can then be partially resolved by various chromatography techniques, namely, gravity chromatography on silica gel (both in the normal and in the RP mode) or Sephadex. For final purification, HPLC is generally necessary, since ingenol esters often represent minor constituents of the diterpenoid bouquet of the plant under investigation. During solid-liquid chromatographic separation, acyl rearrangement can take place, and ingenoids having a free hydroxy group at C-3 and ester groups at C-5 and C-20 are most likely to be isolation artifacts (see Sect. 3.1). The early phytochemical work on ingenoids made extensive use of droplet counter-current chromatography for their purification [8], a technique that minimizes acyl rearrangements. While now obsolete in its original form, this method could benefit from many recent rapid developments in the field [40], and holds potential especially for large scale purifications. However, this possibility is still largely unexploited for the phorboids.

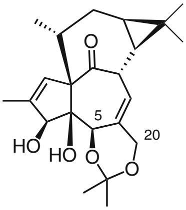
The isolation of ingenol has been detailed from the seed oil of *E. lathyris*, an agricultural commodity (Plate 4), and the methods developed could be applied also to other oily sources. The original method by Hecker [41] involved a series of liquid-liquid and solid-liquid partitions. Thus, a methanolic extract from the seeds

Plate 4 *Euphorbia lathyris* L. at the time of fruit maturation (photograph provided courtesy of Dr. Gianpaolo Grassi, Cra, Rovigo, Italy)

(9.7% yield) was first partitioned between a half saturated NaCl solution and ethyl acetate to remove the polar fractions containing mono- and dimeric coumarins and other polar constituents. The organic phase was then evaporated, and the residue (7.7%) was emulsified with methanol. An oily phase made up by glycerides separated from the emulsion, and, after evaporation, the methanol phase (3.2%) was adsorbed onto silica gel. The cake was then washed sequentially with petroleum ether, to further remove glycerides, and with ethyl acetate, to recover the diterpenoid fraction (0.58%). Zemplén methanolysis of the latter and partitioning between water and ethyl acetate of the residue of the hydrolysis eventually gave a polyol fraction (0.5%) that was adsorbed onto silica gel and then washed with chloroform to remove the ester fraction. Washing with ethyl acetate afforded a purified polyol fraction (0.13%) that was subjected to a sequence of gravity column chromatographic purifications, eventually affording ingenol in an overall 0.025% yield (250 mg/kg of seeds).



51
(*Euphorbia* Factor L1))



52
(ingenol 5.20-acetonide)

A simplified method was later developed [21], based on the possibility of directly separating the diterpene fraction of the oil by partition of a methanolic extract between acetonitrile and cyclohexane, a system originally devised to remove lipophilic tin derivatives in the work-up of Bu_3SnH reductions [42]. The diterpenoid fraction was then treated with 8% KCN in methanol to hydrolyze selectively the macrocyclic diterpenoid esters, of which the major member, the epoxylathyrol ester L_1 (**51**), directly precipitated from the reaction mixture. Ingenol, a more polar compound, could then be easily separated from the macrocyclic esters by gravity column chromatography on silica gel, with an overall isolation yield of ca. 250–600 mg/kg of seeds [21]. The rationale for the chemoselectivity of the hydrolysis is that the presence of nearby free hydroxy groups catalyzes the deacylation of ester groups [43], a condition holding for ingenol esters but not for lathyrol and epoxylathyrol esters. Zemplén methanolysis with freshly prepared sodium methylate in methanol gave lower yields and was less selective for ingenol vs. lathyrol and epoxylathyrol esters, but would, undoubtedly, be more practical for a large-scale process. When obtaining macrocyclic polyesters is not important, a further simplification is possible, directly extracting the crushed seeds with methanolic sodium methylate. In this step, a significant precipitation of the highly crystalline macrocyclic polyols lathyrol and epoxylathyrol takes place, and, after filtration, neutralization, and evaporation, a fraction containing a mixture of ingenol and the partially hydrolyzed macrocyclic diterpene moiety is obtained. This can then be purified by gravity colum chromatography on silica gel [44]. This method minimizes the exposure to ingenol esters, since the crude crushed seeds are extracted with an active solvent that "detoxifies" them, and, comparative experiments with other procedures have highlighted the superiority of this protocol, with yields consistently in the range of 500 mg/kg of seeds [44].

Other sources of ingenol, like the latex of *E. ingens*, turned out not not to be reliable due to large variations in their concentrations [41]. Since all succulent African *Euphorbia* species are now included in the CITES list [45], the trade of *E. ingens* would, in any case, be subjected to severe regulatory limitations. The latex of a South-American chemotype of *E. tirucalli* L., a plant known as "Aveloz" in Brazilian folk medicine, has also been claimed to be an excellent source of ingenol, and used for the development of semi-synthetic antiviral agents (see Sect. 4.2), without, however, disclosing details of the yield and the isolation protocol [46]. "Aveloz" is a popular medicinal plant in Brazil, but in 2011 the Brazilian Agency for Sanitary Vigilance (ANVISA) prohibited its use in herbal medical products because of the presence of co-carcinogenic diterpenoids [47].

Ingenol obtained by hydrolysis of its esters occurs as an amorphous glass, which degrades easily even in the cold, and is better stored as its crystalline 5,20-acetonide, **52**. Alternatively, it has been stored as such in frozen DMSO or in benzene.

2.4 Distribution and Diversity

The distribution of ingenoids is limited to three closely related genera within the Euphorbiaceace family (*Euphorbia*, *Elaeophorbia* and *Mabea*) [48]. The tropical genera *Elaeophorbia* and *Mabea* are small (less than 100 species each), with the genus *Elaeophorbia* often being included in the genus *Euphorbia* [48]. The latter, with 1600–2000 species, depending on the classification system, is the second largest among higher plants after the genus *Astragalus* [49]. Most ingenoids have therefore been reported from plants belonging to the genus *Euphorbia*, to the point that this genus is often quoted as the only source of ingenoids in Nature. For comparison, tigliane derivatives have been reported from twenty-three genera belonging to two families (Euphorbiaceae and Thymelaeaceae) [50]. Ingenol esters occur both in spurges and in cactus-like members of the genus, and no clear trend associating their presence to certain morphological features is evident from the phytochemical literature. Just like phorboids in general, ingenol esters are absent in the New World species of the genus *Euphorbia*. The concentration of ingenoids in plants is generally rather low, often in ppm (mg/kg of) of fresh biomass. The best source of ingenol remains the caper spurge (*E. lathyris*), one of its two original sources, where it occurs in the seeds at a concentration up to ca. 0.5–0.8 g/kg [44]. For comparison, the concentration of phorbol esters in croton oil is an order of magnitude higher [7]. A systematic study on the concentration of ingenol in the latex of various *Euphorbia* species was carried out after hydrolysis of the diterpenoid mixture and GC analysis of the triacetate [51]. Typical concentration levels in the latices analyzed ranged between 1% and 3% [51].