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Tigliane diterpenoids: isolation, chemistry and preliminary biosynthetic studies of a medicinal relevant class of natural compounds

Drug discovery and development
SSD: CHIM/06

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CONTENTS

List of abbreviation

2,2-DMP = 2,2-dimehtoxypropane

Ac_2O = acetic anhydride

CTP = cytidine triphosphate

DCM = dichloromethane

DMAP = 4-dimethylamino pyridine

DMAPP = dimethylallyl pyrophosphate

DXP = 1-deoxy-D-xylulose 5-phosphate

EBC-46 = Tigilanol tiglate

EDC = 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

EtOAc = ethyl acetate

EtOH = ethanol

HRMS = High Resolution Mass

IL = interleukin

IPP = isopentenyl pyrophosphate

MeOH = methanol

MEP = 2-*C*-methyl-D-erythritol 4-phosphate

MS-MS = Mass-Mass spectrometry

MVA = mevalonic acid

NMR = nuclear magnetic resonance

Pe = petroleum ether

PKC = protein kinase C

PMA = see TPA

PPTSA = pyridinium-p-toluensulphonic acid

Py = pyridine

QToFMS = Time-of-flight mass spectroscopy

RTX = resiniferatoxin

SAR = structure-activity relationship

t-BuOOH = *tert*-butylhydroperoxyde

THF = tetrahydrofurane

TPA = 12-*O*-tetradecanoyl phorbol-13-acetate

Scenario background

It is not hard to believe that, even from the birth of modern science, chemists have turned their attention to traditional herbal medicine to discover drugs for the treatment of several diseases. The list of natural compounds isolated from medicinal plants has increased dramatically with the progression of analytics techniques, chromatographic methods, and spectroscopy. After almost two centuries of studies, interest for natural products in drug discovery programs has substantially dwindled in the last 40 years. Possible causes are the intrinsic difficulty of natural product purification from complex matrixes, the establishment of a supply chain, regulatory burdens and the attrition due to the re-discovery of known compounds. [**1**] According to Pharma R&D Annual Review 2019, biotechnological drugs are gaining ground thanks to a better-targeted therapy compared to small-molecules, that, nevertheless, still represent the largest class of current drugs. In this scenario, plants products are at position 11th, stable compared to 2018. Thus, natural products can still be of interest for drug discovery. [**2, Table 1**]

Origin	N. of active products 2019 (2018)	Trend
Plant	266 (248)	↑
Bacterial	43 (51)	↓
Animal	35 (30)	↑
Fungal	27 (28)	↔
Other	24 (-)	↑

Table 1. Pharma R&D Annual Review 2019 on drugs origin

In this context, phorboids hold a special place. These polycyclic diterpenoids, typical of plants from the Euphorbiaceae and Thymelaceae have unique chemistry and biological activity. Thus, certain phorbol esters, a class of tigliane-type phorboids, act as ultra-potent analogues of 2,3-diacylglycerol (DAG) on Protein Kinase C (PKC), a series of serine-threonine kinases involved in several cellular pathways and related to carcinogenesis.

12-*O*-tetradecanoylphorbol-13-acetate (TPA, PMA, 1) is extensively used in biomedical research, while resiniferatoxin (2), a daphnane-type phorboid is under development as an alternative to surgical denervation for intractable pain, ingenol mebutate (3), an ingenane-type phorboid is used for the treatment of actinic keratosis, and tigilanol tiglate (4), is under clinical development for the intratumor treatment of cancers.

Despite their extensive use in biomedical research, and their potent and unique biological profile, phorboids are still largely unexplored in terms of chemistry, biosynthetic origin, and mechanisms of activity. This thesis aims at providing a contribution *en route* to the clarification of these issues.

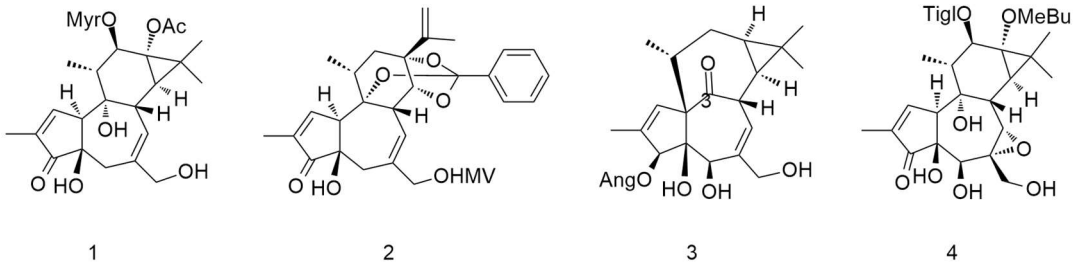


Fig. 1 TPA (**1**), Resiniferatoxin (**2**), Ingenol mebutate (**3**), Tigilanol tiglate (**4**)

Part Ia describes an expeditious isolation method of the parent diterpene polyol phorbol from croton oil (*Croton tiglium* L. oil), and of the basic diterpenoid skeleton of tigilanol tiglate from left-overs from the isolation of this compound from the seeds of *Fontainea picrosperma* C.T.White.

Part 1b presents SAR studies aimed at the exploration of the chemical space of tiglianes, and an exploration of their chemical reactivity.

Part II presents a biosynthetic investigation on the origin of the tigliane skeleton, inspired by the Jakupovic proposal of non-lathyrane derivation of these compounds. The synthesis of labelled precursors is presented as well as a series of feeding experiments on *Fontainea picrosperma*.

References

1. Wright, G. D. (2019). Unlocking the potential of natural products in drug discovery. *Microbial biotechnology*, *12*(1), 55-57.

2. Pharma R&D Annual Review, 2019

Part I: Isolation and chemistry of tigliane diterpenoids

1. Introduction

Tiglianes are a class of diterpenoids, densely functionalized, that shares a 5/7/6/3 hydrocarbon skeleton (**1**). Among all the polycyclic C-20 isoprenoids isolated from Euphorbiaceae and Thymelaceae and named *phorboids*, tiglianes represent the major class in terms of substitutions and variety of the diterpene core. The isolation of the parent alcohol was reported in 1934 by Flaschenträger during the investigation of the vesicant properties of *Croton tiglium* L. seeds oil [**1**]. The compound was named phorbol (**2**) after the name of the plant family (Euphorbiaceae) to which *Croton tiglium* belongs. Speculations and hypothesis regarding the structure of phorbol lingered for years papers in the chemical literature, backed up by degradative studies, [**2**] but only in 1967 Crombie finally elucidated its structure with a crystallographic study on a chloroform solvate of phorbol 20-(5-bromofuroate) registered at – 160 °C [**3**].

The noxious properties of the oil documented in folk medicine turned up the interest of several researchers that, towards the end of nineteenth century, began the investigation on the active fractions of the oil. Dunstan and Boole isolated an ethanol-soluble resinous mass, called *Croton resin,* which retained the irritancy of Croton oil and belying the hypothesis of Buchheim that the biological activity was related to a compound named *Crotonoleic acid* (subsequently revealed a mixture of fatty acids). [**4**] In 1941 Beremblum highlighted the co-carcinogenic properties of Croton oil, testing on mouse skin a subthreshold amount of oil with benzopyrone. [**5**] Only in 1968 with the isolation of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA, PMA, **3**) by Hecker (**Fig. 2**) and co-workers, the first pure tumor-promoting agent was characterized and, quite soon, was intended to be one of the most important agents in biomedical research. [**6**] TPA and other phorbol esters induce activation of PKC, a class of serine-threonine kinase, involved in a host of cellular activities. Mimicking the effect of diacylglycerol (DAG), PKC natural ligand, they can trigger a phosphorylation cascade resulting in a complex network like regulation of proliferation, apoptosis, cytoskeletal structure-function and histamine, IL-2 and cytokines releasing. [**7**]

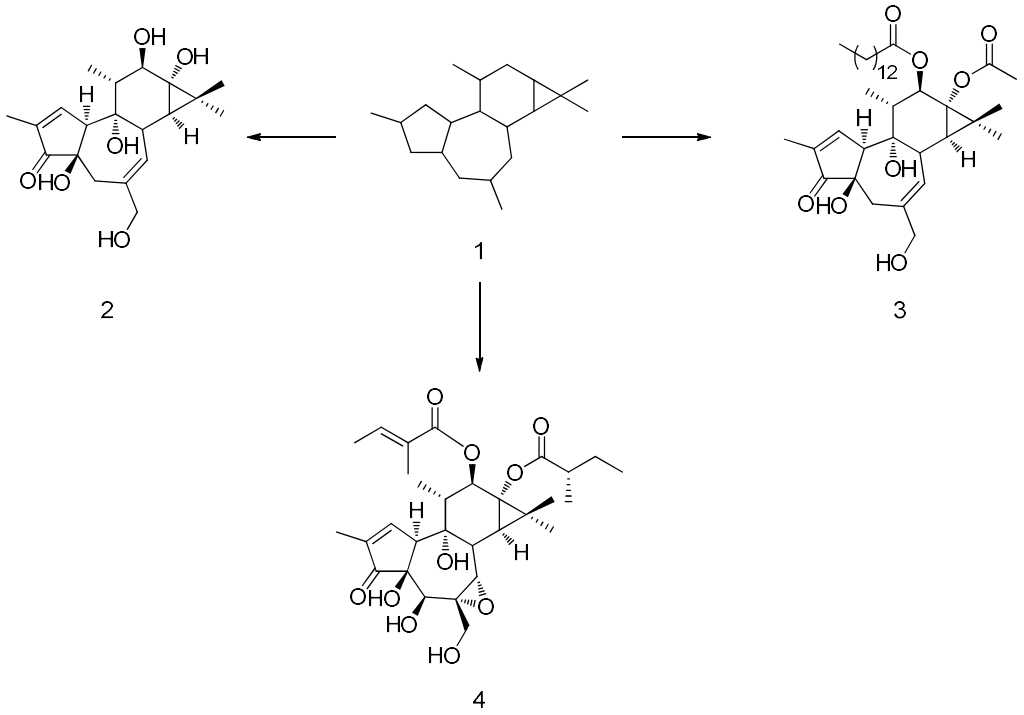


Fig.1.1 Tigliane skeleton (**1**) Phorbol (**2**) TPA (**3**) EBC-46 (**4**)

The ability to promote phenotypic changes in differentiation and/or apoptosis in cell lines and primary malignancies, prompted the use of TPA (PD-616) in clinical trial for treatment of Acute Myelogenous Leukemia or Myelodysplastic Syndrome [**8**] and Hematological malignancies. The first study was terminated for difficulties in patient enrollment while the second confirmed the feasibility of administrate TPA to humans. Despite this achievement, the study was not continued and the attraction towards phorbol esters shaded among the last years of XX century. [9] The interest for phorbol esters was rekindled in 2005 when the Australian company Ecobiotics discovered EBC-46 (Tigilanol tiglate, **4**) from an endemic species belonging to Queensland rainforest, *Fontainea picrosperma* C.T. White. The company was driven by a unique approach that involves collecting information about distribution, habitats and ecological interactions of plants, animals and microbes of Australia's tropical rainforest, named EcoLogic™. [**10**]

PKCs, as well, are back to fashion due to recent considerations about the loss of function and, in some cases, complete inactivation of PKC isozymes mutations in human cancer cells.

Restoring the activity of the enzymes themselves by genome-editing revealed that PKCs work as tumor suppressor agent and, despite the unambiguous tumor promoting activity of phorbol esters, therapies should focus their attention in restoring their activity instead of inhibiting it. [**11**]

1.1 Isolation

1.1.1 *Croton tiglium* L.

Croton tiglium L. is a leafy endemic shrub native to Malabar, Ceylon, Amboina (of the Molucca islands), Philippines, Java and China belonging to the family of Euphorbiaceae. The fruits, ripped after 5 months from blossoming, are a three-celled capsule, each one contains a single seed. [**12**] The oil obtained from solvent extraction or cold squeezing can reach 30-45 %wt on seeds and 50-60%wt on the kernel. [**13**] Croton oil occurs in several folk medicines among South-East Asian country and even in some Africans: for example, in Malaysia the oil is used as purgative, for cancerous sores, carbuncle, colds, fever, paralysis; in Ghana, the seeds were used as fish poisoning while in Sudan as purgative again. [**14**] Describing medicinal plants of Ceylon, Joannes Scott referred to *Jayapāla* (*Croton tiglium* seeds) as the most potent purgative and leaves, acrid as well, source of burning sensation through mouth and throat. [**12**]

The oil was imported by Dutch during the 16th century and in the following centuries lived a fluctuating interest by Europeans. During World War II, Croton oil was used as a denaturing agent for fuel alcohol to power torpedoes motors. USA Navy needed to render the alcohol undrinkable to prevent sailors from its consuming and, consequentially, intoxication and hangover. Sailors developed later a method to distil alcohol from the *denatured* one producing what was later called *Gilly*. [**15**] In the early winter of 1940, the Nazi party of Vidkum Quisling, after taking over of Norway, started to drain the provisions of the country to support German headquarters among Europe. Norwegian partisans, in response, ordered a large shipment of Croton oil from British officials to tamper with it a batch of sardines destined to Saint-Nazaire (a U-boat base with the duty to sink Allied ships bringing supply to England). [**16**]

Nowadays, Croton oil is used in biomedical research for assays like mice ears oedema test as a pro-inflammatory agent. [**17**] In homoeopathy, Tiglium officinale is used in the form of solution or granules in several dilutions (5 CH to 1000 CH) as a remedy for several diseases many of which related to the effect promoted by consumption of the oil itself. An interesting application of the oil was developed in cosmetic surgery as part of a recipe for deep chemical peeling; croton oil (from 0.1 % to 0.8 %, previously max concentration 2.1 %) is combined with USP phenol 88 %, water and Septisol, commercial name of hexachlorophene, an organochlorine disinfectant. After applying the mixture, the acid coagulates and precipitates skin proteins producing a *frost* effect (the way how varying degrees of white occur). Several months later, the skin appears translucent and with a significant wrinkles' reduction. [**18**]

Together with *C. megalocarpus* oil, *C. tiglium* oil was taken in consideration into biodiesel production in perspective to overcome petroleum-based diesel. This specific application influenced the cultivation of the plant in China, where domestication and clone selection were performed to achieve high yields in oil production. [**19**]

Fig. 1.2 *Croton tiglium* drupes (left) and Croton oil (right)

1.1.1.1 Naturally occurring tiglianes in *Croton tiglium* L.

The distribution of tigliane-phorboids in Croton oil was under investigation since the first half of the XX century but, still now, new phorboids are elucidated from seeds, leaves or the oil itself of *Croton tiglium*. Phorbol-like diterpenoids are the most abundant secondary metabolites isolated in the oil.

The tetracyclic system (5/7/6/3) is based on a *trans*-bridgehead bicyclo [4.4.1] undecane core. Traditionally, bi-dimensional representation of the molecules provides for writing the cyclopentenone ring A on the right side with a *trans*-bridge at C-4 and C-10. Most phorboids retain a hydroxy group at C-4 in β-position or, lower percentage, a complete lack of functionalization with an inversion of the configuration (4α-4-deoxy phorbol esters). Ring B is characterized by an allyl alcohol function nestled into the cycloheptane ring; the (*Z*)-oriented olefin side is included between C-6 and C-7 with the methylene group merging out of the plane from C-6. A trans-bridge binds again ring B and C through C-8 and C-9, which possess a tertiary β-hydroxy group. On cyclohexane ring C, it is possible to find the *anti*-oriented glycol function at positions C-12 and C-13, usually esterified and involved in mimicking the acyl chains of DAG in biological systems. Ring D is directly linked to ring C through C-13 and C-14, and it is characterized by a *cis*-oriented β-cyclopropane ring with two methyl group attached at the methylene bridge. The ^{1}H- and ^{13}C-NMR features of phorbol (**2**) are

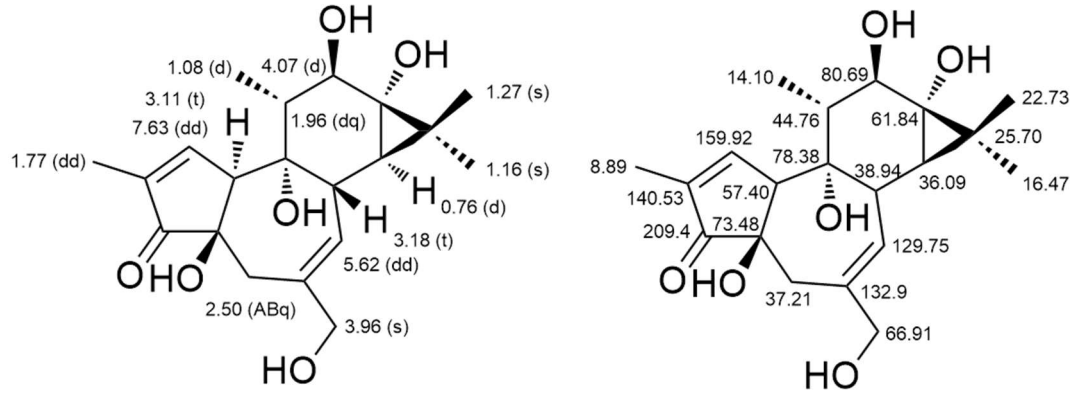
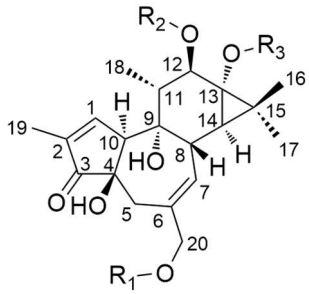


Fig. 1.3 ^{1}H- and ^{13}C-NMR data of phorbol (**2**): (MeOD-*d4*, 400 MHz and 101 MHz, respectively). *J* values: *δ* 7.63 = 2.4,1.4 Hz; 5.62 = 6.0, 2.1 Hz; 4.07 = 10.2 Hz; 3.18 = 5.8 Hz; 3.11 = 2.8 Hz; 2.50 = 19.1 Hz; 1.96 = 10.1, 6.5 Hz; 1.77 = 3.0, 1.4 Hz.

summarized in **Fig. 3**. Some of the most prominent aspects of the NMR spectra are the diastereotopy of protons at C-5 and C-20 whenever the parent alcohol is esterified. Both the AB systems retain a wide *J* coupling (from 18.0 to 10.0 Hz). The electron-withdrawing effect of the enone in ring A reduces the electron density at β-position resulting in a downfield shift of C-1 at δ 7.6 ppm (deshielded). Notably, the cyclopropane proton of C-14, typically the most upfield doublet of the spectra, is around δ 0.90 with a *J* value included between 5.0-6.0 Hz; esterifications at C-12 and C-13 result in an α and γ-downfield and β-upfield shifts. [**20**]

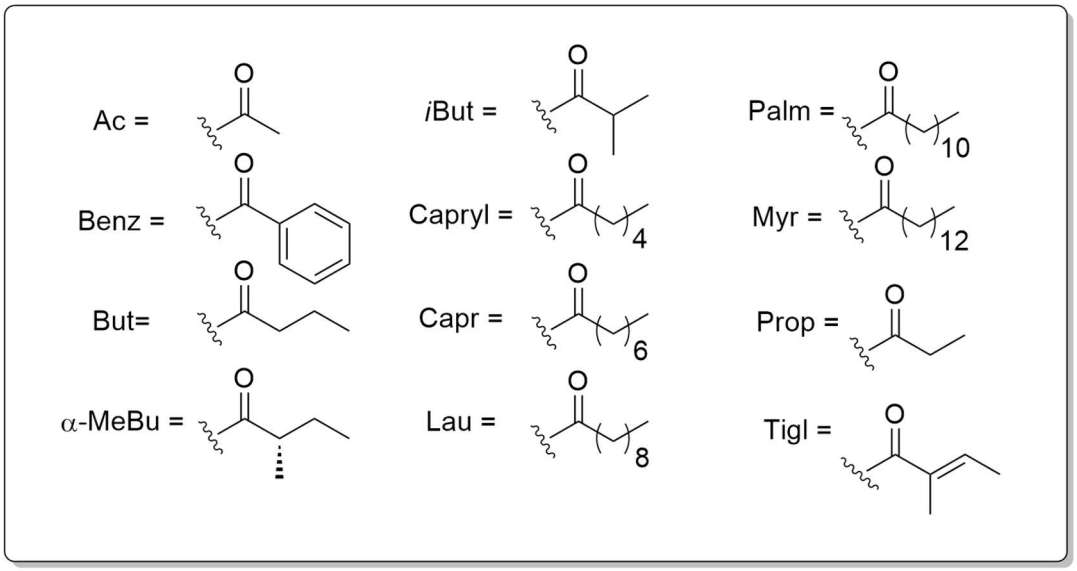
Some of the esters that have been detected so far in Croton oil are presented in the following table:



Compounds	**R_1**	**R_2**	**R_3**
Phorbol (**2**)	H	H	H
Phorbol-12-myristate	H	Ac	H
Phorbol-12-monoacetate	H	Ac	H
Phorbol-12,13-diacetate	H	Ac	Ac
Phorbol 12-acetate-13-tiglate	H	Ac	Tigl
Phorbol 12-acetate-13-decanoate	H	Ac	Capr
Phorbol 12-acetate-13-dodecanoate	H	Ac	Laur
Phorbol 12-tiglate-13-acetate	H	Tigl	Ac
Phorbol 12-decanoate-13-acetate	H	Capr	Ac
Phorbol 12-dodecanoate-13-acetate	H	Laur	Ac
Phorbol 12-tetradecanoate-13-acetate (**3**,TPA,PMA)	H	Myr	Ac
Phorbol 12-hexadecanoate-13-acetate	H	Palm	Ac
Phorbol 12,13-dibutyrate	H	But	But
Phorbol 12-butyrate-13-dodecanoate	H	But	Laur
Phorbol 12-tiglate-13-propionate	H	Tigl	Prop
Phorbol 12-tiglate-13-isobutyrate	H	Tigl	*i*But
Phorbol 12-tiglate-13-butyrate	H	Tigl	But
Phorbol 12-tiglate-13-(*S*)-2-methylbutyrate (**5**)	H	Tigl	αMeBu
Phorbol 12-tiglate13-octanoate	H	Tigl	Capryl
Phorbol 12-tiglate-13-decanoate	H	Tigl	Capr
Phorbol 12-tiglate-13-dodecanoate	H	Tigl	Laur

Phorbol 12-((*S*)-2-methylbutyrate)-13-isobutyrate	H	αMeBu	*i*But
Phorbol 12-((*S*)-2-methylbutyrate)-13-tiglate	H	αMeBu	Tigl
Phorbol 12-((*S*)-2-methylbutyrate)-13-octanoate	H	αMeBu	Capryl
Phorbol 12-((*S*)-2-methylbutyrate)-13-decanoate	H	αMeBu	Capr
Phorbol 12-((*S*)-2-methylbutyrate)-13-dodecanoate	H	αMeBu	Laur
Phorbol 12,13-dibenzoate	H	Benz	Benz
Phorbol 12-decanoate-13-((*S*)-2-methylbutyrate)	H	Capr	αMeBu
Phorbol 12,13-didecanoate	H	Capr	Capr
Phorbol 13,20-diacetate	Ac	H	Ac
Phorbol 13-acetate-20-oleate	Oleic	H	Ac
Phorbol 13-acetate-20-linoleate	Linoleic	H	Ac
Phorbol 13-tiglate-20-linoleate	Linoleic	H	Tigl
Phorbol 12,13,20-triacetate (**4**)	Ac	Ac	Ac
Phorbol 12-tetradecanoate-13,20-diacetate	Ac	Myr	Ac

Table 1.1. Summary of tiglianes isolated from *Croton tiglium* L.



Besides the canonical esters previously reported, other tiglianes from *Croton tiglium* were isolated with different functionalizations, for example, at ring B like in crotignoids A and B with a β-hydroperoxide at C-7 and a transposed double bond between C-5 and C-6. In crotignoids C and D, the peroxidic function is substituted by a β-hydroxy group while in crotignoids E, F and G a keto one occurs. The aldehydic analogue at C-20 was isolated as well from leaves keeping the same structural features of phorbol esters. [**21**] Rare tiglianes that show unusual

rearrangement of cycle D is crotonol A, where a unique C-7/C-14 fused oxetane ring replaces the cyclopropane, and crotonol B that underwent to a ring expansion of ring C resulting in a 5/7/7 system. [**22**] A further complication in the attempt to rationalize the biosynthetic pathway of tiglianes comes in 2016, where a class 4-deoxy-4β-phorbol esters have been isolated from a methanolic extract of the seeds. [**23**]

1.1.1.2 Research paper - An improved preparation of phorbol from Croton oil

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An improved preparation of phorbol from croton oil

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Full Research Paper

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Abstract

Background: Croton oil is the only commercial source of the diterpenoid phorbol (**1a**), the starting material for the semi-synthesis of various diesters extensively used in biomedical research to investigate cell function and to evaluate in vivo anti-inflammatory activity. While efficient chemoselective esterification protocols have been developed for phorbol, its isolation from croton oil is technically complicated, and involves extensive manipulation of very toxic materials like the oil or its native diterpenoid fraction.

Results: The preparation of a crude non-irritant phorboid mixture from croton oil was telescoped to only five operational steps, and phorbol could then be purified by gravity column chromatography and crystallization. Evidence is provided that two distinct phorboid chemotypes of croton oil exist, differing in the relative proportion of type-A and type-B esters and showing different stability to deacylation.

Conclusion: The isolation of phorbol from croton oil is dangerous because of the toxic properties of the oil, poorly reproducible because of differences in its phorboid profile, and time-consuming because of the capricious final crystallization step. A solution for these issues is provided, suggesting that the poor-reproducibility of croton oil-based anti-inflammatory assays are the result of poor quality and/or inconsistent composition of croton oil.

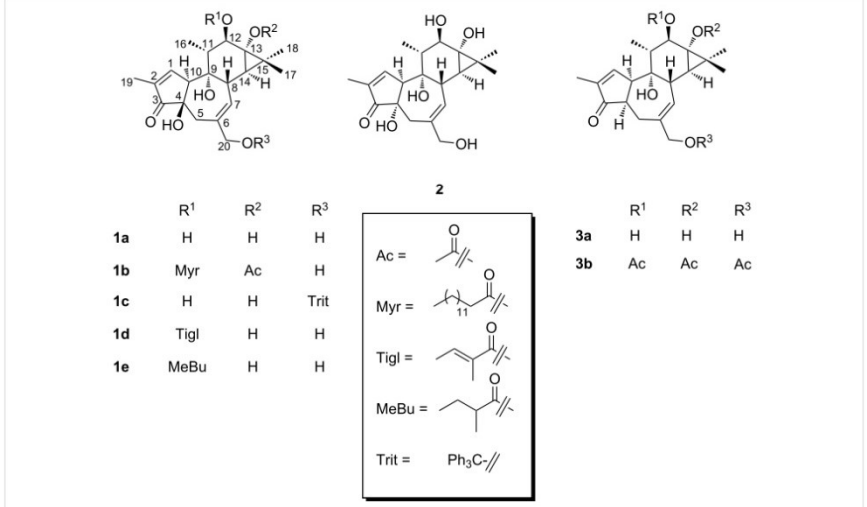
Introduction

Croton oil is obtained by pressing or solvent extraction from the seeds of *Croton tiglium* L., a small tree native to the Far East [1]. The oil is toxic to all living organisms, from bacteria to insects and vertebrates, and its irritancy and cathartic properties are legendary [2]. Croton oil was once used in human medicine as a topical rubefacient and in veterinarian medicine as a strong laxative [1], but nowadays its only medical use is in rejuvenating esthetic surgery in association to blepharoplasty, a prac-

tice that was mastered in the 1960s capitalizing on the potent exfoliating activity of the oil, especially in association to phenol [3].

The extraordinarily obnoxious and vesicant properties of croton oil have fostered studies aimed at the identification of its active principles since the very early developments of organic chemistry. Thus, the first chemical study on croton oil was reported by Pelletier and Caventou, the founding fathers of alkaloid chemistry, in 1818 [4], but the nature of its irritant principles remained obscure and controversial until 1930, when Flaschenträger unambiguously characterized the inflammatory fraction of the oil as a mixture of esters of a crystalline diterpene pentaol, named phorbol (Figure 1, **1a**) after the plant family to whom *C. tiglium* belongs (Euphorbiaceae) [5]. The early studies left their mark in organic chemistry in the well-known names of crotonic and tiglic acids, although, paradoxically, croton oil does not contain crotonic acid, that is only generated in the harsh conditions of the early studies [6]. The structure of phorbol eluded clarification until 1968, when it was eventually elucidated by a low-temperature (−160 °C) crystallographic study on the chloroform solvate of its 20-(5-bromofuroate) [7]. This study solved a riddle that classic degradative studies had proved unable to address because of the tendency of phorbol to skeletal rearrangement and to its idiosyncratic chemical reactivity [6]. By this time, the medicinal use of croton oil had become obsolete, but interest had been rekindled by the discovery of its co-carcinogenic properties by Berenblum in 1941 [8]. The tumor-promoting properties of the oil were associated to a specific class of phorbol diesters, exemplified by phorbol myristate acetate (PMA, TPA, **1b**), having a long-chain and a short-chain acyl residue on the vicinal hydroxy groups on ring C (Type-A esters). The molecular target of PMA was identified in a series of isoforms of PKC, a family of serine/threonine kinases involved in a host of cellular activities [9]. Because of its kinase-activating properties, PMA has become an indispensable tool in the study of cell function, with a single vendor claiming to have sold over 250,000 ampules of TPA since 1980 [10]. PMA has also been clinically investigated as an anticancer agent [11], and, in the wake of the successful development of ingenol mebutate for the management of actinic keratosis, a pre-cancerous condition [12], interest for phorboids in the area of cancer prevention and treatment remains high [13].

Phorbol occurs in croton oil as a mixture of di- and triesters, generally in a ca. 1:2 ratio [6], and therefore isolation involves a deacylation step, critical because of the sensitivity of phorbol to isomerization to 4α-phorbol (**2**) by a base-induced vinylogous retro-aldol mechanism [6]. Furthermore, phorbol strongly retains all kinds of solvents, forming crystalline solvates of limited stability with many common solvents, including ethanol



	R^1	R^2	R^3
1a	H	H	H
1b	Myr	Ac	H
1c	H	H	Trit
1d	Tigl	H	H
1e	MeBu	H	H

	R^1	R^2	R^3
3a	H	H	H
3b	Ac	Ac	Ac

Figure 1: The major diterpene polyols from croton oil [phorbol (**1a**), 4α-phorbol (**2**), 4-deoxy-4α-phorbol (**3a**)] and their derivatives.

[6,10]. Over the past three decades, we have been interested in the chemistry of phorbol, and have practiced its preparation from croton oil, evaluating various strategies to minimize the contact with this very toxic and irritant material, simplifying the purification strategy, and improving the storage of the final product. After summarizing the methods previously described in the literature, we describe in detail the protocol we have developed and its modulation according to differences in the composition of commercial croton oil.

Results and Discussion

The process developed by Flaschenträger [5] and then basically used by Hecker [6] and Crombie [14] in their classic studies on the structure of phorbol is based on the repeated extraction of croton oil with methanol to separate phorbol esters from triglycerides, and on the use of barium hydroxide as a base for the hydrolysis, using long (4–5 days) reaction times under mild basic conditions (pH ca. 8–9). After partition between ether and water, and precipitation of barium as a sulfate, the water phase is exhaustively extracted with ether and ethyl acetate, and then evaporated. Phorbol is extracted from the residue with hot methanol and, after filtration of the inorganic salts and evaporation, the methanol extract is crystallized from ethanol. There are two major issues with this protocol. The first one is the repeated handling of croton oil, a very toxic and obnoxious material, during the methanol extraction step, that requires several hours of stirring and then of resting to achieve a good phase separation, and the second one is the capricious crystallization of phorbol from the transesterification reaction mixture, a viscous oil for the presence of glycerol, that requires extended periods (four weeks according to ref. [6]). Furthermore, the unstable ethanol solvate has to be transformed into a more stable hydrate by recrystallization from hot water, a step that also requires a long time (one week according to ref. [6]). Also of concern is the overall number of operational steps (a series of over fifteen partitions, separations and evaporations), while the recovery of the more lipophilic and abundant phorbol triesters by methanol extraction of the oil is problematic, since these compounds are strongly retained in the triglyceride phase and multiple extractions are necessary to transfer them into the methanol phase, with an estimated loss of ca 50% [15]. To cope with the difficulties of the crystallization steps, protocols based on the purification of a crude mixture of ethanol solvates of phorboids by reverse-phase preparative flash chromatography or by countercurrent chromatography were developed [15-17]. The recovery of the triesters could be improved by first treating croton oil with acidic methanol to selectively remove the 20-acyl group. Partition between methanol and hexane afforded a crude mixture of phorbol diesters, that was next tritylated. After deacylation by basic treatment and chromatography, 20-tritylphorbol (**1c**) was obtained, as a hydrate, in sufficient purity to be used as starting material for the esterification [18]. This method was originally developed by Bernd Sorg of the Deutsches Krebsforschung Zentrum (Heidelberg, Germany), who kindly shared it with other researchers in the field. In our hands, it was simpler and more efficient than the original process developed by Flaschenträger, but it was, nevertheless, problematic for the multigram isolation of phorbol due to the extensive handling of the toxic croton oil and the ultra-toxic mixture of phorbol diesters obtained in the acidic transesterification step. Furthermore, the tritylation of the crude mixture of phorbol diesters suffered from interference from variable amounts of glyceryl mono- and diesters co-extracted with phorbol diesters and, presumably, already occurring in the native oil.

Since phorbol and its monoesters are not toxic, an "anticipation" of the deacylation step could afford a reaction mixture amenable to handling under normal laboratory conditions, also securing the recovery of phorbol triesters, difficult to selectively extract from the oil with a polar solvent. This strategy is not new in conception [16,19], but its implementation needs improvement in the recovery of phorbol to be practical. Thus, the water solution of phorbol and glycerol obtained after hydrolysis of the oil and washing with organic solvents must be evaporated with care, maintaining an acidic pH to minimize epimerization to 4α-phorbol [19]. Next, the recovery of phorbol from the resulting ca 10% solution of phorboids in glycerol is difficult under both normal- and reverse-phase silica gel chromatography, that fail to separate phorbol from 4α-4-deoxyphorbol (**3a**) because of their similar chromatographic behavior and of the presence of glycerol. As a result, the week-long crystallization from water was still necessary [19], a process that, tediousness aside, we found was accompanied by partial epimerization to 4α-phorbol (**2a**) and erosion of the overall final yield.

To streamline the recovery of phorbol in the deacylation step, croton oil was treated with sodium methylate. By replacing hydrolysis with transesterification, it was possible to remove fats from the detoxified reaction mixture by extraction with petroleum ether, making dilution with water unnecessary. Provided that the pH of the reaction did not exceeded 13, retroaldol epimerization was also negligible, and undetectable by TLC control. Evaporation of methanol was straightforward and gave a solution of phorboids in glycerol as a dark thick oil that was subjected to liquid–liquid partition to recover phorbol from the glyceryl matrix. After considerable experimentation, we found that glycerol could be efficiently removed from a tetrahydrofuran (THF) solution of the transesterification residue by repeated washings with acidified brine. 1-Butanol and 1,4-dioxane were far less selective, affording extracts heavily contaminated by glycerol, also giving problems of foaming during their evaporation. The rationale for the selective partition be-

tween THF, a low-boiling and easily removed solvent, and water is unclear. The use of THF was inspired by the work of Seebach on the solubility of peptides in ether-type organic solvents in the presence of certain alkaline cations [20], and it is not unconceivable that the interaction with sodium ions substantially diversifies the relative polarity of glycerol and phorbol, making it possible to selectively partition them. In this way, the preparation of the diterpene polyol fraction was telescoped to only five operational steps (treatment of croton oil with sodium methylate, extraction with petroleum ether, evaporation, partition between THF and brine, and evaporation of THF). Purification of phorbol from the THF extract by gravity column chromatography (GCC) was then straightforward, affording a semicrystalline ca. 5:1 mixture of phorbol (**1a**) and 4α-4-deoxyphorbol (**3a**). The two compounds had very similar chromatographic behavior, but could be efficiently separated by exploiting the efficient solubility of **3a** in ethyl acetate, a solvent where phorbol is insoluble. Thus, after trituration with ethyl acetate, filtration, and washing, phorbol could be obtained as an off-white powder (6.0 g, 1.2% from croton oil), sufficiently pure for further chemical modification and devoid of **3a**. The ethyl acetate solvate of phorbol is a powder with limited stability (weeks) also at low temperature, but crystallization from methanol afforded large crystals of the more stable methanol solvate. While the ethanol solvate of phorbol degrades in a few days even at low temperature [21], the methanol solvate could be stored for at least four months at 4 °C without any significant degradation.

Transesterification with sodium methylate, and presumably also with barium hydroxide under the milder conditions of the Flaschenträger protocol, could not remove the α-branched acyl group of type-B phorbol esters, and a mixture of phorbol 12-monoesters, mainly phorbol 12-tiglate (**1d**) and phorbol 12-(2-methylbutyrate) (**1e**) was obtained from the early chromatographic fractions. The mixture of 12-acyl phorbols that had resisted global transesterification could not be further hydrolyzed without epimerization to 4α-phorbol (**2**) and extensive degradation, and accounted for ca 30% of the amount of phorbol obtained from the transesterification. The recovery of phorbol from the monoesters **1d** and **1e** was, however, possible after tritylation of the primary 20-hydroxy group (vide infra).

While this method worked well with different batches of croton oil, with consistent yields of phorbol as EtOAc solvate in the range of 1%, some samples gave a lower yield (0.2–0.3%) because of incomplete transesterification. Furthermore, the crude transesterification mixture was devoid of significant amounts of 4α-4-deoxyphorbol (**3a**), and contained as major constituent a mixture of partially hydrolyzed esters, mainly phorbol 12-tiglate (**1d**) and phorbol 12-(2-methyl)butyrate (**1e**). Thus, the ^{1}H NMR spectrum (methanol-d_4) of the crude phorboid fraction, while showing the deshielded signals of the tiglate methine (δ ca. 6.80) and of H-1 of phorbol at δ ca. 7.60, lacked the singlet of H-1 of 4α-4-deoxyphorbol at δ ca. 7.20. Hydrolysis of the 12-monoesters failed under a variety of conditions, including hydrazinolysis for the tiglate residue, and required conditions too basic for the survival of phorbol. On the other hand, tritylation of the primary 20-hydroxy group had a surprising stabilizing effect toward basic degradation, making it possible to remove the remaining ester group. 20-Tritylphorbol (**1c**) [18] obtained in this way could be directly used for the synthesis of specific esters, or, alternatively, deprotected with acidic methanol (pH 3) to phorbol. The reasons for this trityl-induced stabilization are unclear, an educated guess being that the bulky trityl group could hinder oxidative reactions based on oxygen attack to the ring B double bond, a major degradation pathway for phorbol derivatives, especially under basic conditions [22].

Taken together, these observations revealed that two chemotypes of croton oil exist. The high-yielding oil contains mainly type-A phorbol di- and triesters. These phorboids have a long-chain acyl group bound to the secondary hydroxy group at C-12 and a short chain acyl group bound to the 13-hydroxy group, and are easily transesterified to phorbol. Conversely, the low-yield chemotype is dominated by type-B phorbol esters, where the long-chain ester group is located at the tertiary 13-hydroxy group, and branched acyl groups are bound to the 12-hydroxy. These branched acyl groups are not significantly removed by transesterification in the pH range of stability of phorbol, even at the more basic conditions of our protocol compared to the classic Flaschenträger method. Furthermore, 4α-4-deoxyphorbol derivatives are not contained in significant amounts in this chemotype.

Croton oil is used as a reference for in vivo anti-inflammatory assays, like the mouse-ear erythema assay, and it is tempting to suggest that the notoriously poor-reproducibility of the data from this assay [23] might be related also to differences in the composition of croton oil, since the irritancy of phorbol esters is critically dependent on their acylation profile [6]. However, the native phorboid profile of croton oil is still poorly characterized in terms of analytical profile [24], and the recovery of the native highly lipophilic phorboid esters from the lipid matrix of the oil remains a challenge. We hope that our observations will foster studies aimed at developing analytical methods to better characterize and quantify the diterpenoid profile of this oil, whose extraordinarily irritant properties have not only generated scientific interest, but also found a place in history [25] and literature [26].

Experimental

General experimental procedures: ^{1}H and ^{13}C NMR spectra for the mixtures **1d**/**1e** were measured on a Bruker 700 Anance III HD (700.43 MHz; 176.13 MHz). Chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δH = 7.24, δC = 77.0). Silica gel 60 (70–230 mesh) for gravity column chromatography (GCC) was purchased from Macherey-Nagel (Düren, Germay). Aluminum-coated Merck 60 F254 (0.25 mm) plates were used for TLC, visualizing the spots by UV inspection and/or staining with 5% H_2SO_4 in ethanol and heating. All solvents were of analytical grade, and were purchased from Aldrich, while croton oil was supplied by Adipogen Life Sciences (San Diego, USA). References samples of the batches used in this study are kept at the Novara laboratories.

Phorbol from croton oil

a) Croton oil rich of type-A esters: In a 2 L round-bottom flask, freshly prepared 0.3 N sodium methylate in methanol was added dropwise (ca. 10 mL/min) to a magnetically stirred mixture of croton oil (LKT Laboratories, batch number 2597837, 500 mL) and methanol (50 mL) until the pH reached a value of 12–12.5 (pH strips, 0.5 pH unit resolution). Approximatively 1 L of methylate solution was necessary to reach and stabilize this pH value, and during the addition the amber color of the oil initially faded, and next darkened to eventually become black when the pH was strongly basic (>10). The course of the transesterification was followed by TLC, monitoring the appearance of the spot of phorbol [(EtOAc/MeOH 96:4 as eluent, direct deposition from the reaction mixture, R_f (phorbol) = 0.14)], and the lack of formation of 4α-phorbol (R_f = 0.09 in the same eluent system). After stirring overnight, the reaction mixture, whose pH was now around 11.5, was transferred into a 3 L separatory funnel, neutralized with a few drops of glacial acetic acid, and extracted with petroleum ether (5 × 300 mL). The upper phase was initially deep yellow, but its color faded with the successive extractions, while the lower phase remained dark colored. Evaporation of the lower methanol phase gave a viscous black residue that was dissolved in THF (250 mL) and washed with brine until the lower water phase was almost colorless (5 × 250 mL). The combined water phases were back-extracted with THF (ca. 100 mL), and the pooled THF phases were dried with sodium sulfate and then evaporated. A semi-solid residue was obtained (ca. 30 g), then purified by GCC on silica gel (250 g). The column was packed with petroleum ether/EtOAc 5:5 and the amount of EtOAc was gradually increased. Elution with petroleum ether/EtOAc 2:8 gave a crude fraction of phorbol monoester (8.9 g, see infra or the characterization). Elution was next continued with EtOAc and finally with EtOAc/MeOH 9:1 to afford a mixture of phorbol (**1a**) and 4α-4-deoxyphorbol (**3a**) as a semi-solid orange paste (ca. 15 g). The paste was triturated with EtOAc (150 mL) and the suspension was cooled two hours at the refrigerator temperature and next suction filtered to obtain phorbol as a slightly oatmeal-colored powder (6.0 g, 1.2% from the oil). Recrystallized from hot MeOH (35 mL) afforded 1.07 g of large colorless crystals of a methanol solvate.

The mother liquors from the trituration with EtOAc were evaporated, dissolved in pyridine (20 mL), and then treated with Ac_2O (20 mL) and DMAP (cat.). After 1 h, the reaction was worked up by the addition of a few drops of methanol to destroy the excess Ac_2O and of 2 N H_2SO_4, and next extracted with EtOAc. After drying and evaporation, the residue was purified by GCC on silica gel using petroleum ether/EtOAc 8:2 as eluent to afford 12,13,20-triiacetyl-4α-4-deoxyphorbol (**3b**, 1 g) [27] and 12,13,20-triacetylphorbol (500 mg) [27].

b) Croton oil rich of type-B esters: The oil (Alexis Biochemicals, batch number 350-089-0000, 500 mL) was processed as above. The de-glycerinated THF crude phorboid mixture was separated by GCC to afford 9.0 g of a mixture of phorbol monoesters and 1.0 g crude phorbol, that, when analyzed by ^{1}H NMR was devoid of 4α-4-deoxyphorbol. A portion (1.0) of the phorbol monoesters mixture was further purified by GCC to obtain an orange powder, that was then washed with ether to afford a colorless product (400 mg). This, when analyzed by ^{1}H NMR, was a mixture of the phorbol monoesters **1d** and **1e** (ca. 2:1 ratio).

Phorbol-12-tiglate (1d): ^{1}H NMR (700.43 MHz, $CDCl_3$) δ 7.56 (s, 1H, H-1), 5.63 (m, 1H, H-7), 4.84 (d, J = 9.8 Hz, 1H, H-12), 4.03 (m, 1H, H-20b), 3.98 (m, 1H, H-20a), 3.17 (br d, J = 2.5 Hz, 1H, H-10), 3.09 (m, 1H, H-8), 2.54 (m, 1H, H-5b), 2.44 (m, 1H, H-5a), 1.77 (dd, J = 2.9,1.3 Hz, 3H, H-19), 2.15 (m, 1H, H-11), 1.01 (s, 3H, H-17), 1.16 (s, 3H, H-16), 1.03 (s, 3H, H-18), 0.90 (m, 1H, H-14), 6.85 (qd, J = 7.1, 1.3 Hz, 1H, H-3'), 1.78 (m, 3H, H-4'), 1.80 (d, J = 1.09 Hz, 1H, H-5'), 4.93 (br s, 1H, 9-OH); ^{13}C NMR (176.13 MHz, $CDCl_3$) δ 208.80 (s, C-3), 160.28 (s, C-1), 140.74 (s, C-6), 133.41 (s, C-2), 129.29 (d, C-7), 79.17 (s, C-9), 87.36 (d, C-12), 73.49 (s, C-4), 67.97 (t, C-20), 60.83 (s, C-13), 56.77 (d, C-10), 43.50 (d, C-11), 38.99 (d, C-8), 38.74 (t, C-5), 35.19 (d, C-14), 27.69 (s, C-15), 22.27 (q, C-16), 17.05 (q, C-17), 16.08 (q, C-18), 10.14 (q, C-19), 170.51 (s, C-1'), 138.66 (s, C-3'), 128.13 (s, C-2'), 14.51 (q, C-4'), 12.05 (q, C-5').

Phorbol-12-(2-methylbutyrate) (1e): ^{1}H NMR (700.43 MHz, $CDCl_3$) δ 7.55 (br s, 1H, H-1), 5.63 (br d, J = 5.1 Hz, 1H, H-7), 4.83 (d, J = 10.0 Hz, 1H, H-12), 4.03 (d, J = 12.9 Hz, 1H, H-20b), 3.98 (d, J = 12.9 Hz, 1H, H-20a), 3.16 (br d, J = 3.3 Hz, 1H, H-10a), 3.09 (br d, J = 5.6 Hz, 1H, H-8a), 2.54 (d, J = 19.1 Hz, 1H, H-5b), 2.44 (d, br, J = 16.3 Hz, 1H, H-5a), 1.80

(dd, J = 2.9, 1.3 Hz, 3H, H-19), 2.11 (dd, J = 10.0, 6.72 Hz, 1H, H-11), 1.04 (s, 3H, H-17), 1.17 (s, 3H, H-16), 1.00 (d, J = 6.5 Hz, 3H, H-18), 0.89 (d, J = 7.6 Hz, 1H, H-14a), 2.39 (td, J = 13.9, 7.0 Hz, 1H, H-2'), 1.65 (m, 1H, H-3'), 1.48 (tt, J = 13.7, 7.4 Hz, 1H, H-3'), 1.13 (d, J = 6.9 Hz, 1H, H-5'), 0.90 (t, J = 6.0 Hz, 3H, H-4'); ^{13}C NMR (176.13 MHz, $CDCl_3$) δ 208.77 (s, C-3), 160.18 (d, C-1), 140.84 (s, C-6), 133.42 (s, C-2), 129.17 (d, C-7), 79.12 (s, C-9), 87.41 (d, C-12), 73.47 (s, C-4), 67.92 (t, C-20), 60.89 (s, C-13), 56.82 (d, C-10), 43.28 (d, C-11), 38.96 (d, C-8), 38.70 (t, C-5), 35.14 (d, C-14), 27.63 (s, C-15), 22.37 (q, C-16), 17.24 (q, C-17), 15.92 (q, C-18), 10.13 (q, C-19), 179.68 (s, C-1'), 41.31 (d, C-2'), 26.90 (t, C-3'), 11. 67 (q, C-4'), 16.47 (q, C-5').

Hydrolysis of the mixture of 1d/1e: A portion of the mixture of monoesters (3.0 g) was dissolved in pyridine (30 mL) and treated with trityl chloride (11.4 g) and cat. DMAP. After stirring overnight at room temp., the reaction was worked up by dilution with EtOAc (50 mL) and washing with 2 N H_2SO_4/brine (10:1, 100 mL). After drying and evaporation, the residue was purified by GCC on silica gel (petroleum ether/EtOAc 4:6 as eluent) to afford 3.14 g of a mixture of 20-tritylphorbol monoesters. The latter was dissolved in methanol, and 0.3 N sodium methylate was added dropwise until pH reached 12.5. After stirring overnight at room temp., the reaction was worked up by neutralization with 2 N H_2SO_4, dilution with brine, and extraction with CH_2Cl_2. Evaporation of the solvent left a solid residue, that was purified by GCC on silica gel (75 g, petroleum ether/EtOAc 3:7 as eluent) to afford 930 mg of 20-trityl phorbol. The latter could be directly used for the preparation of specific 12,13-diesters. Alternatively, it was dissolved in methanol (15 mL) and acidified to pH 3 with a few drops of 70% $HClO_4$. After 30 min, the reaction was worked up by neutralization with NaOAc and evaporation. The residue was purified by GCC on silica gel (15 g) using EtOAc/MeOH 95:5 as eluent, to afford 460 mg phorbol, that was triturated with EtOAc, eventually affording 295 mg of a colorless powder.

Supporting Information

Supporting Information File 1

ESI-HRMS and ^{1}H and ^{13}C NMR spectra of compounds **1d** and **1e**.
[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-13-133-S1.pdf]

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1.1.2 *Fontainea picrosperma* C.T. White

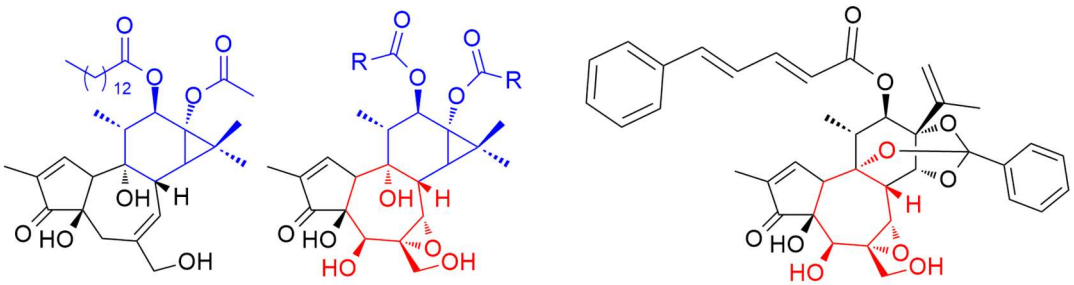
Fontainea picrosperma C.T. White is a sub-canopy tree endemic of the northeastern forests of Australia. The genus Fontainea consists of only nine species mostly distributed between eastern Australia, Papua New Guinea, New Caledonia and Vanuatu. [**24**] The fruits, succulent orange/red drupes, reach maturation between December and January and undergo to scatter-hoarding by cassowaries, giant tailed rats and musky rat kangaroos. [**25**]

During the collection of data for understanding the interactions between plants, animals and microbes of the Atherton Tablelands rainforests, the Australian pharmaceutical company, QBiotics Ltd., noticed that *F. picrosperma* fruits were or deprived of them fleshes (seed keeps untouched) or completely regurgitated by wild animals whenever the seeds were broken. The further analysis of the bioactive fraction from the kernel revealed a quite huge percentage of diterpenoids esters, structurally related to tigliane but with some analogy with daphnanes. A plethora of 5β-hydroxy-6,7α-epoxy phorbol esters were addressed to be the main compounds, implied in the offensiveness of the kernel and that justify the missed *zoochory*. Even if 5β-hydroxy-6,7α-phorbol esters are not an entirely new sub-group in tigliane type diterpenoid, none of the previous one isolated present a hydroxy (and its relative acyl decoration) at C-12. The oil obtained by solvent extraction (usually methanol) is closed to 10-13 % from undried frozen kernel and, of this, up to 6 % are 5β-hydroxy-6,7α-phorbol esters. A complete phytochemical composition is not established yet due to privileged researches on the bioactive fractions and therefore to tiglianes diterpenoids.

Fig. 1.6 *Fontainea picrosperma* C.T.White fruits' detail.

1.1.2.1 Isolation of 5β-hydroxy-6,7α-epoxy-5,20-acetonide phorbol from *F.picrosperma* oil

The strategy aimed to reduce the incredible variety of C-12,13 esters to only one molecular identity optimized in 1.2.1.2, was applied as first at the methanolic extract of *F. picrosperma* kernel but treatment with sodium methylate completely degraded all the epoxytiglianes. This observation was quite surprising because of the hydrolytic behavior with both TPA (tigliane ester) and mezerein (daphane ester), whose share structural elements with the epoxytiglianes (**Fig. 7**), give a precise conversion to the parent polyol (respectively, phorbol and 12-hydroxydaphetoxin). [**2, 26**]



(3) (10)

Fig. 1.7 Structural homologies between TPA (3), a general 5β-hydroxy-6,7α-epoxy tiglianes and mezerein (10)

This *Gestalt* effect suggests that the chemistry of these compounds goes beyond the one expected from the sum of the isolated structural elements and a new approach has to be developed.

In order to reduce possible base-induced rearrangement at ring B (Payne rearrangement**, Fig. 8**), the secondary alcohol at C-5 and primary one at C-20 were protected in an acetonide using both acetone and 2,2-dimethoxypropane (ratio 2:1) as reagent in the presence of an excess of the mild acid pyridinium *p*-toluenesulfonate (PPTSA), to generate the protected ester with a satisfactory yield. The obtained acetonide resulted stable in basic conditions and transesterification can be achieved with Cs_2CO_3 in methanol, a surprisingly mild condition.

This protocol proved a convenient source of the key intermediate **11** to sustain structure-activity studies and increase the yield of extraction of tigliane skeleton for further biosynthetic investigation (close to 8 % w/w on crude extract).

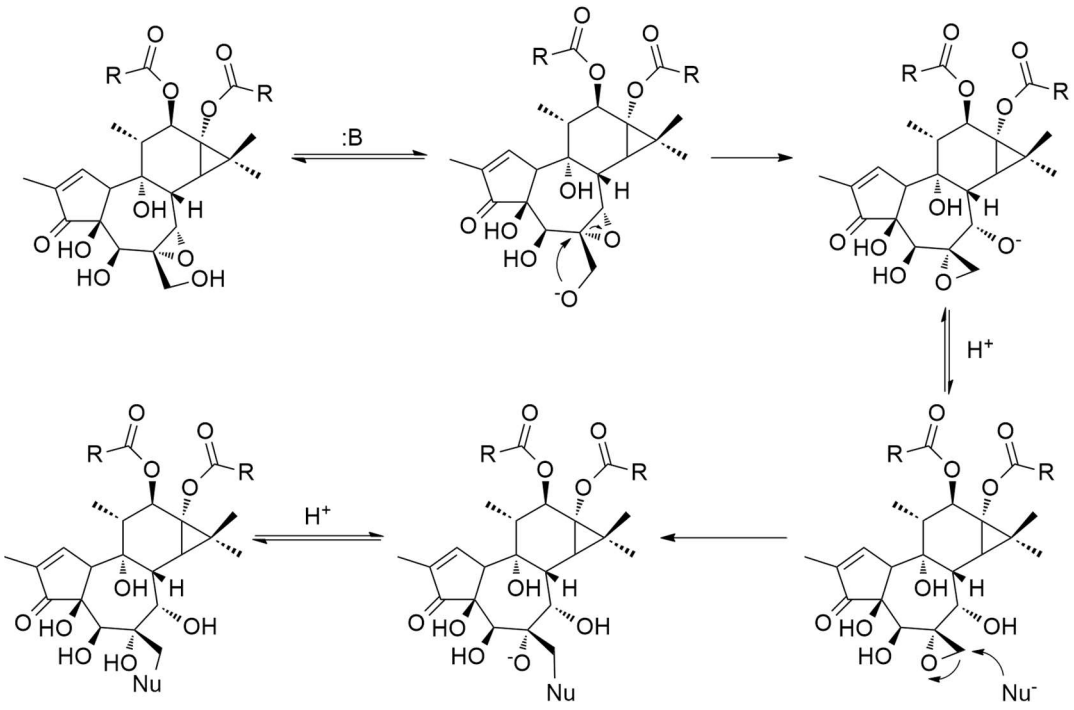


Fig. 1.8 Payne rearrangement catalysed by base transesterification approach

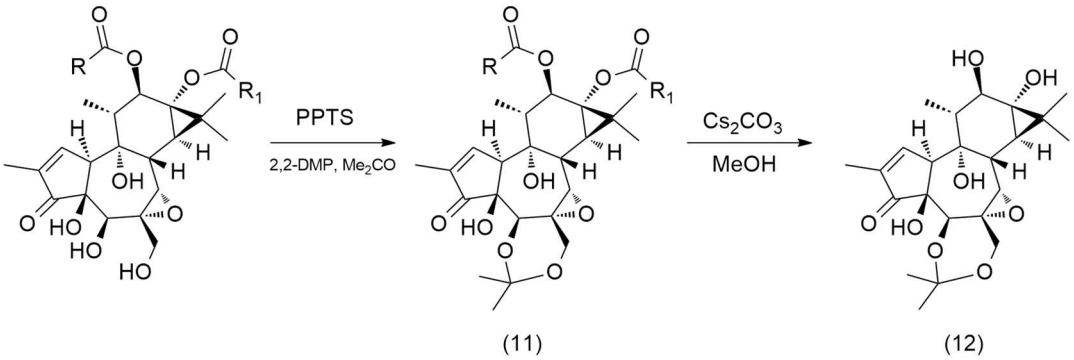


Fig. 1.9 Preparation of 5β-hydroxy-6,7α-epoxy-5,20-acetonide phorbol from *F.picrosperma* oil

1.2.2.2 Supporting information

Chemicals were purchased from Alfa Aesar (Kandel, Germany), Fluorochem (Hadfield, UK) or Sigma Aldrich Chemie (Steinheim, Germany) and used without any further purification. Solvents were purified by distillation and dried according to standard methods. All non-aqueous reactions were performed under an inert atmosphere (nitrogen) in flame-fired flasks. Thin-layer chromatography was performed with 0.2 mm precoated plastic sheets TLC silica gel 60 F_{254} (Merck) and visualized by UV inspection (254 nm) and stained with 5 % H2SO4 in methanol before heating. Column chromatography was carried out using Merck silica gel 60 (70-230 mesh). ^{1}H and ^{13}C NMR spectra were recorded with Pollux Bruker AV III Prodigy (400 MHz) spectrometer and referenced against solvent signals (^{1}H-NMR residual proton signals: $CDCl_3$ δ=7.26; ^{13}C-NMR: $CDCl_3$ δ=77.16). Starting material was supplied by INDENA s.p.a (Milano, Italy) for QBiotics Limited (Yungaburra, Australia) as side-cuts from GMP and non-GMP production of EBC-46 (Tigilanol tiglate).

Synthesis of 12,13-diacyl-6,7α-epoxyphorbols-5,20-acetonide (11): To a solution of F. *picrosperma* oil side cuts (10.0 g, IDT Silica 3A-4D EBC concentrate 13.03.2015) in acetone (54 mL) and 2,2-dimethoxypropane (27 mL), pyridinium-*p*-toluenesulfonate (2.7 g) was added at room temperature. After 4 days, the reaction was diluted with H_2O and extracted EtOAc. The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo to afford 10.82 g of **11**. TLC (petroleum ether/ethyl acetate 6:4): R_f= 0.67.

Synthesis of 5β-hydroxy-6,7α-epoxy phorbol 5,20-acetonide (12): To a solution of **11** (10.82 g) in MeOH (5 mL), a freshly prepared solution of Cs_2CO_3 in MeOH (20 mL, 0.3 M) was slowly added at room temperature. The color of the reaction changes from orange to dark brown. After 3 days, the reaction was diluted with H_2O (2.8 mL to get a ratio MeOH:H_2O 9:1) and extracted several times with petroleum ether. The aqueous phase was acidified with H_2SO_4 1 M to pH ≈ 3, dilute with brine and extracted with EtOAc. The combined organic layers were dried over Na_2SO_4. After evaporation, the residue was purified by gravity column chromatography on silica gel (pe/EtOAc 2:8) affording **12** (1.94 g) as yellowish amorphous

solid. Trituration with Et_2O afforded an oat-meal colored powder (1.25 g). TLC (petroleum ether/ethyl acetate 6:4): R_f= 0.22. ^{1}H NMR (400 MHz, MeOD-d_4) δ 7.73 (dt, J = 2.8, 1.3 Hz, 1H), 4.20 (d, J = 13.0 Hz, 1H), 4.13 (s, 1H), 4.10 (d, J = 9.9 Hz, 1H), 3.82 (p, J = 2.7 Hz, 1H), 3.46 (d, J = 13.0 Hz, 1H), 3.15 (d, J = 7.1 Hz, 1H), 3.09 (s, 1H), 1.82 (dd, J = 2.9, 1.3 Hz, 3H), 1.75 (dq, J = 9.7, 6.5 Hz, 1H), 1.52 (s, 3H), 1.46 (s, 3H), 1.37 (s, 3H), 1.23 (s, 3H), 1.06 (d, J = 6.5 Hz, 3H), 1.03 (d, J = 7.1 Hz, 1H).^{13}C-NMR (101 MHz, MeOD-d_4): δ = 208.05 (C_q), 162.77 (CH), 135.21 (C_q), 102.24 (C_q), 82.53 (CH), 78.39 (C_q), 74.10 (C_q), 70.07 (CH), 66.61 (CH_2), 66.59 (CH), 63.40 (C_q), 62.74 (C_q), 52.66 (CH), 48.68 (CH), 37.83 (CH), 36.94 (CH), 27.75 (CH_3), 24.42 (CH_3), 23.72 (CH_3), 22.92 (CH_3), 17.96 (CH_3), 16.16 (CH_3), 10.09 (CH_3).

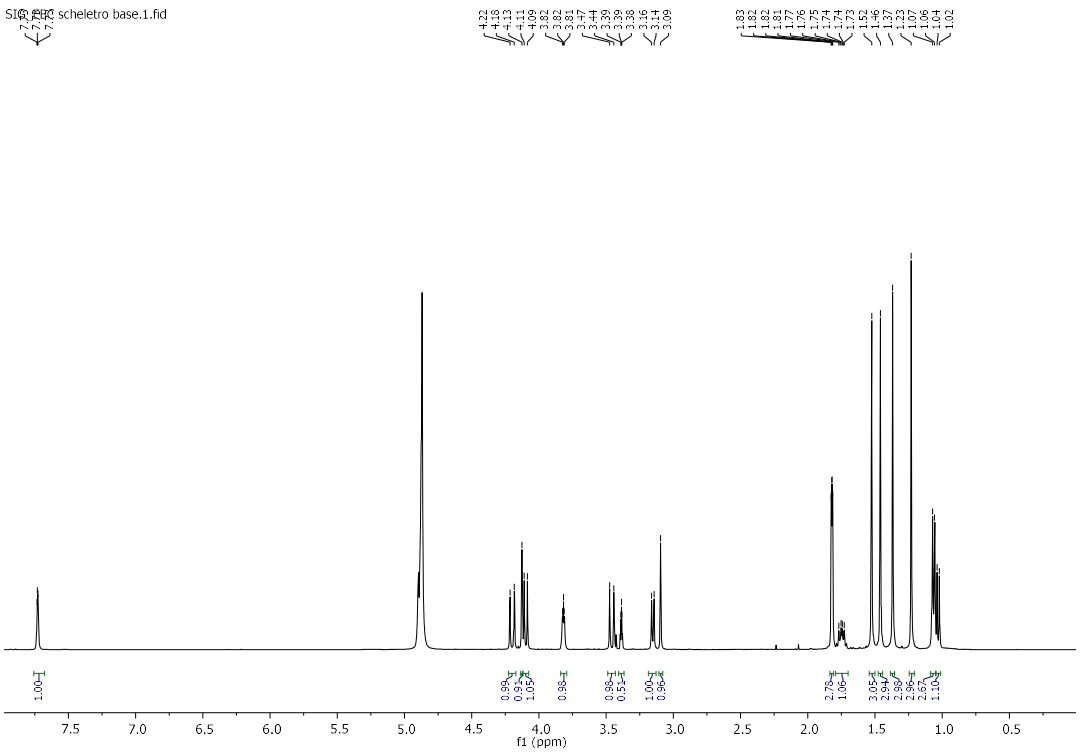


Fig. 1.10 ^{1}H-NMR spectra of **12**

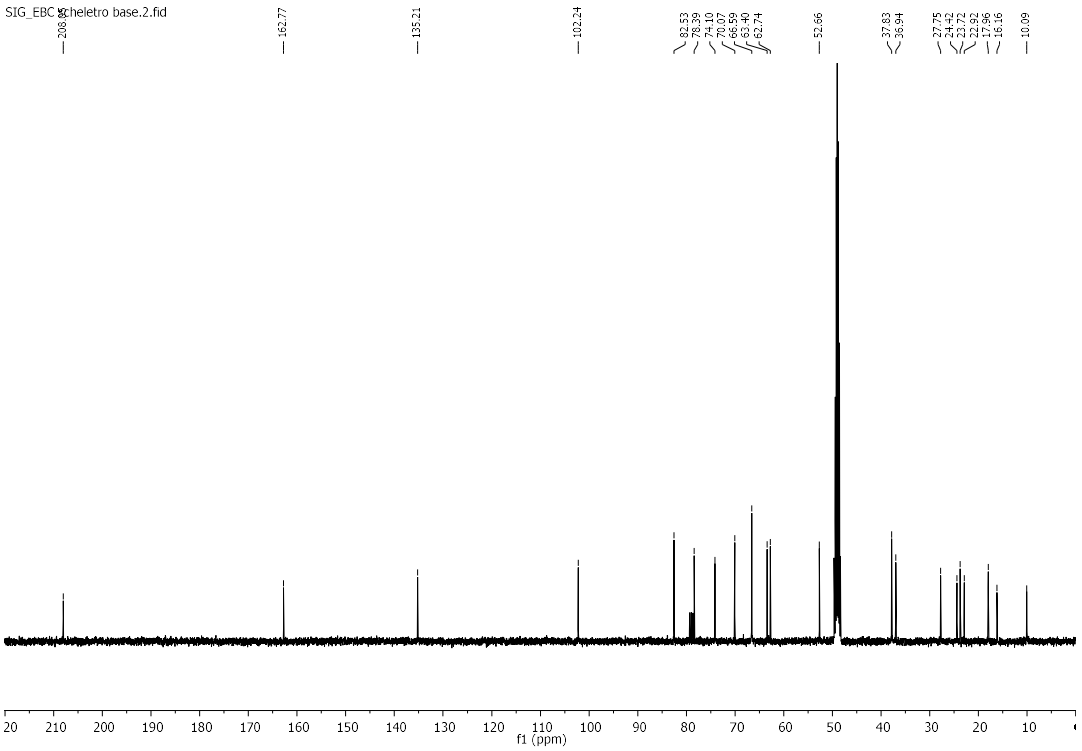


Fig. 1.11 ^{13}C-NMR spectra of **12**

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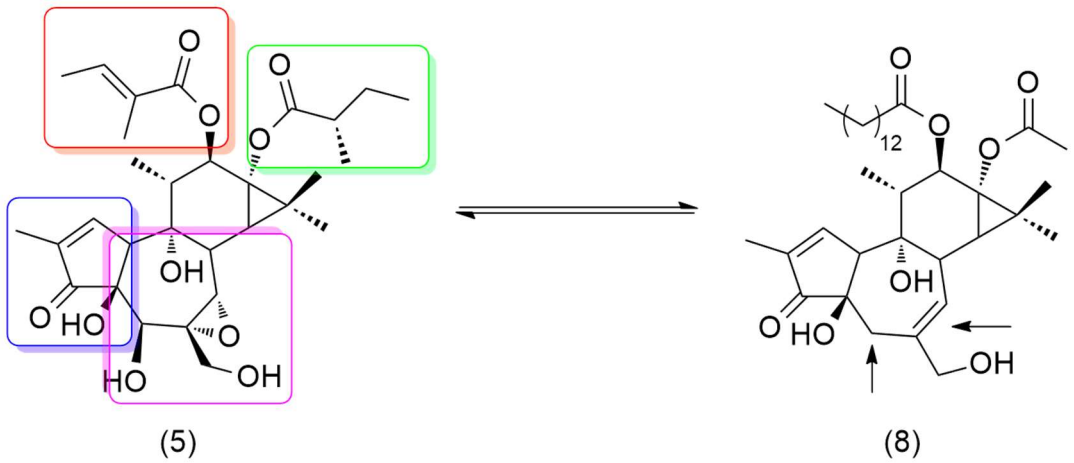
1.2 Chemistry of tiglianes: SAR studies on phorbol esters

The unique biological profile exploited by 5β-hydroxy-6,7α-epoxytiglianes compared to other phorbol esters was at the base of the challenging rationale aimed to design hybrid molecules between the two experimental drug candidate, TPA and EBC-46. The chemical space around the esters at C-12 and C-13 was explored in previous activities. It led to the conclusions that significant cytotoxicity is favored by α-branching whenever the two acyl groups are different and higher, or at least comparable, if the acylation profile consists in a symmetric pair of linear aliphatic acids having at least six carbons each. Thus, the seven-membered ring B was investigated in terms of the occurrence or lack of the natural substituents: β-hydroxy at C-5 and 6,7-α epoxide. [**Fig. 10**]



(5) (8)

Fig. 1.12 Analogies and differences between EBC-46 (**5**) and TPA (**8**)

1.2.1 Esterifications in phorbol

The isolated parent alcohol phorbol (**2**) or 5β-hydroxy-6,7α-epoxy phorbol 5,20-acetonide (**12**) can be selectively esterified at position C-12, C-13 and C-20. The primary allylic alcohol at C-20 is the most reactive and, in order to have access to the diol on ring C, must be protected. Traditionally, protection is carried by acylation, as an acetate, or with trimethylsilyl chloride to afford the 20-trityl ether. Other silyls protecting group have shown weak stability at C-20 and often are extruded during work-up. The protected derivative appears more stable than the parent alcohol suggesting the importance of shielding the allylic alcohol in case the product won't be previously crystallized. Both the protecting group, by the way, can be easily removed by acid solvolysis in methanol. Once protected, phorbol-20 trityl ether or 20-acyl phorbol gives access at positions C-12 and C-13 that, for example, in Steglich conditions (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide, EDC) and DMAP (4-dimethylaminopyridine) provides the mono- or di-esters. Although the expected product from mono-esterification would be the ester at C-12 (as secondary alcohol is most reactive than the tertiary at C-13), the isolated one is the 13-acyl ester.

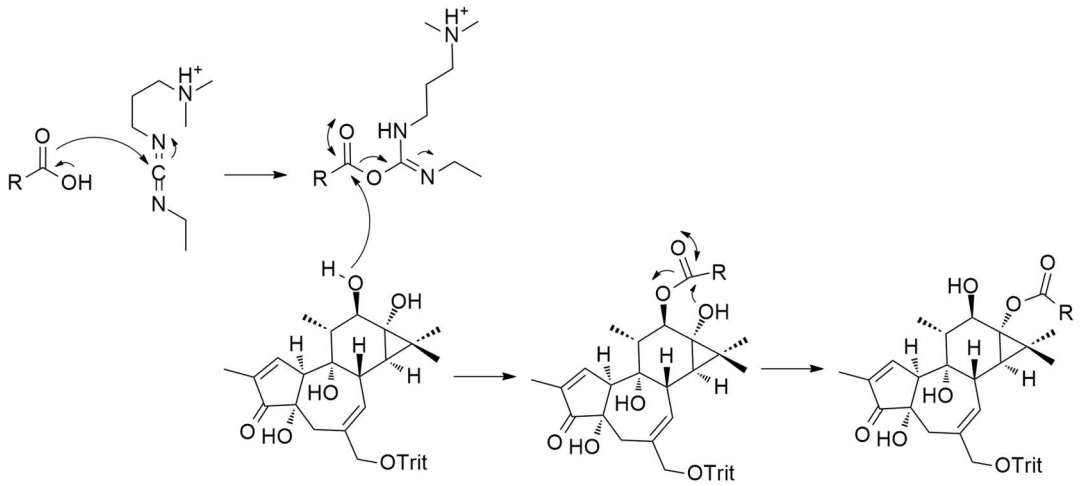


Fig. 1.13 Esterification course in tiglianes. The anchimeric effect of C-12 to esterify C-13

What is believed is that the activated acid is firstly esterified at C-12 and, subsequentially, the lone pair of electrons on the vicinal oxygen proceed via a nucleophilic attack at neighbour-carbonyl to de-acylate it and re-esterify itself. This migration has essential consequences in

the development of a synthesis of phorbol derivatives, as exemplified by the synthesis of 12-tigloyl-13-(*S*)-methylbutyril phorbol, the analogue of the drug candidate EBC-46. (**Fig. 1.11**) Esterification of aliphatic acids or not particularly hindered ones take place under Steglich conditions with variable yields. However, as soon as a hindered substituent has to be used, the previous conditions do not produce any results. After several trials, it was observed a successful functionalization under Yamaguchi conditions with 2,4,6-trichlorobenzoyl chloride and diisopropylethylamine (DIPEA).

The synthesis of **16** proceeds starting from phorbol (2), isolated as in paragraph 1.1.1.2, protected with trityl chloride at C-20. The first esterification was led under Steglich conditions with (S)-(+)-2-methylbutyric acid, EDC and a catalytic amount of DMAP. Conversely, esterification at C-13 was done with Yamaguchi reaction affording 15 but, during the work-up of the reaction, the slightly acidic condition induces removal of the protecting group. The residual protected derivate was deprotected with perchloric acid in MeOH to afford product 16. (**Fig. 1.12**)

Despite the accessible synthesis shown, preliminary bioactivity studies reveal an indiscriminate activation of PKC as canonical phorbol esters, suggesting that the selectively could arise from modification at ring B, like the 5β-hydroxy group or the 6,7α-epoxide.

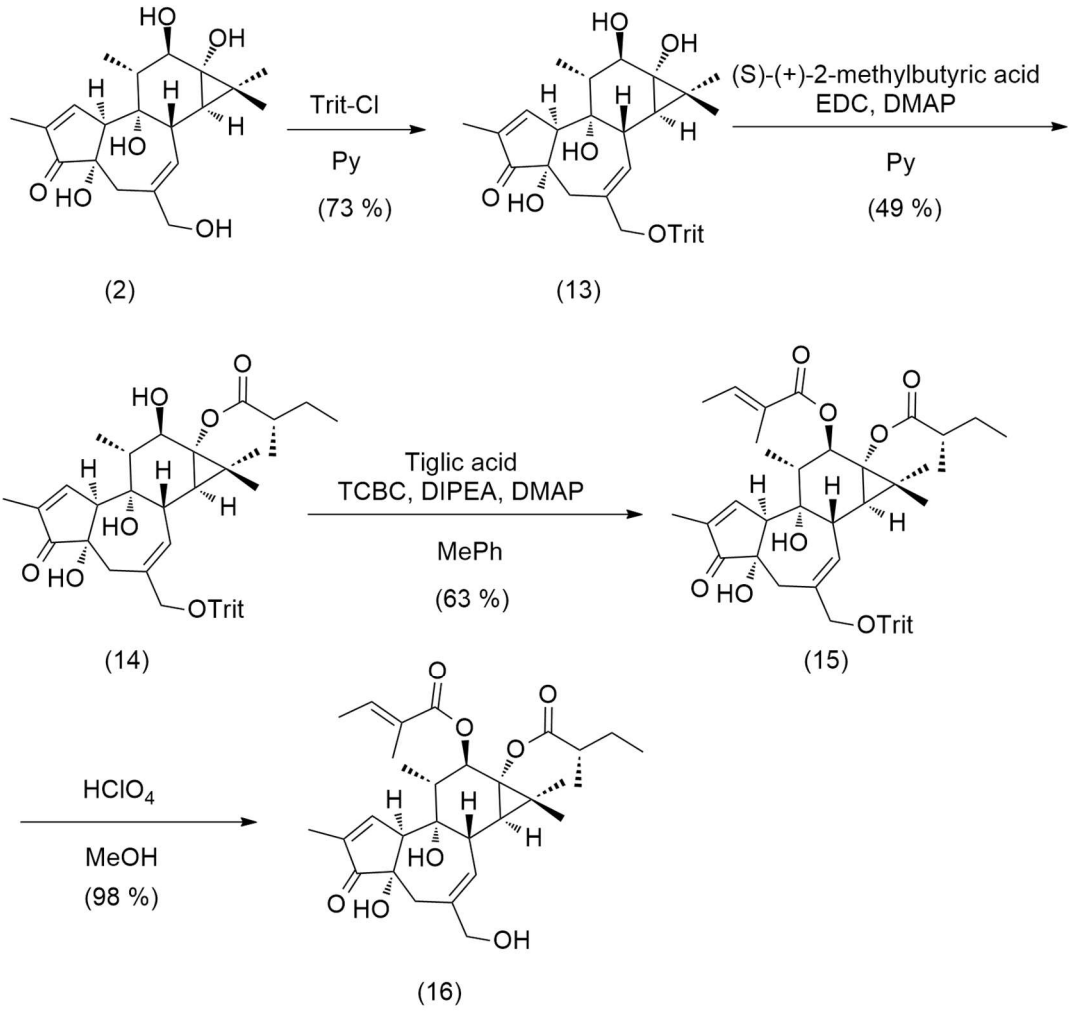


Fig. 1.14 Synthesis of EBC-46 analogue with phorbol

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1.2.2 Allylic oxidation on ring B

Oxidation is one of the most critical chemical transformations in natural products total synthesis both as for stabilization addition on a vicinal functional group and for simplify further modifications by the modification of the new framework. C-H oxidation of hydrocarbon is generally complicated and often require special conditions to achieve chemo-, regio- and stereoselectivity. [2]

As first, we have decided to investigate oxidative conditions on 12,13-diacyl-20-trityl phorbol, aware of the intrinsic difficulty already described for ingenane diterpenoids. Protection of the primary alcohol is just as compulsory at C-20, in order to avoid oxidation to the corresponding aldehyde, as it is protection at C-12 and C-13. An α-ketol rearrangement was described by Hecker in 1969 to produce *abeo*-phorbols whenever PCC it is used as oxidizing agent. (**Fig. 1.13**)

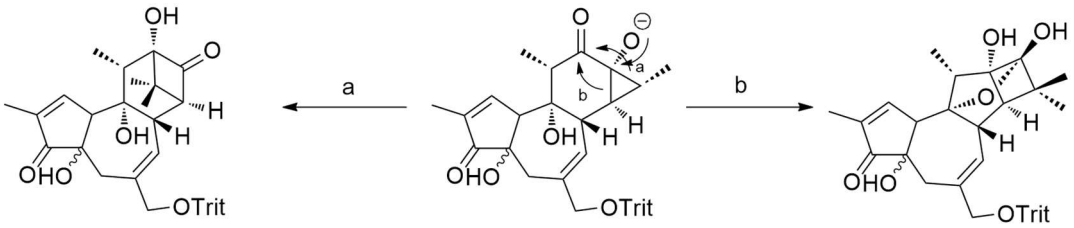

Fig. 1.15 α-ketol rearrangement PCC mediated on 12-dehydro-20-trityl phorbol

Therefore, phorbol (2) was protected at C-20 with triphenylmethyl (Trityl) chloride (10) and esterified at C-12 and C-13 with Ac_2O/Py to achieve 12,13-diacetyl-20-trityl phorbol (11). The intermediate **11** was used as starting material for allylic oxidation reactions like SeO_2/*t*-BuOOH and Phenylselenynic anhydride, but none of the mentioned procedures led to the allylic alcohol. Interestingly, the reactions with selenium derivatives promoted the removal of the protecting group and the oxidation of the primary alcohol to the corresponding aldehyde.

A change in the protecting group was then evaluated in terms of stability under the reported conditions, but the product isolated was the same. (**Fig. 1.14**)

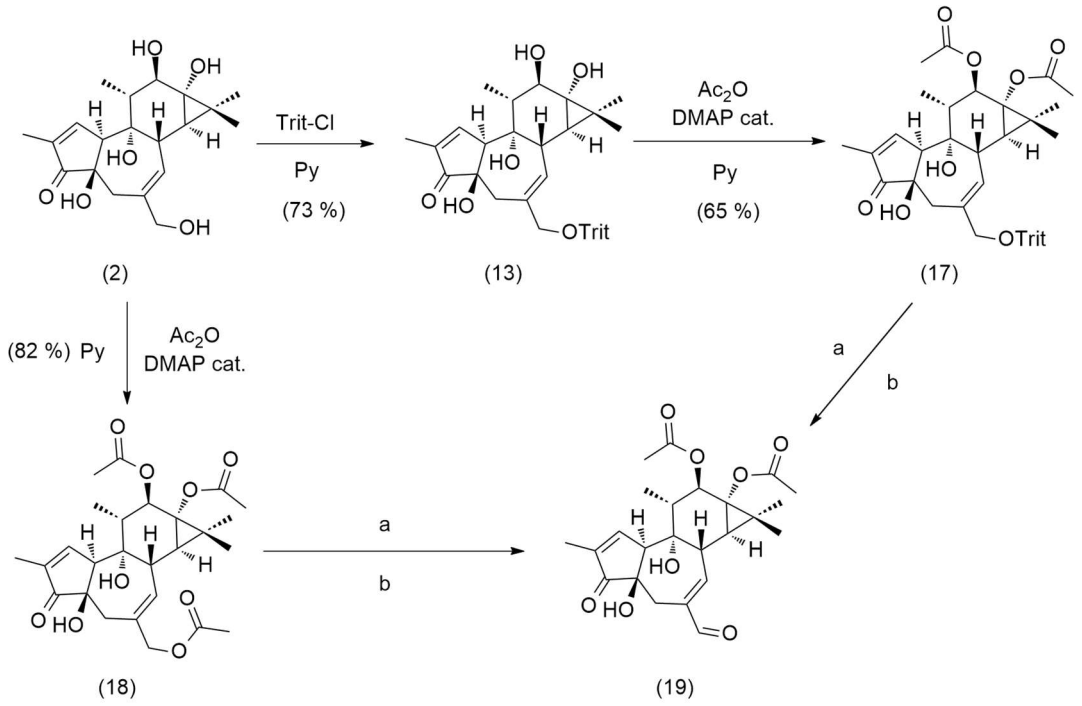

Fig. 1.16 Unexpected synthesis of 12,13-diacetyl-20-formyl phorbol

Chromium (VI)-based allylic oxidations are used as well in natural products total synthesis. From CrO_3 to more stable PCC and PDC, these reagents accomplish perfectly insertion of a keto group in hydrocarbon scaffold as accomplished by Nicolau and co-workers in 1994 in total synthesis of taxol [3]. Therefore, product 17 has been used in the conditions reported (30 eq PCC, 20 eq NaOAc), but no significant product was obtained. By simple TLC analysis was evident the removal of the protecting group, followed by a decomposition of the starting material. The slight acidity of PCC that it is generally quenched by adding sodium acetate could have promoted the deprotection at C-20 inducing subsequentially a decomposition of the residue.

Another trial was performed with $Pd(OH)_2$ on carbon/*t*-BuOOH/K_2CO_3, but in this case, a complete lack of reactivity was observed.

We hypothesize that the “cage-like” conformation could represent an obstacle to target ring B in phorbol, so we have thought to flatten the *trans*-bridged cyclic system to expose better the 7-member ring by reduction of the enone system on ring A and protection of the diol C-3 and

C-4 as an acetonide. The first step, after acylation of the reactive hydroxy groups, was a Luche reduction to achieve the β-hydroxy allylic alcohol on ring B. In fact, in order to reach proper protection of the 1,2-diol system a *syn* stereoisomerism would be preferred. The diol 20 was protected 2,2-dimethoxypropane:acetone (1:2 ratio) with pyridinium-*p*-toluenesulfonate in catalytical amount. (**Fig. 1.15**)

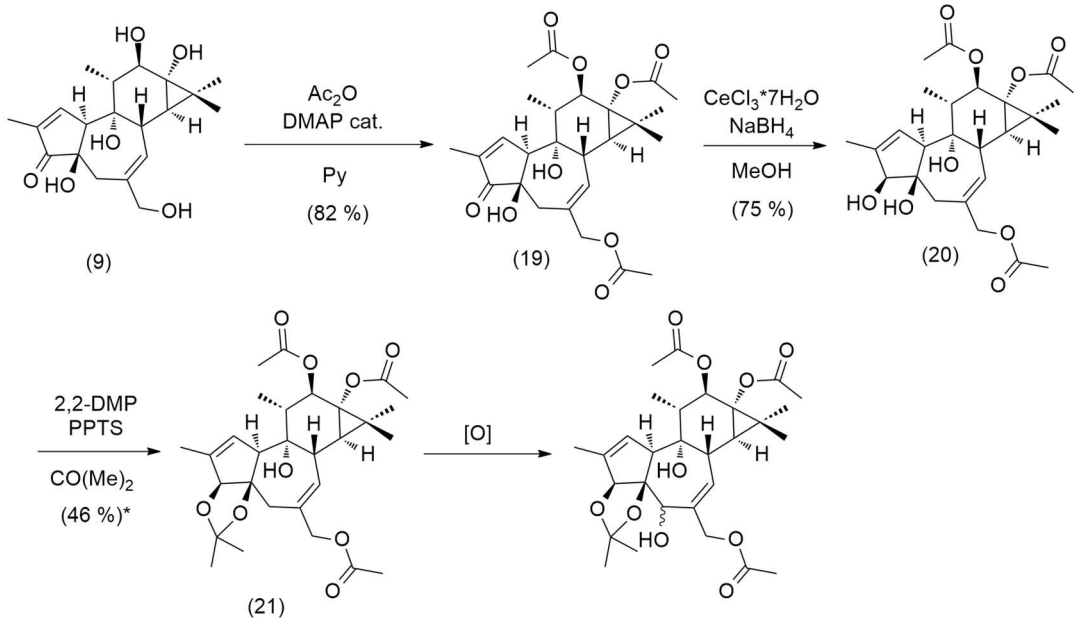

Fig. 1.17 Strategy to oxidize C-5 position in 12,13,20-triacetyl phorbol-3,4-acetonide

Product **21** was used, as first, in the same condition of the expedited reaction tried on the triacetate *wild-type*. Reactions with selenium do not bring to any reactivity while, interestingly, PCC/NaOAc (Nicolau's conditions) affords products from the oxidation at C-10. (**Fig. 1.16**)

The oxidation of the C-10 allyl alcohol (**Fig. 1.17, a**) might induce a ring opening through the deprotonation at C-11, allowing the obtainment of an intermediate *seco* phorbol esters. The enol group at C-9 can rearrange allowing the elimination of an acetate to get **22a**. Conversely, the activated alcohol as chromate ester (**Fig. 1.17, b**) can transforms the alkene into an epoxide fostering the attack on the more substituted carbon. The re-oxidation to the corresponding ketone at C-7 leads to **22b. [5]**

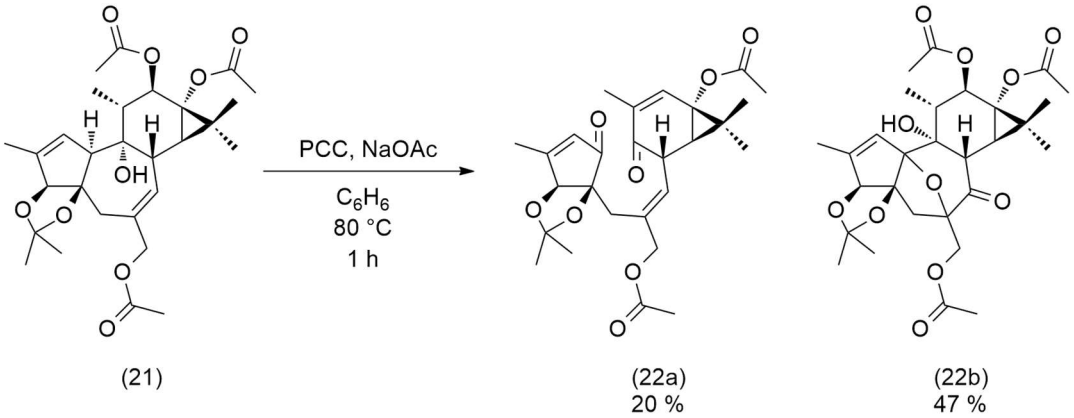

Fig. 1.18 PCC/NaOAc allylic oxidation of **21**

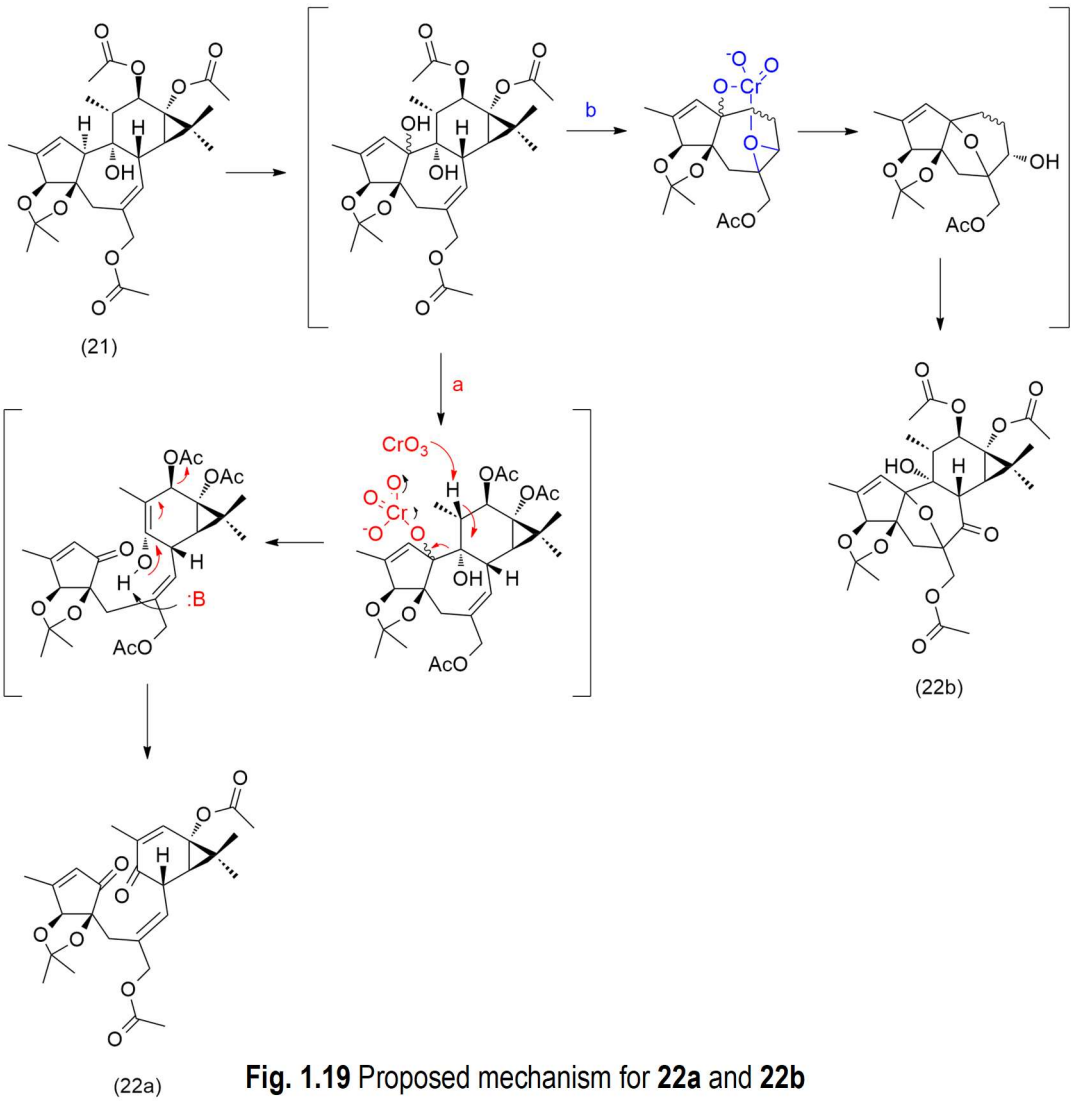

Fig. 1.19 Proposed mechanism for **22a** and **22b**

Possibly, flattening of the bicyclic ring exposes better the system to oxidative condition, paving the way for the discoveries of new reactions around the chemistry of tiglianes. Alternative oxidations will be taken in considerations to reach the desired goal.

1.2.3 Deoxygenation on 5β-hydroxy-6,7α-epoxy phorbol 5,20-acetonide

The second strategy among the synthesis of EBC-46 analogues intended to elucidate substructures that retain biological selectivity was deoxygenation of the epoxide. It is known that epoxidation of phorbol esters with peroxy acids (like m-chloroperbenzoic acid) produces selectively β-epoxide and that α-epoxidation, the natural one, can be achieved using vanadium-based reactions in the presence of chiral ligand. [**6**]

In order to synthesize complementary molecules for our library, we decided to start from the isolated 5β-hydroxy-6,7α-epoxy phorbol 5,20-acetonide (**12**). As first, protection at position C-12 and C-13 was achieved by acylation with hexanoic acid (**23**). Then, a series of reactions were attempted to produce the olefinic derivative.

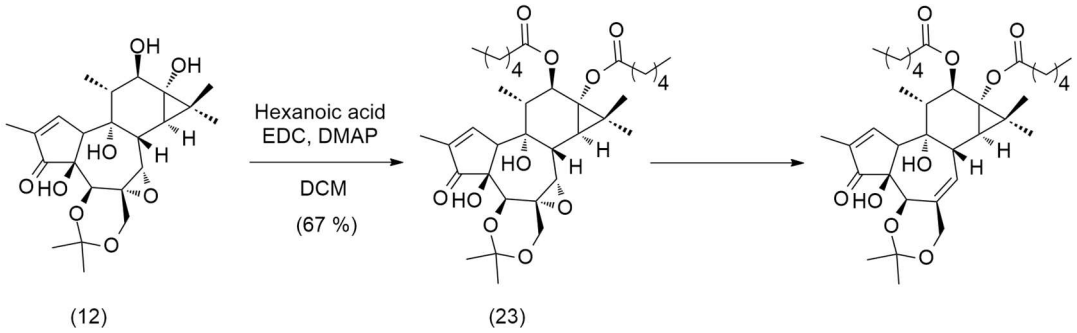

Fig. 1.20 Synthetical strategy to synthesize 12,13-dihexanoyl phorbol 5,20-acetonide

Based on previous laboratory experience on deoxigenative chemistry of triterpenoids, the reaction with iodine with triphenylphosphine was experienced as first. The reaction, at room temperature under inert atmosphere, produces selectively the iodohydrine derivative with 88 % of yield. Interestingly, taking inspiration by Garlaschelli and Vidari upon the reduction of epoxide to alkenes, iodohydrine intermediate should be formed as first, and a subsequent E_2-iodo elimination with triphenylphosphine oxide formation should take place. [7]

The reaction in dichloromethane, in a dry or wet atmosphere, produces only the iodohydrine 5,20-acetonide derivative but, if it is performed in acetonitrile, beside the epoxide opening the cleavage of the acetonide is prompted by *in situ* formations of HI.

Conditions	Product
TPP, I_2	24: 12,13-dihexanoyl-6-hydroxy-7-iodo phorbol-5,20 acetonide (88 %)
Zn, AcOH, AcONa, NaI (rt or Δ)	Not react
SmI_2	26: unknown structure
Zn, $CuSO_4$, 80 °C	Not react
Metrioxorhenium (VII), TPP	Not react
NaI, Me_3SiCl	Starting material decomposition
TPP, I_2, K_2CO_3	Not react
$CeCl3*7H_2O$, $(EtO)_3P$ (rt or Δ)	Not react
NaI, $(EtO)_3P$	Not react
***p*-TSA*H_2O, NaI**	24, 25
AcOH, NaI	Not react
$(EtO)_3P$, microwave	Not react
n-BuLi, $SnCl_2$, Me_3Al (0°C to rt)	Not react

Table 1.1 Attempted reaction to deoxygenate 5β-hydroxy-6,7α-epoxy phorbol 5,20-acetonide

Noteworthy is the deoxygenation mediated by samarium iodide, which it is prepared in situ by reaction of samarium with 1,2-diiodoethane in dry THF (Kagan method). The latter was let it react with 23, and complete conversion of starting material was observed but the isolated product was not the expected olefin. A further rearrangement of phorbol occurred and the structure is still under investigation to establish the proper connectivity.

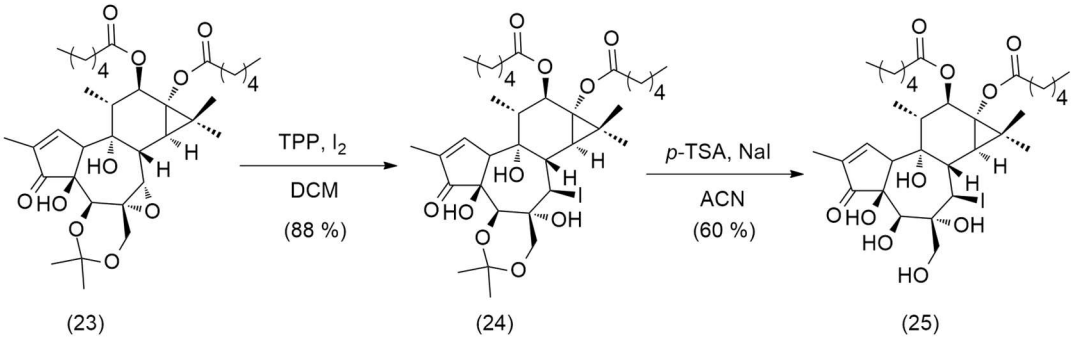

Fig. 1.21 Synthetical strategy to synthesize 12,13-dihexanoyl phorbol 5,20-acetonide

Due to intrinsic difficulties to deoxygenate directly 23, a two-step strategy was then evaluated to reduce the iodohydrine derivative into the corresponding alkenes. Again, trying to access the tigliane skeleton represents an obstacle and the expedited conditions degrade, rearrange or cleavage the protecting group as shown in table 1.2.

Conditions	**Product**
$SnCl_2$, $POCl_3$	Starting material decomposition
Zn, EtOH, 80 °C	27: unknown structure
Mesyl chloride, TEA	Not react
***p*-TSA, NaI**	12,13-dihexanoyl-6-hydroxy-7-iodo phorbol

Table 1.2 Attempted reaction to reduce the iodohydrine 24

In conclusion, the recent chemistry under developing aimed to synthesize a library of compounds with or without functional groups that naturally occurs in the experimental drug tigilanol tiglate (EBC-46) reveal an unexpected series of products never described so far in the chemistry of phorbol (tiglianes). Further efforts are required on the basis of all the strategies tried up to now.

1.2.4 Supporting information

Chemicals were purchased from Alfa Aesar (Kandel, Germany), Fluorochem (Hadfield, UK) or Sigma Aldrich Chemie (Steinheim, Germany) and used without any further purification. Solvents were purified by distillation and dried according to standard methods. All non-aqueous reactions were performed under an inert atmosphere (nitrogen) in flame-fired flasks. Thin-layer chromatography was performed with 0.2 mm precoated plastic sheets TLC silica gel 60 F_{254} (Merck) and visualized by UV inspection (254 nm) and stained with 5 % H2SO4 in methanol before heating. Column chromatography was carried out using Merck silica gel 60 (70-230 mesh). ^{1}H and ^{13}C NMR spectra were recorded with Pollux Bruker AV III Prodigy (400 MHz) spectrometer and referenced against solvent signals (^{1}H-NMR residual proton signals: $CDCl_3$ δ=7.26; ^{13}C-NMR: $CDCl_3$ δ=77.16).

Synthesis of 20-trityl phorbol (13): To a solution of phorbol (682.6 mg, 1.873 mol, 1 eq) in pyridine (7 mL), triphenylmethyl chloride (2.61 g, 9.365 mol, 5 eq) and DMAP were added at room temperature. After stirring it for 3 h, the reaction was diluted with H_2SO_4 1 M aq. sol and extracted three times with EtOAc. The combined organic layers were dried over Na_2SO_4. After evaporation, the residue was purified by gravity column chromatography on silica gel (pe/EtOAc 4:6) affording 13 (830 mg, 1.368 mol, 73 %) as white amorphous powder. TLC (EtOAc): R_f= 0.52.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.6 (dd, *J* = 2.7, 1.4 Hz, 1H), 7.5-7.2 (m, 15 H), 5.55 (d, *J* = 4.0 Hz, 1H), 4.14-4.10 (m, 1H), 3.59 (*J* =12.5 Hz, 2H), 3.04 (t, *J* = 5.1 Hz, 1H), 2.92-2.87 (m, 1H), 2.45 (d, *J*= 20.5 Hz, 1H), 2.35 (d, *J*= 19.0 Hz, 1H), 2.12 (s, 1H), 1.86 (dq, *J* =11.0, 6.4 Hz, 1H), 1.79-1.76 (m, 3H), 1.32 (s, 3H), 1.19 (s, 3H), 1.07 (d, J= 6.5 Hz, 3H), 0.80 (d, J= 5.1 Hz, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.1, 159.8, 143.9, 139.0, 133.0, 128.7, 128.5, 127.7, 126.7, 87.0, 81.5, 78.4, 73.6, 68.8, 62.5 , 57.6, 45.2, 39.5, 39.0, 36.2, 26.8, 23.4, 17.4, 15.1, 10.3.

Synthesis of 13-(*S*)-2-methylbutiryl-20-trityl phorbol (14): To a solution of (*S*)-(+)-2-methylbutyric acid (107.6 µL, 0.989 mmol, 1.2 eq) in DCM (5 mL), EDC (189.6 mg, 0.989 mmol, 1.2 eq) and DMAP were added at room temperature. When EDC was completely

dissolved, a solution of 13 (500 mg, 0.824 mmol, 1 eq) in DCM (10 mL) was added dropwise. After stirring it overnight, the reaction was diluted with brine and extracted three times with DCM. The combined organic layers were dried over Na_2SO_4. After evaporation, the residue was purified by gravity column chromatography on silica gel (pe/EtOAc 6:4) affording 14 (278.9 mg, 0.403 mmol, 49 %) as pale-yellow amorphous powder. TLC (petroleum ether/ethyl acetate 7:3): R_f= 0.13. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.6 (s,1H), 7.5-7.35 (m, 6H), 7.32-7.20 (m, 9 H), 5.56 (d, *J* = 4.0 Hz, 1H), 3.99 (d, *J* =10.3 Hz, 2H), 3.6 (s, 2 H), 3.22 (d, *J* = 16.3 Hz, 1 H), 3.12 (m, 1 H), 3.10 (d, *J* = 2.7 Hz), 2.4 (d, *J* = 16.3 Hz), 2.5 (m, 1 H), 1.98 (s, 3 H), 1.75 (s, 3 H), 1.5 (m, 2 H), 1.24 (s, 3H), 1.20 (s, 3H), 1.10 (s, 3 H), 0.98-0.87 (m, 7 H).^{13}C NMR (75 MHz, $CDCl_3$) δ 194.46, 172.03, 144.85, 144.40, 141.57, 138.30, 128.92, 128.07, 127.77, 126.4, 86.66, 79.36, 75.88, 74.63, 65.2, 64.72, 40.95, 42.8, 41.19, 40.6, 38.96, 26.86, 20.2, 23.38, 25.1, 11.8, 16.36, 10.66, 10.32.

Synthesis of 12-tigloyl-13-(*S*)-2-methylbutyril-20-trityl phorbol (15): To a solution of tiglic acid (289.8 mg, 2.895 mmol, 20 eq) and diisopropylethylamine (504.26 µL, 2.895 mmol, 20 eq) in toluene (3 mL), 2,4,6-trichlorobenzoyl chloride (452.3 µL, 2.895 mmol, 20 eq) was added. After stirring it overnight, the reaction was filtered and the residue was evaporated *in vacuo*. The crude was re-dissolved in toluene and added dropwise to a pre-mixed solution of 14 (100 mg, 0.148 mmol, 1 eq) and DMAP (17.7 mg, 0.148 mmol, 1 eq) in toluene (2 mL). The reaction was stirred overnight at reflux and, after completion, diluted EtOAc and washed with H_2SO_4 1 N, $NaHCO_3$ sat. sol. and brine, dried over Na_2SO_4 and concentrated *in vacuo.* Purification of the crude was reached by column chromatography on silica gel (pe/EtOAc 8:2) affording **15** (70.4 mg, 0.091 mmol, 63 %) as yellow amorphous powder. TLC (petroleum ether/ethyl acetate 9:1): R_f= 0.57. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.6 (s, 1H), 7.3-7.2 (m, 15 H), 6.82 (dd, *J* = 15.0, 7.0, Hz, 1H), 5.7 (d, *J* = 4.0 Hz, 1H), 5.45 (d, *J* = 10.13 Hz, 1H), 4.5 (br s, 1H), 4.11 (s, 2 H), 3.3 (m, 1 H), 3.25 (m, 1H), 2.7-2.3 (AB, *J* = 16.3 Hz, 2 H), 2.35 (m, 1 H), 2.2 (m, 1 H), 1.90 (s, 3H), 1.85 (d, *J* = 7.0 Hz, 3 H), 1.75 (s, 3 H), 1.4 (m, 2 H), 1.1 (s, 3H), 1.05 (s, 3H),1.0 (s, 3H) 0.95-0.7 (m, 7 H).^{13}C NMR (75 MHz, $CDCl_3$) δ 194.46, 172.03, 167.46, 144.85, 144.40, 141.58, 138.92, 138.30, 128.92, 128.07, 127.77, 127.57, 126.4, 86.66, 79.36,

75.88, 74.7, 65.2, 64.71, 43.3, 41.19, 40.95, 40.6, 38.96, 26.86, 25.1, 23.38, 20.2, 18.33, 16.36, 15.47, 11.8, 10.33.

Synthesis of 12-tigloyl-13-(S)-2-methylbutyril phorbol (16): To a solution of 15 (70 mg, 0.09 mmol, 1 eq) in MeOH (2 mL), an aqueous solution of $HClO_4$ 60 % (3.3 µL, 0.033 mmol, 0.37 eq) was added at room temperature. After stirring overnight at room temperature, the reaction is quenched with sodium acetate to pH 7, dilute with EtOAc and washed with brine. The collected organic phases were dried over Na_2SO_4 and concentrated *in vacuo.* Purification of the crude was reached by column chromatography on silica gel (pe/EtOAc 4:6) affording **16** (46.8 mg, 0.088 mmol, 98%) as pale-yellow amorphous powder. TLC (petroleum ether/ethyl acetate 6:4): R_f= 0.20. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.57 (d, *J* = 2.1 Hz, 1H), 6.82 (qd, *J* = 7.0, 1.7 Hz, 1H), 5.87 (s, 1H), 5.72 – 5.67 (m, 1H), 5.45 (d, *J* = 10.2 Hz, 1H), 5.00 (t, *J* = 1.9 Hz, 0H), 4.93 – 4.86 (m, 1H), 4.08 – 3.89 (m, 3H), 3.27 (d, *J* = 6.3 Hz, 1H), 3.26 – 3.18 (m, 1H), 2.59 (d, *J* = 19.1 Hz, 2H), 2.48 (d, *J* = 19.0 Hz, 1H), 2.41 – 2.27 (m, 1H), 2.17 (dq, *J* = 10.1, 6.5 Hz, 1H), 2.05 (s, 1H), 1.82 (s, 1H), 1.80 – 1.76 (m, 3H), 1.73 (d, *J* = 1.6 Hz, 2H), 1.28 (s, 3H), 1.26 – 1.22 (m, 2H), 1.20 (s, 3H), 1.12 (d, *J* = 7.1 Hz, 4H), 0.92 (t, *J* = 7.5 Hz, 3H), 0.87 (d, *J* = 6.5 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 209.43, 179.28, 167.70, 161.15, 140.65, 137.56, 132.93, 129.42, 128.64, 78.54, 73.83, 68.12, 65.47, 56.20, 43.51, 41.39, 39.12, 38.59, 36.72, 26.30, 26.18, 23.95, 17.15, 16.30, 14.59, 14.54, 12.37, 11.74, 10.22.

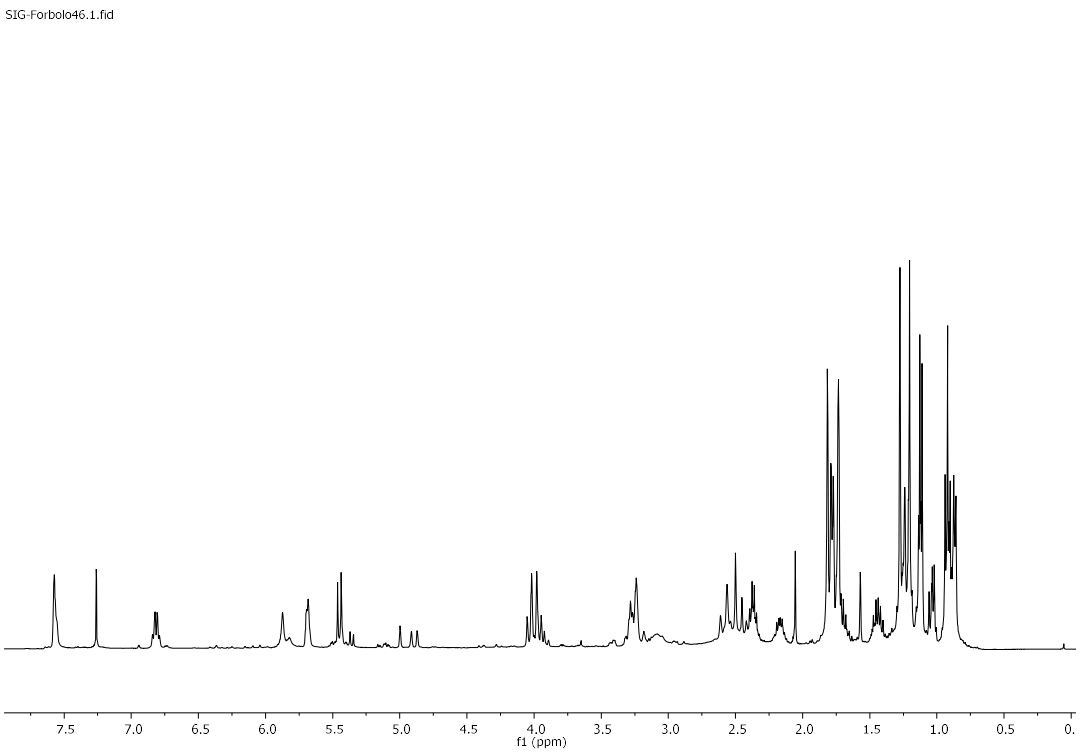

Fig. 1.22 ^{1}H-NMR spectra of **16**

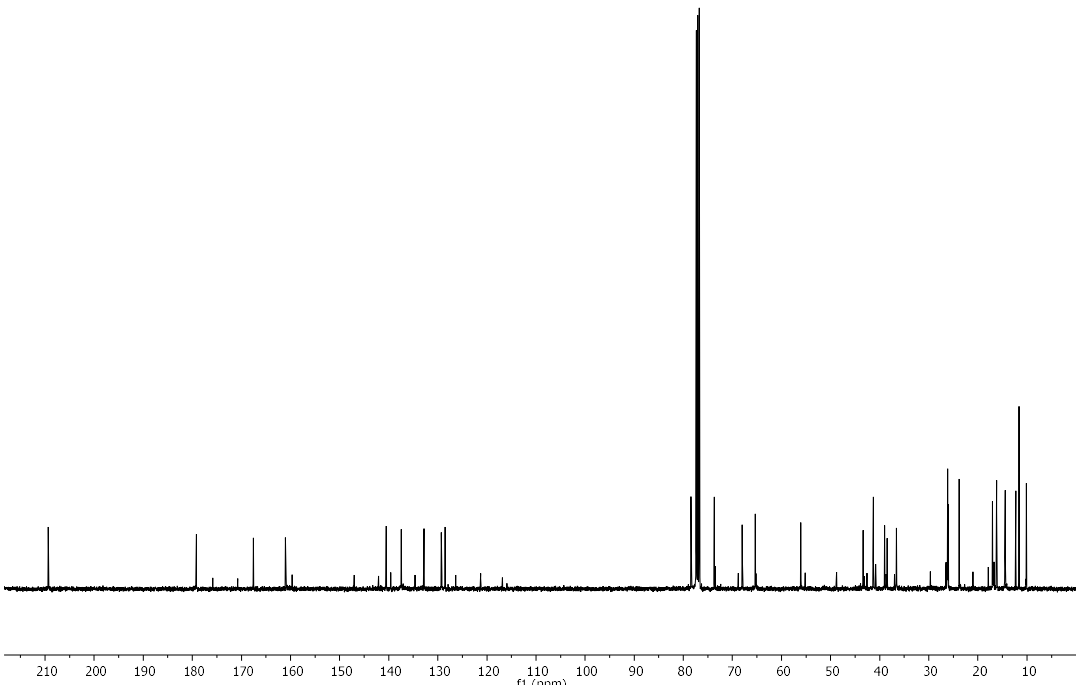

Fig. 1.23 ^{13}C-NMR spectra of **16**

Synthesis of 12,13-diacetyl-20-trityl phorbol (17): To a solution of **13** (500 mg, 0.824 mmol, 1 eq) in pyridine (5 mL), Acetic anhydride (392.9 µL, 4.12 mmol, 5 eq) and a catalytic amount of DMAP were added. After stirring overnight, the reaction is diluted with EtOAc and washed several times with H_2SO_4 1 N. The collected organic phases were dried over Na_2SO_4 and concentrated *in vacuo.* Purification of the crude was reached by column chromatography on silica gel (pe/EtOAc 8:2) affording **17** (307 mg, 0.5356 mmol, 65 %) as a white amorphous powder. TLC (petroleum ether/ethyl acetate 6:4): R_f= 0.76. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.6 (s, 1 H), 7.4-7.35 (m, 6H), 7.3-7.1 (m, 9 H), 5.69 (d, *J* = 5.9 Hz, 1H), 5.37 (d, *J* = 10.3 Hz, 1H), 3.54 (s, 2 H), 3.10 (m, 1H), 3.06 (m, 1H), 2.50-2.15 (AB, J = 19 Hz, 2H), 2.10 (s, 3H), 2.06 (s, 3H), 1.77 (d, J = 1.5 Hz, 3H), 1.23 (s, 3H), 1.17 (s, 3H), 1.05 (d, J = 5.2 Hz, 1H),0.9 (d, *J*= 6.5 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 209.25, 173.68, 171.04, 161.07, 143.98, 138.19, 132.63, 129.49, 128.69, 127.93, 127.92, 127.80, 127.25, 127.00, 86.89, 77.94, 73.83, 69.03, 65.64, 60.42, 56.10, 42.92, 39.25, 39.02, 36.24, 25.71, 23.92, 21.11, 21.08, 21.06, 16.73, 14.38, 14.21, 10.13.

Synthesis of 12,13,20-triacetyl phorbol (18): To a solution of 2 (500 mg, 1.37 mmol, 1 eq) in pyridine (5 mL), Acetic anhydride (653 µL, 6.85 mmol, 5 eq) and a catalytic amount of DMAP were added. After stirring overnight, the reaction is diluted with EtOAc and washed several times with H_2SO_4 1 N. The collected organic phases were dried over Na_2SO_4 and concentrated *in vacuo.* Purification of the crude was reached by column chromatography on silica gel (pe/EtOAc 7:3) affording **18** (556.4 mg, 1.134 mmol, 82 %) as a white amorphous powder. TLC (petroleum ether/ethyl acetate 6:4): R_f= 0.76. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.6 (s, 1 H), 5.7 (m, 1H), 5.25 (d, *J* = 10.3 Hz, 1H), 5.1 (m, 1 H), 4.46-4.43 (AB, *J* =13.0 Hz, 2 H), 2.6-2.42 (AB, J = 19.0 Hz, 2H), 3.2 (m, 1 H), 2.2 (m, 1 H), 2.12 (s, 3H), 2.11 (s, 3H), 2.05 (s, 3H), 1.8 (s, 3H), 1.25 (s, 3H), 1.23 (s, 3H), 0.9 (m, 3H), 0.8 (d, J = 5.2 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 209.1, 174.2, 171.2, 171.4, 161.4, 136.0, 133.3, 132.9, 78.4, 77.2, 69.8, 66.0, 73.9, 56.4, 43.3, 39.6, 39.1, 36.5, 26.1, 24.2, 21.2, 21.4, 17, 14.8, 10.3

General procedure for 12,13-diacetyl-20-formyl phorbol (19): To a solution of SeO_2 (0.5 eq) in DCM, a solution of *tert*-butylhydroperoxide 70 % (2 eq) was added. After 30 minutes, a solution of **17** or **18** in DCM was added dropwise. After stirring overnight at room temperature,

the reaction was diluted with DCM and washed with brine. The collected organic phases were dried over Na_2SO_4 and concentrated *in vacuo.* Purification of the crude was reached by column chromatography on silica gel (pe/EtOAc 8:2) affording **19** (35 % from **17,** 19 % from **18**) as a pale yellow oil. TLC (petroleum ether/ethyl acetate 7:3): R_f= 0.29. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.45 (s, 1H), 7.58 (dd, *J* = 2.5, 1.4 Hz, 1H), 6.73 (dd, *J* = 5.8, 2.3 Hz, 1H), 5.44 (d, *J* = 10.3 Hz, 1H), 3.64 (t, *J* = 5.6 Hz, 1H), 3.08 (q, *J* = 2.3, 1.8 Hz, 1H), 3.00 – 2.87 (m, 1H), 2.49 (d, *J* = 19.7 Hz, 1H), 2.26 – 2.18 (m, 1H), 2.15 (s, 3H), 2.11 (s, 4H), 1.81 (dd, *J* = 2.9, 1.3 Hz, 4H), 1.28 (d, *J* = 4.6 Hz, 11H), 1.26 (dd, *J* = 2.9, 1.0 Hz, 2H), 0.93 (d, *J* = 6.5 Hz, 3H), 0.89 (d, *J* = 9.8 Hz, 1H).^{13}C NMR (101 MHz, $CDCl_3$) δ 208.35, 208.32, 193.58, 174.05, 171.06, 160.16, 156.79, 143.40, 133.65, 78.98, 77.48, 77.16, 76.84, 76.64, 72.83, 65.43, 56.20, 43.26, 41.29, 35.78, 34.49, 32.08, 29.85, 29.51, 26.03, 23.87, 22.84, 21.22, 21.14, 16.76, 14.56, 14.27, 10.24.

Synthesis of 3β-hydroxy-12,13,20-triacetyl phorbol (20): To a solution of **18** (300 mg, 0.611 mmol, 1 eq) in MeOH (3 mL), $CeCl_3$*$7H_2O$ (227.6 mg, 0.611 mmol, 1 eq) and NaBH4 (81 mg, 2.14 mmol, 3.5 eq) were added. After stirring for 7 minutes, the reaction was diluted with $CHCl_3$ and washed with NH_4Cl sat. sol and brine. The collected organic phases were dried over Na_2SO_4 and concentrated *in vacuo.* Purification of the crude was reached by column chromatography on silica gel (pe/EtOAc 8:2) affording **20** (225.3 mg, 0.457 mmol, 75 %) as a colourless oil. TLC (petroleum ether/ethyl acetate 6:4): R_f= 0.40. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.71 (d, *J* = 1.9 Hz, 1H), 5.37 (d, *J* = 10.4 Hz, 1H), 4.58 (d, *J* = 1.1 Hz, 1H), 4.47 (q, *J* = 12.5 Hz, 2H), 4.12 (q, *J* = 7.1 Hz, 2H), 3.09 (s, 1H), 2.90 (t, *J* = 5.7 Hz, 1H), 2.82 – 2.73 (m, 1H), 2.60 (d, *J* = 17.9 Hz, 1H), 2.17 (s, 0H), 2.09 (s, 2H), 2.08 (s, 2H), 2.07 (s, 2H), 2.04 (s, 2H), 1.74 – 1.69 (m, 2H), 1.59 (s, 1H), 1.44 (s, 2H), 1.28 (s, 2H), 1.26 (s, 1H), 1.24 (s, 1H), 1.23 (s, 2H), 1.21 (s, 3H), 1.02 (d, *J* = 4.8 Hz, 1H), 0.92 (d, *J* = 6.4 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.54, 171.08, 170.84, 135.08, 133.68, 132.67, 129.80, 96.10, 88.59, 78.09, 77.23, 68.94, 65.38, 60.42, 58.13, 43.05, 42.84, 39.93, 35.92, 29.44, 28.46, 25.44, 23.91, 21.14, 21.04, 16.44, 14.25, 14.23, 13.48.

Synthesis of 12,13,20-triacetyl phorbol-3,4-acetonide (21): To a solution of 20 (146.5 mg, 0.2976 mmol, 1 eq) in a mixture of acetone and 2,2-dimethoxypropane (2:1 ratio, 1.5 mL:0.75

mL), pyridinium-*p*-toluene sulfonate was added. After stirring overnight at 50 °C, the reaction was diluted with $CHCl_3$ and washed with brine. The collected organic phases were dried over Na_2SO_4 and concentrated *in vacuo.* Purification of the crude was reached by column chromatography on silica gel (pe/EtOAc 8:2) affording **21** (73 mg, 0.457 mmol, 46 %) as a yellow oil. TLC (petroleum ether/ethyl acetate 6:4): R_f= 0.72. ^{1}H NMR (400 MHz, $CDCl_3$)δ 5.76 – 5.71 (m, 1H), 5.37 (d, *J* = 10.4 Hz, 2H), 4.62 (d, *J* = 1.4 Hz, 1H), 4.06 (d, *J* = 4.0 Hz, 2H), 3.36 (dt, *J* = 3.4, 1.7 Hz, 1H), 3.26 (d, *J* = 8.9 Hz, 1H), 2.72 (d, *J* = 16.3 Hz, 1H), 2.09 (s, 5H), 2.09 (s, 5H), 2.04 (s, 5H), 1.71 (dd, *J* = 2.8, 1.3 Hz, 5H), 1.44 (s, 1H), 1.37 (s, 4H), 1.27 (s, 1H), 1.24 (s, 10H), 1.21 (s, 2H), 1.19 (s, 4H), 0.92 (d, *J* = 5.9 Hz, 2H), 0.88 (d, *J* = 6.4 Hz, 5H).^{13}C NMR (101 MHz, $CDCl_3$) δ 173.54, 171.08, 170.84, 135.08, 133.68, 132.67, 129.80, 111.15, 96.10, 88.59, 78.09, 77.23, 68.94, 65.38, 60.42, 58.13, 43.05, 42.84, 39.93, 35.92, 29.44, 28.46, 25.44, 23.91, 21.14, 21.08, 21.04, 16.44, 14.25, 14.23, 13.48.

Synthesis of 22a and 22b: To a solution of **21** (250 mg, 0.456 mmol, 1 eq) in Benzene (3 mL), PCC (884.65 mg, 4.104 mmol, 9 eq) and sodium acetate (224.43 mg, 2.736 mmol, 6 eq) were added at room temperature. After stirring it for 1 h at 80 °C, the reaction was worked by by filtration on celite with toluene. The residue was dried under *vacuo* and purified by gravity column chromatography on silica gel (pe/EtOAc 8:2) affording **22a** (45 mg, 0.08 mmol, 18 %) and (pe/EtOAc 7:3) **22b** (125 mg, 0.216 mmol, 47%) both as colourless oil. TLC (petroleum ether/ethyl acetate 7:3): R_f(**22a**)= 0.43, R_f(**22b**)= 0.31.

22a) ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.74 (q, *J*= 1.5 Hz, 1H), 6.01 (d, *J* = 1.6 Hz, 1H), 5.78 (d, J = 10.2 Hz, 1 H), 4.80 (s, 1 H), 4.65 (s, 2 H), 3.32 (d, J = 9.85 Hz, 1 H), 2.98-2.51 (ABs, J = 14.89 Hz, 2 H), 2.28 (s, 3 H), 2.09 (s, 3 H), 2.08 (s, 3 H), 1.81 (d, J = 1.26 Hz, 3 H), 1.43 (s, 3 H), 1.26 (s, 3 H), 1.23 (s, 3 H), 0.87 (s, 3 H). ^{13}C-NMR (101 MHz, $CDCl_3$): *δ* 205.50, 195.19, 175.17, 171.22, 170.79, 142.16, 132.86, 132.71, 129.78, 129.33, 114.04, 86.19, 84.25, 67.86, 62.52, 42.65, 33.37, 30.55, 30.46, 28.61, 28.24, 22.07, 21.12, 21.10, 17.47, 16.19, 15.21.

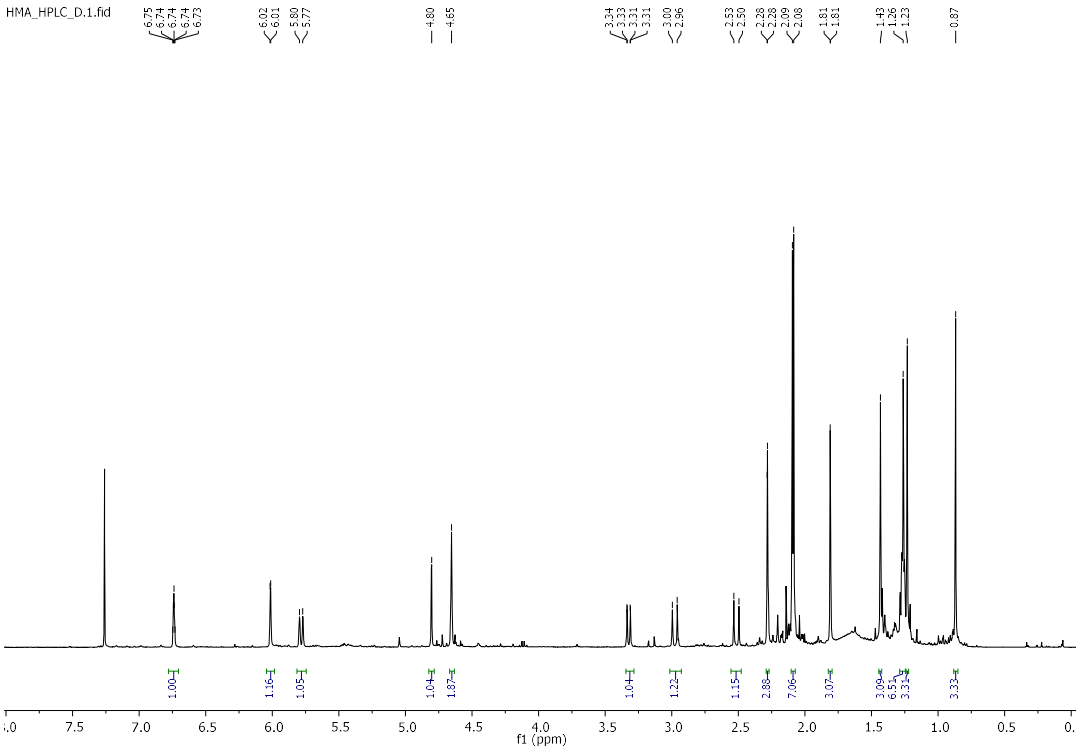

Fig. 1.24 ^{1}H-NMR spectra of **22a**

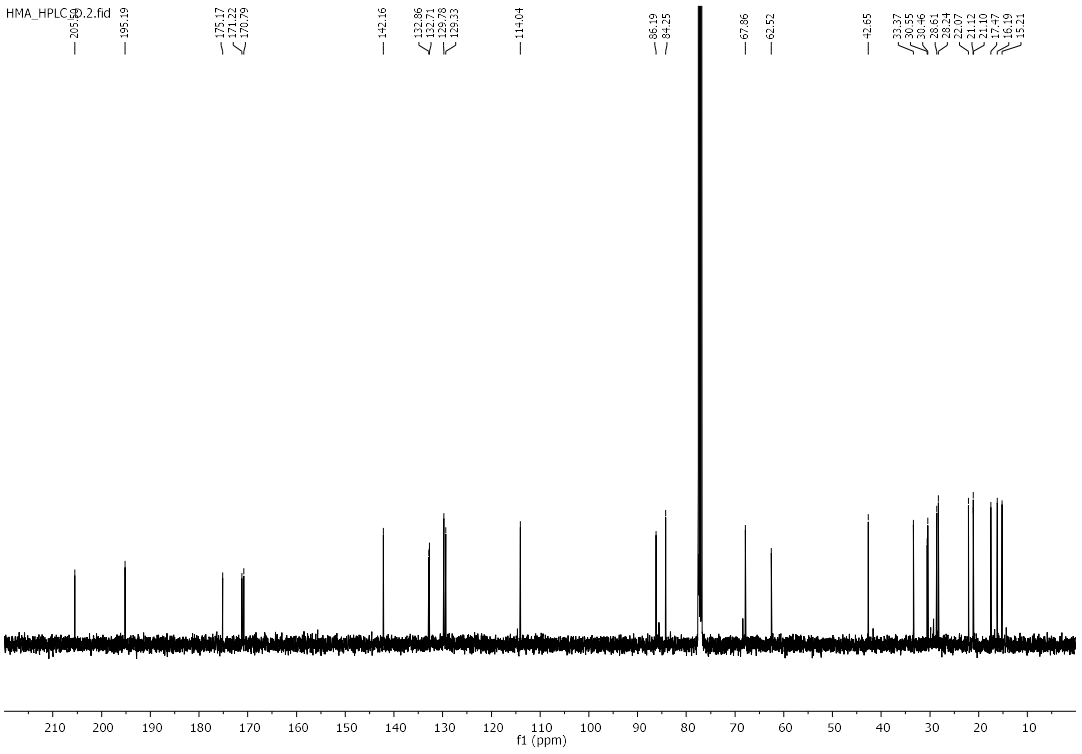

Fig. 1.25 ^{13}C-NMR spectra of **22a**

22b) ^{1}H NMR (400 MHz, $CDCl_3$) δ ^{13}C-NMR (101 MHz, $CDCl_3$): *δ* 5.67 (s, 1H), 5.37 (d, *J* = 10 Hz, 1 H), 4.80 (s, 1 H), 4.32-4.23 (ABs, *J* = 11.60 Hz, 2 H), 3.02 (d, *J* = 5.30 Hz, 1 H), 2.66-2.23 (ABs, *J* = 14.12 Hz, 2 H), 2.01 (s, 3 H), 2.00 (s, 3 H), 1.98 (s, 3 H), 1.77 (s, 3 H), 1.72 (d, *J* = 4.79, 1 H), 1.33 (s, 3 H), 1.16 (s, 3 H), 1.12 (s, 3 H), 0.82 (d, *J* = 6.31 Hz, 3 H). ^{13}C-NMR (101 MHz, $CDCl_3$): *δ* 202.28, 172.98, 171.18, 170.72, 143.28, 127.45, 113.44, 97.74, 95.53, 94.83, 85.48, 81.15, 76.48, 65.40, 63.05, 48.41, 44.41, 41.68, 27.17, 27.11, 26.94, 23.51, 21.27, 21.12, 21.03, 16.31, 14.53, 13.16.

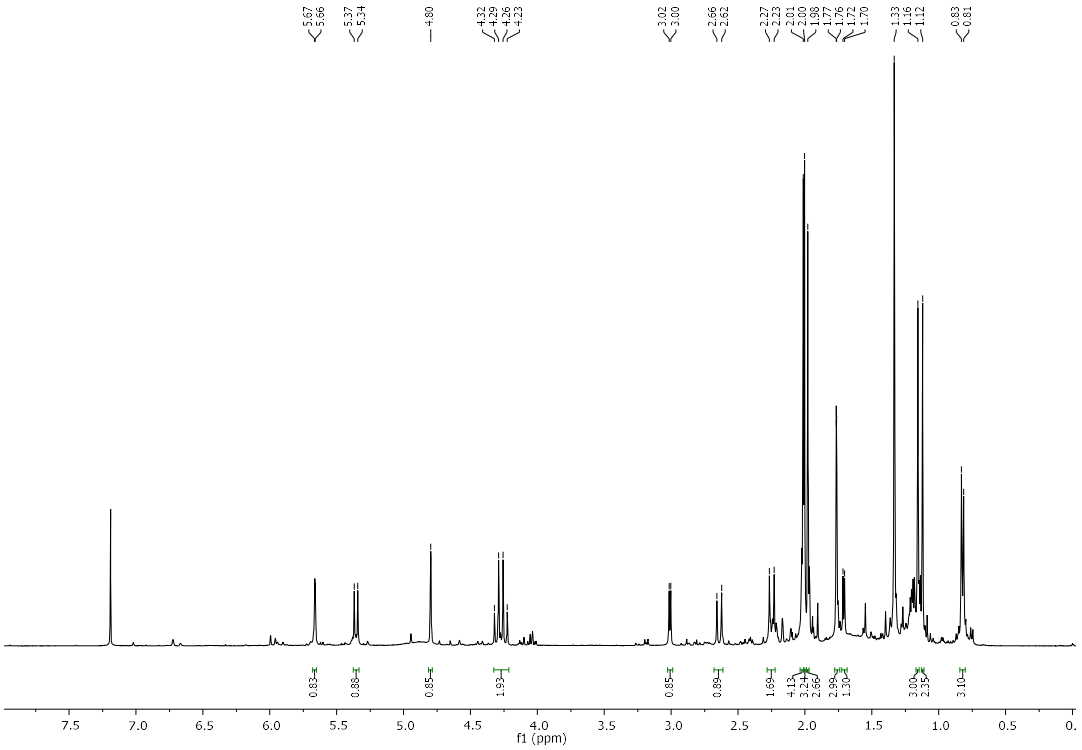

Fig. 1.26 ^{1}H-NMR spectra of **22b**

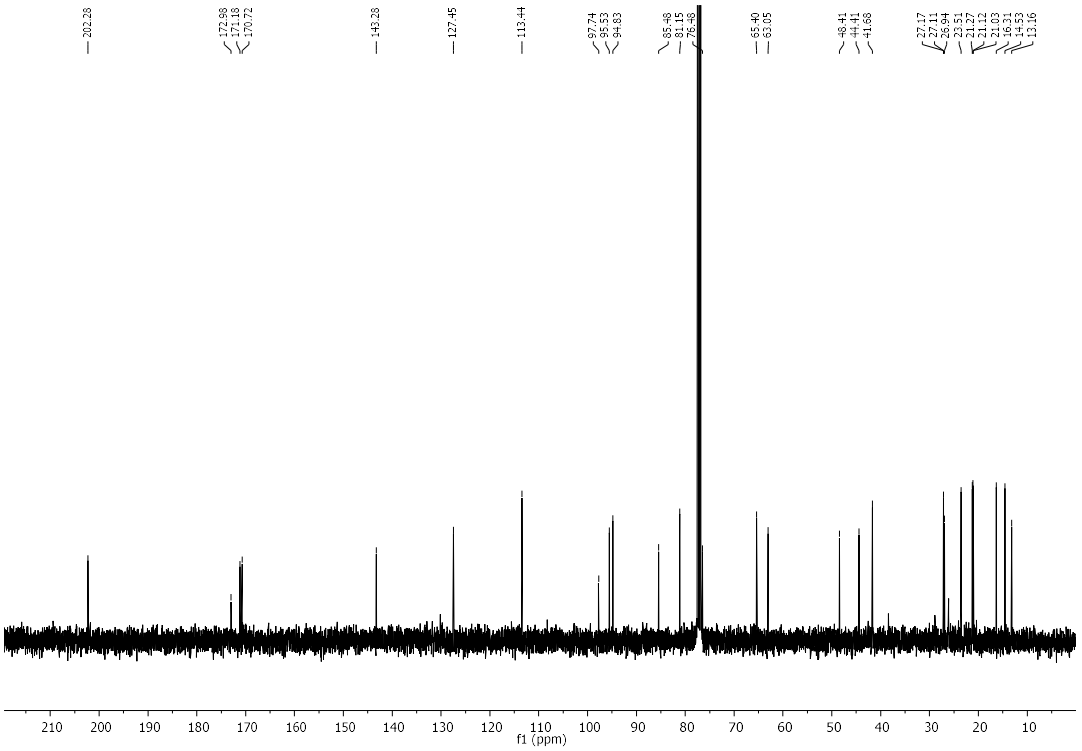

Fig. 1.27 ^{13}C-NMR spectra of **22b**

Synthesis of 12,13-dihexanoyl- 5β-hydroxy-6,7α-epoxy phorbol-5,20-acetonide (23): To a solution of hexanoic acid (2.05 mL, 16.41 mmol, 5 eq) in DCM (15 mL), EDC (16.41 mmol, 5 eq) and DMAP were added at room temperature. When EDC was completely dissolved, a solution of 12 (1.43 g, 3.28 mmol, 1 eq) in DCM (5 mL) was added dropwise. After stirring it overnight, the reaction was diluted with brine and extracted three times with DCM. The combined organic layers were dried over Na_2SO_4. After evaporation, the residue was purified by gravity column chromatography on silica gel (pe/EtOAc 8:2) affording 24 (1.65 g, 2.60 mmol, 79 %) as white amorphous powder. TLC (petroleum ether/ethyl acetate 6:4): R_f= 0.84.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 (dd, *J* = 2.7, 1.5 Hz, 1H), 5.72 (s, 1H), 5.35 (d, *J* = 10.0 Hz, 1H), 4.09 (s, 1H), 3.99 (t, *J* = 2.8 Hz, 1H), 3.92 (d, *J* = 12.9 Hz, 1H), 3.56 (d, *J* = 12.9 Hz, 1H), 3.39 (s, 1H), 3.12 (d, *J* = 6.6 Hz, 1H), 2.96 (d, *J* = 1.1 Hz, 1H), 2.37 – 2.20 (m, 4H), 1.87 (dq, *J* = 9.9, 6.4 Hz, 1H), 1.72 (dd, *J* = 2.9, 1.3 Hz, 3H), 1.59 (pd, *J* = 7.4, 3.8 Hz, 4H), 1.46 (s, 3H), 1.43 (s, 3H), 1.32 – 1.24 (m, 8H), 1.22 (s, 3H), 1.20 (s, 3H), 0.86 (td, *J* = 6.9, 2.7

Hz, 6H), 0.80 (d, *J* = 6.5 Hz, 3H). ^{13}C-NMR (101 MHz, $CDCl_3$): *δ* 206.18 (C_q), 176.11 (C_q), 173.51 (C_q), 161.47 (CH), 133.93 (C_q), 101.14 (C_q), 76.88 (C_q), 76.70 (CH), 72.70 (C_q), 68.56 (CH), 65.71 (C_q), 65.65 (CH), 65.41 (CH_2), 60.26 (C_q), 49.20 (CH), 45.46 (CH), 36.28 (CH), 35.62 (CH), 34.55 (CH_2), 34.27 (CH_2), 31.23 (CH_2), 31.21 (CH_2), 26.37 (C_q), 24.94 (CH_2), 24.91 (CH_2), 24.22 (CH_3), 23.80 (CH_3), 22.38 (CH_2), 22.35 (CH_2), 22.19 (CH_3), 17.17 (CH_3), 15.02 (CH_3), 14.01 (CH_3), 13.93 (CH_3), 10.03 (CH_3).

Synthesis of 12,13-dihexanoyl-5β-hydroxy-6α-hydroxy-7β-iodo phorbol-5,20-acetonide (24): In a flamed round-bottomed flask iodine (176.39 mg, 1.39 mmol, 1 eq) was dissolved in dry DCM (2.5 mL) under nitrogen. After complete dissolution of iodine, triphenylphosphine (364.58 mg, 1.39 mmol, 1 eq) was added. The reaction colour turns from deep purple to yellow. After stirring at room temperature for 5 minutes, a solution of 23 (879 mg, 1.39 mmol, 1 eq) in DCM (10 mL) was added. The reaction was stirred overnight at room temperature, turning from yellow-orange to colorless. After completion, it was quenched by MeOH, dilute with DCM and washed with a saturated aqueous solution of $Na_2S_2O_3$ (x3) and brine (x1). The combined organic phases were dried over Na_2SO_4. Purification of the crude was reached by column chromatography on silica gel (pe/EtOAc 8:2) affording **24** (927.5 mg, 1.22 mmol, 88 %) as white amorphous powder. TLC (petroleum ether/ethyl acetate 8:2): R_f= 0.5. TLC control (petroleum ether/ethyl acetate 8:2): R_f= 0.34.^{1}H NMR (400 MHz, C_2D_6CO) δ 7.63 (dd, *J* = 2.4, 1.4 Hz, 1H), 5.41 (s, 1H), 5.28 (d, *J* = 2.9 Hz, 1H), 5.25 (d, *J* = 10.6 Hz, 1H), 5.19 (d, *J* = 0.9 Hz, 1H), 5.11 (d, *J* = 8.6 Hz, 1H), 4.48 (d, *J* = 8.3 Hz, 1H), 3.94 (d, *J* = 2.9 Hz, 1H), 3.76 (d, *J* = 8.3 Hz, 1H), 3.69 – 3.65 (m, 1H), 2.29 – 2.18 (m, 4H), 2.18 – 2.10 (m, 1H), 2.05 (dq, *J* = 10.5, 6.5 Hz, 1H), 1.92 (p, *J* = 2.2 Hz, 3H), 1.68 (d, *J* = 5.9 Hz, 1H), 1.61 (dd, *J* = 2.8, 1.4 Hz, 3H), 1.54 – 1.46 (m, 4H), 1.44 (s, 3H), 1.26 (s, 3H), 1.23 – 1.19 (m, 10H), 1.18 (s, 4H), 1.13 (s, 3H), 0.77 (t, *J* = 7.4 Hz, 9H), 0.74 (d, *J* = 6.7 Hz, 4H).^{13}C NMR (101 MHz, C_2D_6CO) δ 208.24, 176.11, 173.02, 161.82, 133.22, 111.69, 88.98, 76.42, 75.68, 74.62, 73.79, 66.26, 52.12, 46.67, 44.65, 44.34, 42.59, 33.96, 33.91, 30.97, 30.93, 27.49, 27.44, 26.09, 24.70, 24.16, 22.33, 22.13, 22.10, 16.32, 14.25, 13.37, 13.31, 9.15.

Synthesis of 12,13-dihexanoyl-5β-hydroxy-6α-hydroxy-7β-iodo phorbol (25): To a solution of 24 (100 mg, 0.131 mmol, 1 eq) in ACN:H_2O (2,5 mL), an aqueous solution of $HClO_4$ 60 % (3 µL, 0.05 mmol, 0.37 eq) was added at room temperature. After 4 h a white precipitate is formed, the reaction is filtered on sintered glass funnel and the filtrate was washed with H_2O, affording 25 (83.1 mg, 0.115 mmol, 88 %) as white amorphous powder. TLC (petroleum ether/ethyl acetate 6:4): R_f= 0.3. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.72 (s, 1H), 5.90 (s, 1H), 5.40 (d, *J* = 9.0 Hz, 1H), 5.25 (d, *J* = 10.1 Hz, 1H), 4.45 (d, *J* = 11.1 Hz, 1H), 3.83 (s, 1H), 3.78 (d, *J* = 11.1 Hz, 1H), 3.13 – 3.07 (m, 1H), 2.26 (dtd, *J* = 13.2, 7.5, 2.7 Hz, 5H), 2.02 – 1.91 (m, 2H), 1.80 (d, *J* = 6.2 Hz, 1H), 1.74 (t, *J* = 1.8 Hz, 3H), 1.56 (td, *J* = 7.4, 2.8 Hz, 4H), 1.25 (dt, *J* = 5.7, 1.9 Hz, 5H), 1.23 (s, 5H), 1.13 (s, 3H), 0.83 (td, *J* = 6.9, 2.5 Hz, 7H), 0.78 (d, *J* = 6.5 Hz, 3H).^{13}C NMR (101 MHz, $CDCl_3$) δ 209.58, 176.75, 173.58, 164.18, 134.48, 79.67, 77.89, 77.36, 76.18, 75.84, 73.63, 67.03, 66.70, 52.75, 52.34, 45.53, 45.04, 39.21, 34.59, 34.44, 31.30, 31.28, 26.62, 24.98, 24.31, 22.78, 22.46, 22.42, 17.33, 15.15, 14.09, 14.03, 10.03.

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Part II: Biosynthetic studies on tigliane skeleton

2. Terpenes

Terpenoids are the largest class of secondary metabolites isolated in living organisms. They take their name from the German word *Terpentin* (turpentine in English), from which they were first isolated, and thanks to the extensive efforts of Otto Wallach (Nobel prize, 1910) and Leopold Ruzicka (Nobel prize, 1989) we are now able to rationalize terpenes scaffolds under the flag of the *Biogenetic Isoprene Rule*. [**1**] After isolation of isoprene in 1961 by Sanadze and Dolidze as a decomposition product, the term *hemiterpenes* came into use to refer at the active biological precursors isopentenyl pyrophosphate (IPP) and its isomer dimethylallylpyrophosphate (DMAPP). [**2**] Condensation of the C-5 units commonly occur in a head-to-tail way, generating C-10 (monoterpenes), C-15 (sesquiterpenes), C-20 (diterpenes), C-25 (sesterpenes), C-30 (triterpenes), C-35 (sesquarterpenes), C-40 (tetraterpenes). By the way, in the last decade, several enzymes belonging to the class of prenyl diphosphate synthases rationalize complex pattern in higher terpenoids that cannot be explained following a simple head-to-tail condensation. These "irregular terpenoids" are quite rare in nature and often specific for certain genera or species and for this reason they can be used as chemo-taxonomical marker to identify unknown species. Generation of the C-5 units in plants can be achieved through two different pathways: a cytosolic one known as **mevalonate pathway** (or HMG-CoA reductase pathway) common in eukaryotes, archaea and some bacteria and a plastidic pathway called mevalonate-independent pathway or **2-*C*-methyl-d-erythritol-4-phosphate/1-deoxy-d-xylulose-5-phosphate pathway (MEP/DOXP)**.

2.1 Mevalonic acid pathway

Mevalonate pathway (MVA) represents a series of enzymatic reactions that occurs in fungi, plant cytoplasm, animals, most eukaryotes, archaea and some eubacteria to obtain isoprenoids from the activated acetyl-coenzyme A (**1**, Acetyl-CoA). The acetyl-coA thiolase (AACT) catalyses the extension of two units of acetyl-CoA through a Claisen condensation followed by a decarboxylative step to obtain acetoacetyl-coA (**2**). A third molecule of acetyl-

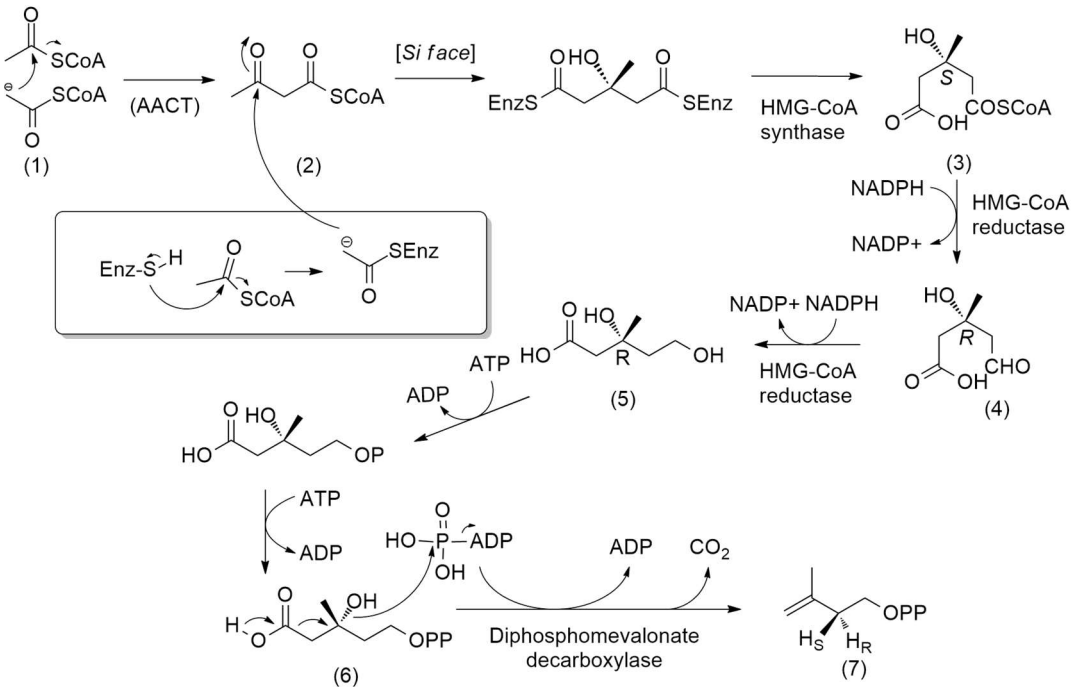
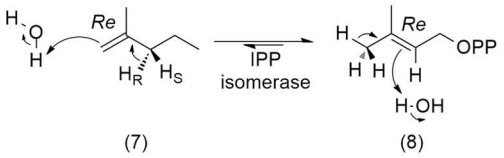


Fig. 2.1 Mevalonic acid pathway to IPP

coA reacts with the enzyme itself to enhance the aldol condensation with acetoacetyl-coA that, after hydrolysis by HMG-CoA synthase, release (*S*)-β-hydroxy-β-methylglutaryl coenzyme A (**3**, HMG-CoA). The sequential reductions of the thioester group by HMG-CoA reductase produces the aldehydic derivative (**4**) followed by the primary alcohol one (**5**), consuming two molecules of NADPH. The final product, (*R*)-(+)-mevalonic acid, is then pyrophosphorylated at C-5 hydroxy group by two ATP to produce mevalonic acid 5-pyrophosphate (**6**, MVAA) in two steps. The further decarboxylative dehydration step catalysed by diphosphomevalonate decarboxylase is promoted by phosphorylation at the tertiary antiperiplanar hydroxy group to yield the final isopentenyl pyrophosphate (**7**, IPP). (**Fig. 2.1**)

Isomerization of IPP is a process that requires IPP isomerase and water to catalyse the antarafacial 1,3-allylic rearrangement to produce γ,γ-dimethylallyl pyrophosphate (DMAPP). [**Fig. 2.2**]



(7) (8)

Fig. 2.2 Isomerization of IPP to DMAPP

2.2 Mevalonate-independent pathway: 2-*C*-methyl-D-erythritol-4-phosphate (MEP)

Toward the end of the 1980s, Rohmer and co-workers speculated a possible mevalonate-independent pathway for isoprenoids biosynthesis after feeding experiment with labelled acetate, glucose, pyruvate and erythrose. Further investigations unearthed the occurrence of this pathway in bacteria and chloroplasts of green algae and higher plants. [**4,5**]

Glyceraldehyde-3-phosphate (**9**) and pyruvic acid (**10**), intermediates mainly produced by glycolysis, are condensed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS) to yield 1-deoxy-D-xylulose-5-phosphate (**11**, DXP). In order to address the proper conversion of DXP by IspC reductoisomerase into 2-C-methyl-D-erythritol-4-phosphate (**12**), two different mechanisms were postulated: the first one is a retroaldol-aldol rearrangement that produces a molecule of glycolaldehyde phosphate and 1,2-dihydroxy-1-propene as intermediates, while the second one is α-ketol rearrangements. Both the pathways consume a molecule of NADPH. Compound **12** is cytidinylated by CTP through the activity of IspD, phosphorylated at the tertiary alcohol and cyclize to produce a 2,4-cyclodiphosphate with the elimination of CMP. IspG promotes the removal of the phosphates affording 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate (**13**), which is later converted in IPP (**7**). (**Fig. 2.3**)

Despite the notable importance of IPP isomerase to convert IPP to DMAPP, the production of the primary hemiterpenes can proceed independently via the resonance stabilized allylic carbocation of **13**.

Thanks to ^{13}C-D-glucose experiments, it was possible to prove that the two distinct pathways have different cellular localization: mevalonate pathway occurs in cytoplasm and it is involved in the biosynthesis of sesquiterpenes, triterpenes and sterols, while DXP pathway takes place

in chloroplast (plastids) and it is focused on the biosynthesis of monoterpenes and diterpenes. [**3**]

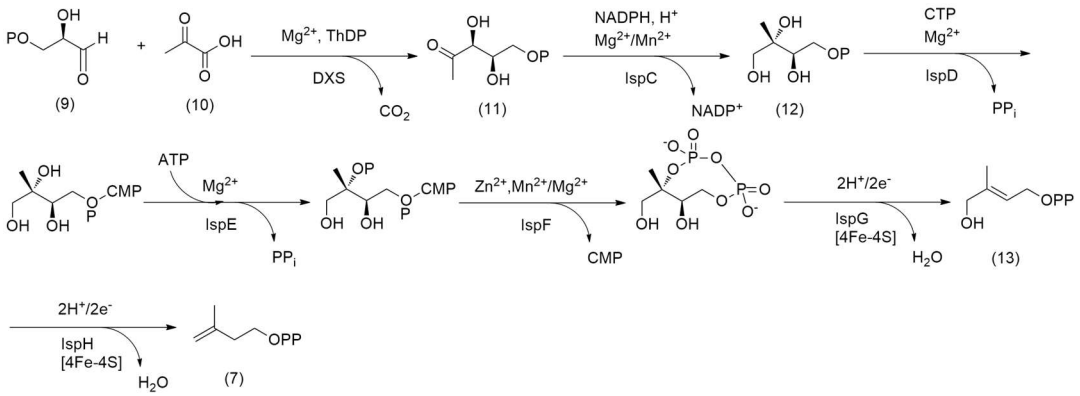


Fig. 2.3 MEP pathway

Even if the gene pool is separated inside the cell environment, IPP, DMAPP and other linear precursors of isoprenoids biosynthesis can be exchanged according to the "Cross-talk theory" and this could be an explanation of the late discovery of DXP pathway in feeding experiments. [**6**]

2.3 Isoprenoid elongation: Diterpenes

Isoprenoid polymerization proceeds through the activity of a class of enzyme called *prenyltransferase* which promotes the ionization of the allyl pyrophosphate group in DMAPP (**7**) by means of Mg^{2+} ion in the catalytic site. The electrophilic attack of IPP exomethylene C-3,4 to the DMA^{+} carbocation occurs from the *Re* face (β) of the double bond with a final *pro-R* proton elimination from the opposite face (α). Thus, the final product geranyl pyrophosphate (**14**) possess an *E* configuration at the newly formed double bond. (**Fig. 2.4 a**) The iteration of the process with four units of IPP (DMAPP) produces a molecule of (*E,E,E*)-geranylgeranyl pyrophosphate (GGPP, **19**), the linear precursor in the biosynthesis of diterpenes. Particularly noteworthy is the fact that the reaction proceeds through a *head-to-tail* propagation where as

the *head* is going to be considered the isopropylene group of DMAPP and as *tail* the carbon atom carrying the pyrophosphate group. [3]

Most isoprenoids follow the *head-to-tail* propagation, however other polymerizations have been observed in nature from the reaction of IPP and DMAPP or their higher homologues. For example, lavandulol (**15**) and chrysantemic acid (**16**), two monoterpenoids that occur, respectively, in lavender essential oil and *Chrysantemum cinerariaefolium* (Trevir.) Vis extract, are produced from a *head-to-middle* condensation. [**7,8**] Anisotomenoic acid (**17**) and anisotomenol (**18**) (from *Anisotome* spp) are produced by coupling of two units of geranyl pyrophosphate in a *head-to-head* fashion. [9]

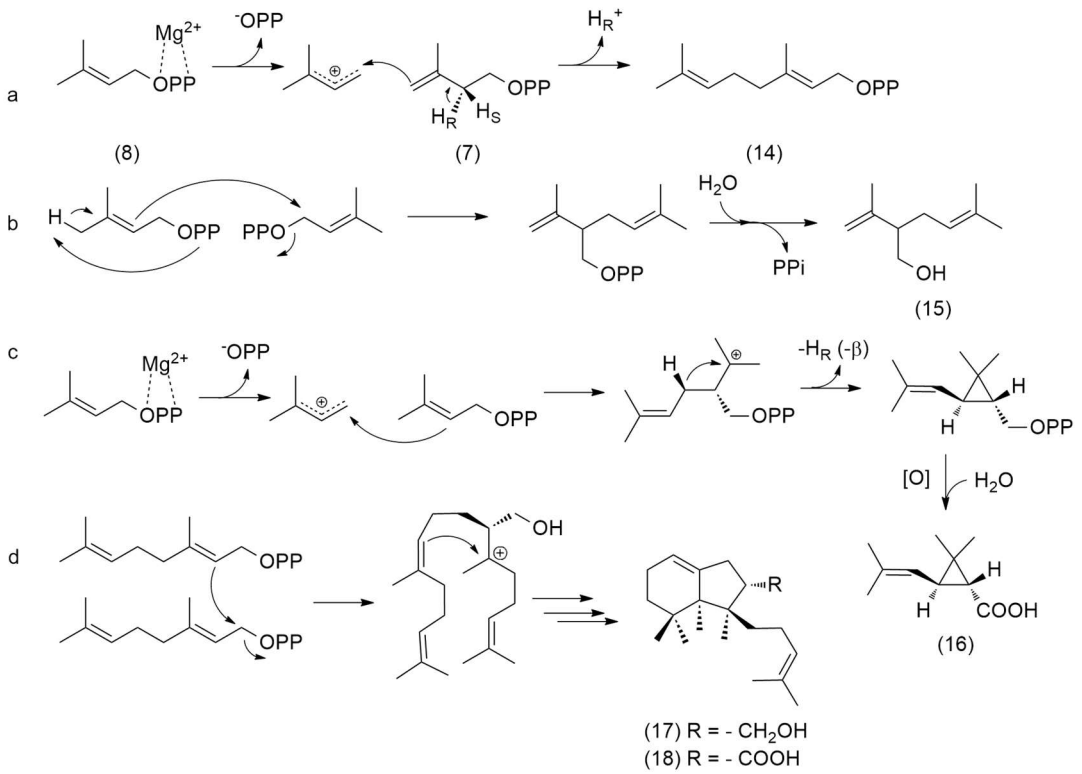


Fig. 2.4 Examples of propagation: a) *head-to-tail* to produce GPP b) *head-to-middle* for LPP and lavandulol c) *head-to-middle* in chrysanthemic acid d) *head-to-head* in anisotomenes

Diterpenoid structural diversity arises from cascade reactions of the tetraprenyl precursor **19** catalyzed by a class of enzyme known as terpene cyclases. These enzymes can be broadly divided into two

categories according to the chemistry that triggers the initial carbocation formation. Type I cyclases are metal-dependent enzymes that catalyze the formation of the allylic carbocation at C-1 by leaving of the terminal phosphate group. On the other side, class II cyclases induce the protonation at C-C double bond at the isopropylidene group producing a tertiary carbocation. The latter interacts with remaining π orbital to generate macrocyclic diterpenoid. [10]

Euphorbiaceae family are excellent producers of macrocyclic and polycyclic diterpenoids with complex frameworks. As shown in **Fig. 2.5**, cyclization of GGPP (**19**) by class II terpenes cyclases produces abietanes (**20**), atisanes (**21**), pimaranes (**22**), kauranes (**23**), and trachylobanes (**24**) while the same process catalyzed by class I enzymes fall to, fo cembranes (**25**), casbanes (**26**) and jatrophanes (**27**).

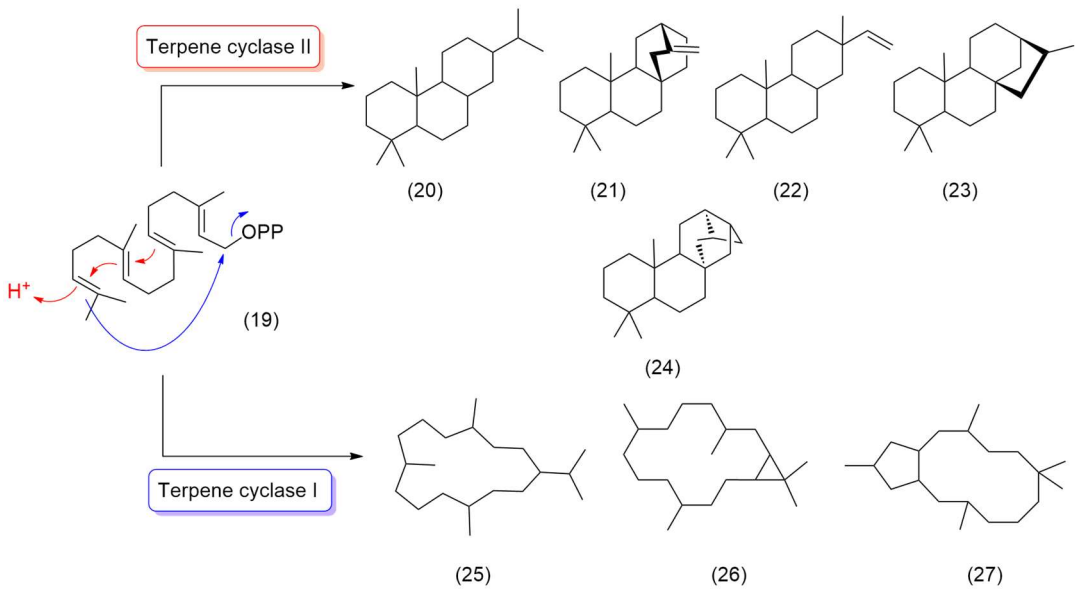


Fig. 2.5 Examples of cyclization promoted by terpene cyclase I (blue) and terpene cyclase II (red)

2.4 Topological biogenesis for tigliane diterpenoids

Tigliane diterpenoids biosynthesis represents one of the most intriguing “black-box” in natural products chemistry. From a structural point of view, phorboids are polycyclic diterpenoids but, according to the previous described enzymatic reactions, the early stages aimed to rationalize the biosynthesis can be easily connected to a type I cyclization. By a topological standpoint,

GGPP (19) cyclization mediated by the enzyme casbene synthase bring to casbene (**27**), a bicyclic diterpenoid [12.1.0] isolated from a cell-free extract of *Ricinus communis* exposed to fungi. Further ring closures between C-6 and C-10 (casbene numbering) foster the synthesis of the lathyrane skeleton (formerly lathyrene, **27**). By lathyranes, it would be possible to achieve the jatrophane frame by the opening at the cyclopropane ring coming back to a bicyclic system. On the other side, closure at C-8 and C-14 (lathyrane numbering) represents the keystone to produce the tigliane skeleton (**29**). Breaking the cyclopropane ring might afford to rhamnopholanes (**31**) and daphnanes (**32**) while a ring expansion at ring C could rationalize the biosynthesis of ingenanes (30). (**Fig. 2.6**)

Despite the easy way to interpret the carbon-carbon connectivity between phorboids, several mechanical issues lay a shadow on the derivation of tiglianes from lathyranes:

1) **Occurrence**: Tigliane diterpenoids are well spread among Euphorbiaceae and Thymelaceae families while the isolation of lathyranes is ascribed up to now to few species of genera Euphorbia and Jatropha. If tiglianes should derive from lathyranes, the occurrence of the seconds' ones should be more marked.

2) **Hydroxylation at C-13:** All natural tiglianes are characterized by a hydroxy group on the cyclopropane ring (C-13, tiglianes numbering) as well as ingenane while no lathyranes isolated so far contain this functionalization as shown in phorbol (**2**) and lathyrol (**33**). Carbon hydroxylation in a biological system is an iron radical-mediated process that would afford a cyclopropylalkyl radical instead of a cyclopropyl radical. The instability of the radical would, therefore, generate a 3-butenyl radical with, eventually, an opening of the cyclopropane ring.

3) **Functional group displacement**: The closure keystone between C-8 and C-14 (lathyrane numbering) would require a previous functionalization at the lathyrane skeleton such as to rationalize the mechanism in term of nucleophilic/electrophilic properties. Again, lathyranes isolated so far contain generally a carbonyl group at C-14 and either a hydroxy group, electrophilic or a series of unfunctionalized methylenes. Moreover, most of them show an (*E*)-double bond between C-12 and C-13 that, besides preventing spatial interaction between the substituents at C-8, C-14, should undergo to isomerization in tiglianes since (*E*)-double bond can exist in eight-membered or higher systems. [12]

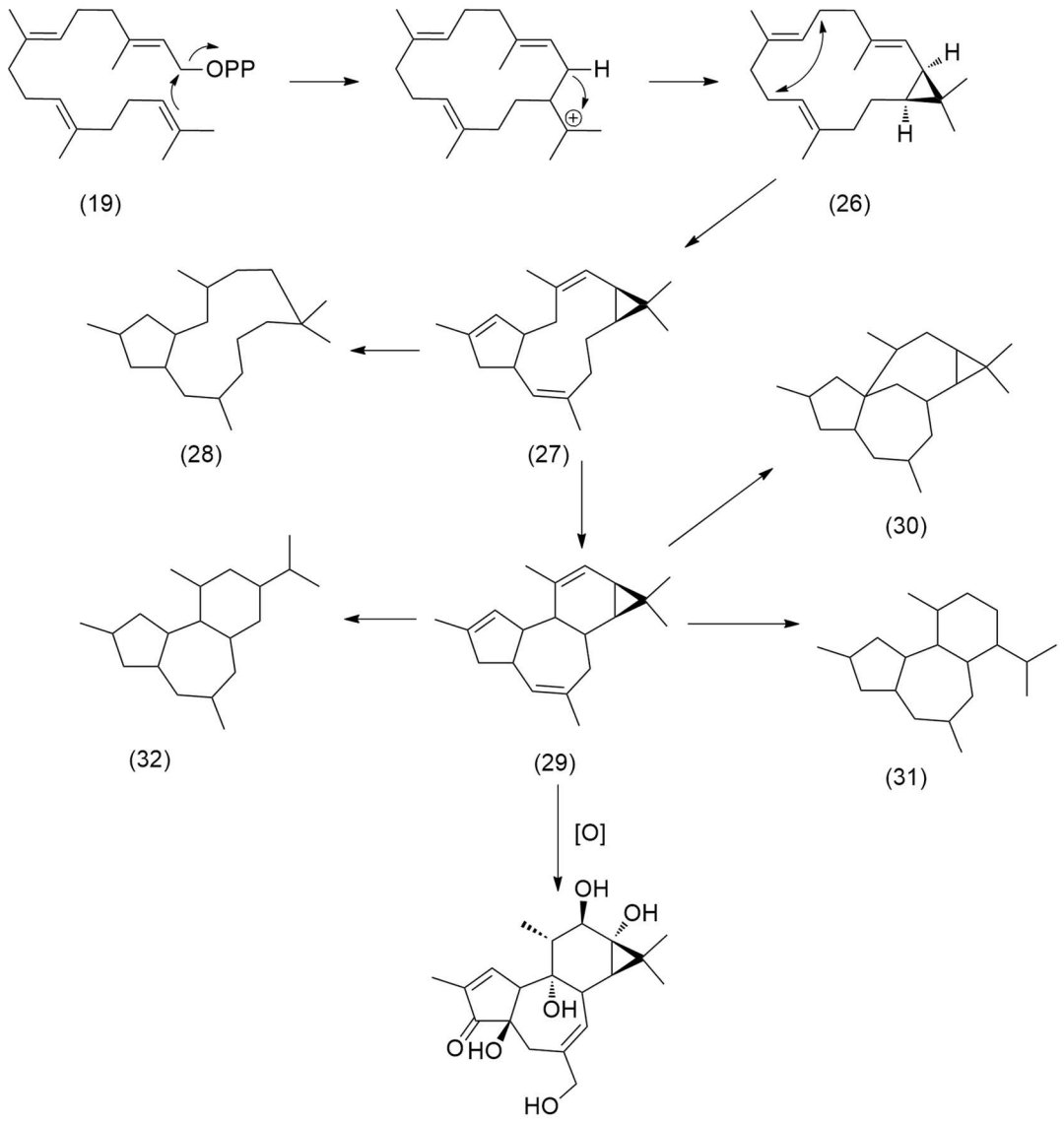


Fig. 2.6 Topological biogenetic proposal for phorboids from lathyrane skeleton

Taken together, all of these observations reveal a kind of incompatibility between the direct biogenetic connection of lathyranes and tiglianes in flat models commonly shown in literature and textbook.

2.5 Jasmin Jakupovic biogenetic proposal for phorboids

An elegant proposal aimed to solve the riddle of the questionable relationship between tiglianes and lathyranes was proposed by prof. Jasmin Jakupovic (1949-2005) in the late 2000s. Unfortunately, his premature demises foreclosed the spreading of his idea among the natural products chemistry community and this concept was relegated to laboratory meetings and a PhD thesis. [**13**]

Starting from a retro-synthetical analysis, the opening of the cyclopropane ring could be done by direct protonation at C-13 or C-8, establishing a logical connection of the casbane skeleton to the cembrane one. If the newly generated cembranyl cation promotes the folding at C-13 (tigliane numbering), the produced casbene would be incompatible in terms of *cis* stereochemistry at the formed cyclopropane and leave unsolved the hydroxylation at same

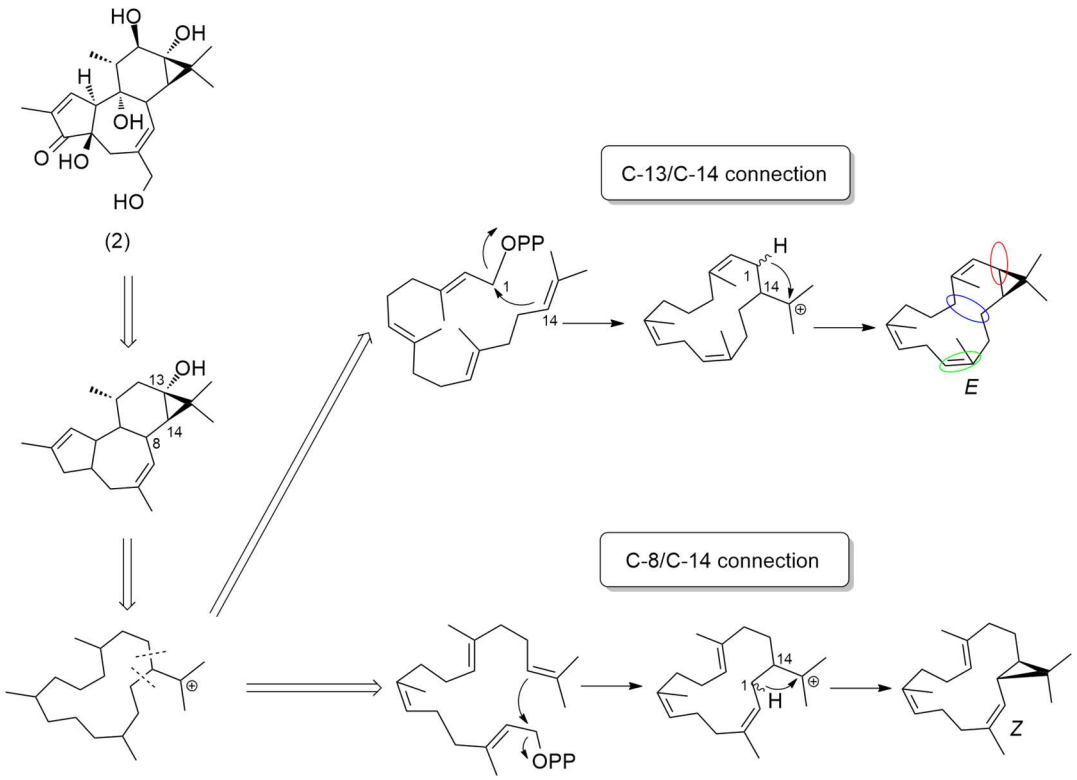


Fig. 2.7 Retro-synthetical analysis of tigliane skeleton

one concurrently with the stereochemistry of the ring B double bond, *E* in the precursor and *Z* in tiglianes.

To solve this incompatibility should be invoked an unusual closure by formal insertion of the side-chain cation center into a C(8)-H sigma bond that only after an oxidative rearrangement would result in the proper scaffold. This mechanism is associated with an inversion of the stereochemistry of double bond at C-2,3 (GGPP numbering) that can be explained by a classic isomerization of the canonical methylallyl phosphates of GGPP to an α-dimethylvinyl phosphates (geranyl-linalyl pyrophosphate, GLPP).

So, the electrophilic attack proceeds on the *si*-face of the *head* of the tetraprenyl precursor by the vinyl group *synclinal* to the pyrophosphate to generate an opposite configuration of the double bond and the unusual localized cyclopropane ring. After cyclopentane ring A formation, possibly by an aldol-like condensation that promotes the isomerization of the double bond, the former lathyrane generated by the process has got the proper locations (Δ^5 and Δ^{12}) shared by tiglianes, ingenanes and rhamnopholanes.

The oxidative step aimed to introduce a ketone in α-position at the cyclopropane represents the keystone in Jakupovic proposal. Protonation at the carbonyl group triggers the formation of an unstable cyclopropylmethyl cation which is quenched by the endocyclic double bond and induces the transposition of the cyclopropane ring at C-13 to generate the controversial hydroxy ring D. A final quenching with water introduces the tertiary alcohol at C-9. All the other steps to achieve phorbol or EBC-46 can be rationalized as a series of oxidative steps and final acyl decoration with fatty acids, shorter or longer, linear or branched. [**12**]

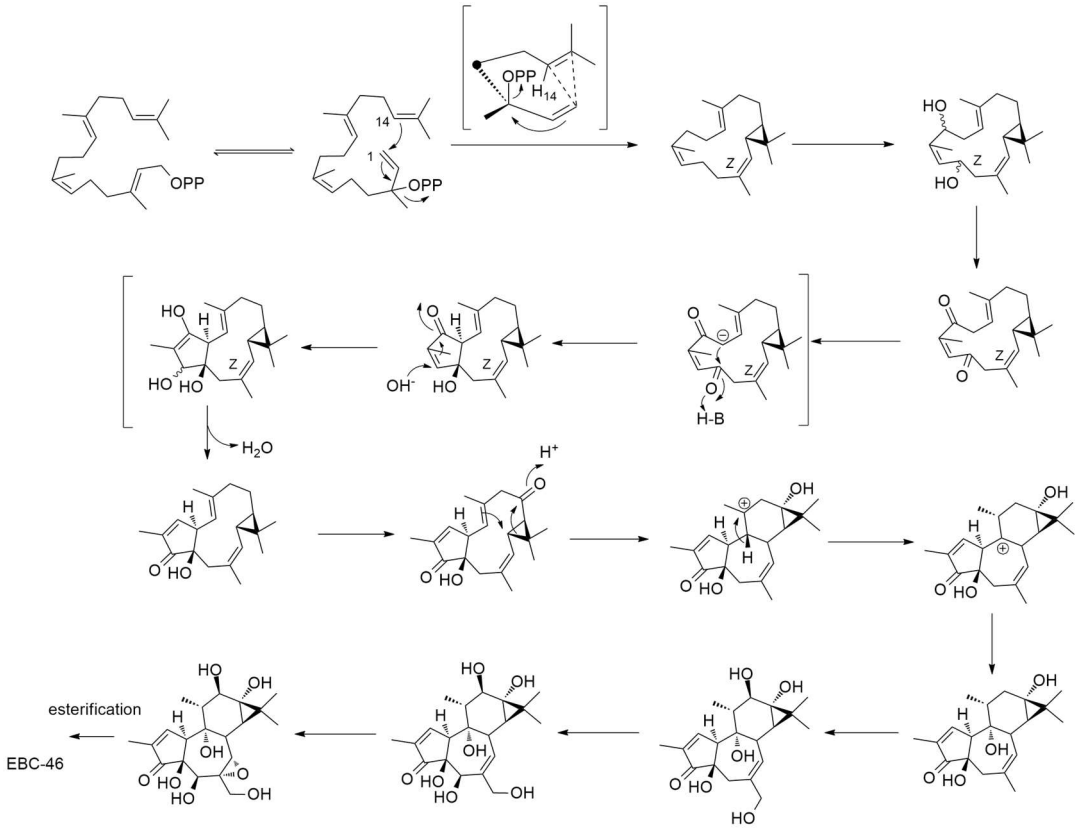


Fig. 2.8 Jakupovic proposal of tigliane skeleton biogenesis

2.6 Synthesis of labelled precursors to elucidate tigliane biogenetic pathway

Labelling organic molecules with isotopes to highlight metabolic pathways provided since their first application in the first half of the XX century, new insights in biosynthesis research. With a concurrent rising of analytical techniques like NMR and MS, it was possible to switch from radioactive nuclei like ^{14}C and ^{3}H and their degradative analysis to ^{13}C and ^{2}H isotopes, more stable and easier to handle. Incorporation of labelled atoms in molecules understudy can provide important details to the stereochemical course of the biosynthetic pathway.[**14**]

In order to determine whether Jakupovic proposal occurs in plants belonging to Euphorbiaceous family, a series of labelling experiments with ^{13}C and ^{2}H atoms are here taken in consideration. In fact, once the labelled atom is incorporated during the downstream process to synthesize the metabolite, the enrichment from the natural occurrence of carbon-13 and deuterium (respectively, 1.1 % and 0.015%) can be easily detected by NMR spectroscopy by augmented intensity in the signals (relative abundance), coupling constants between the marked positions and, in ^{13}C-NMR spectra, a slightly shift of the signals generated by labelled atoms towards lower fields.

The synthesis of labelled GGPP (**19**) at position C-1 would eventually provide insight about the way how the cyclization takes place, as mentioned in **Fig. 2.7,** but it would not give any detail about the hydroxycyclopropane ring formation. Furthermore, geranylgeraniol is not efficiently taken up by plant cells. Thus, assuming that usually plant diterpenes are produced via the MEP pathway, a series of 1-deoxy-D-xylulose (**11**) isotopomers have been synthesized as a precursor for GGPP. Even if this carbohydrate can be used for MEP pathway as well as thiamine biosynthesis and pyridoxal phosphate [**15**], an increase of its concentration seems to enhance the activity of 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), key enzyme in the production of IPP/DMAPP [**16**] and feeding downstream intermediates, like MEP, do not produce any incorporation possibly to the missing phosphorylative machinery. [**17**]

The synthesis of isotopomers of **11** labelled at C-5 with ^{13}C and ^{2}H was performed starting from commercial propargyl alcohol which is protected with *tert*-butyldimethylsilyl chloride. Alkyne deprotonation proceeds with *n*-BuLi and insertion of the labelled substrate (*p*-formaldehyde) followed by reduction to the corresponding allylic alcohol **35**. Oxidation with PCC, compared to the previous attempt with Swern oxidation, reduces the loss of deuterium under 10 %. The enantioselective reduction with (*R*)- or (*S*)- alpine borane affords **37a-b**, which enantiomeric excess was evaluated by esterification with the chiral α-methoxy-α-trifluoromethylphenylacetic acid (Mosher's ester). The newly generated allylic alcohol was protected by a benzyl group and treatment with TBAF provided the deprotection of the opposite silane. **39** was oxidized by IBX and, later, Grignard reaction with methylmagnesium bromide and its re-oxidation resulted in the methyl ketone. Thus, Sharpless dihydroxylation with AD-mix β and a catalytic hydrogenation with Pd/C afforded (5*R*)-(5-^{13}C,5-^{2}H)-1-deoxy-1-D-xylulose (**44a**) and (5*S*)-(5-^{13}C,5-^{2}H)-1-deoxy-1-D-xylulose (**44b**). (**Fig. 2.9**)

Synthesis of the remaining isotopomer of **11** was performed by esterification of fumaric acid (**45**) with trimethylsilyl chloride and subsequent reduction to the diol **47**. Sever issues occurred during work-up of the reaction due to the high polarity of the diol and therefore its retention in water phase. Monoprotection with benzylbromide was achieved employing silver (I) chloride and the obtained product **48c** follows the synthetical strategy developed for the synthesis of **44a** and **44b.**

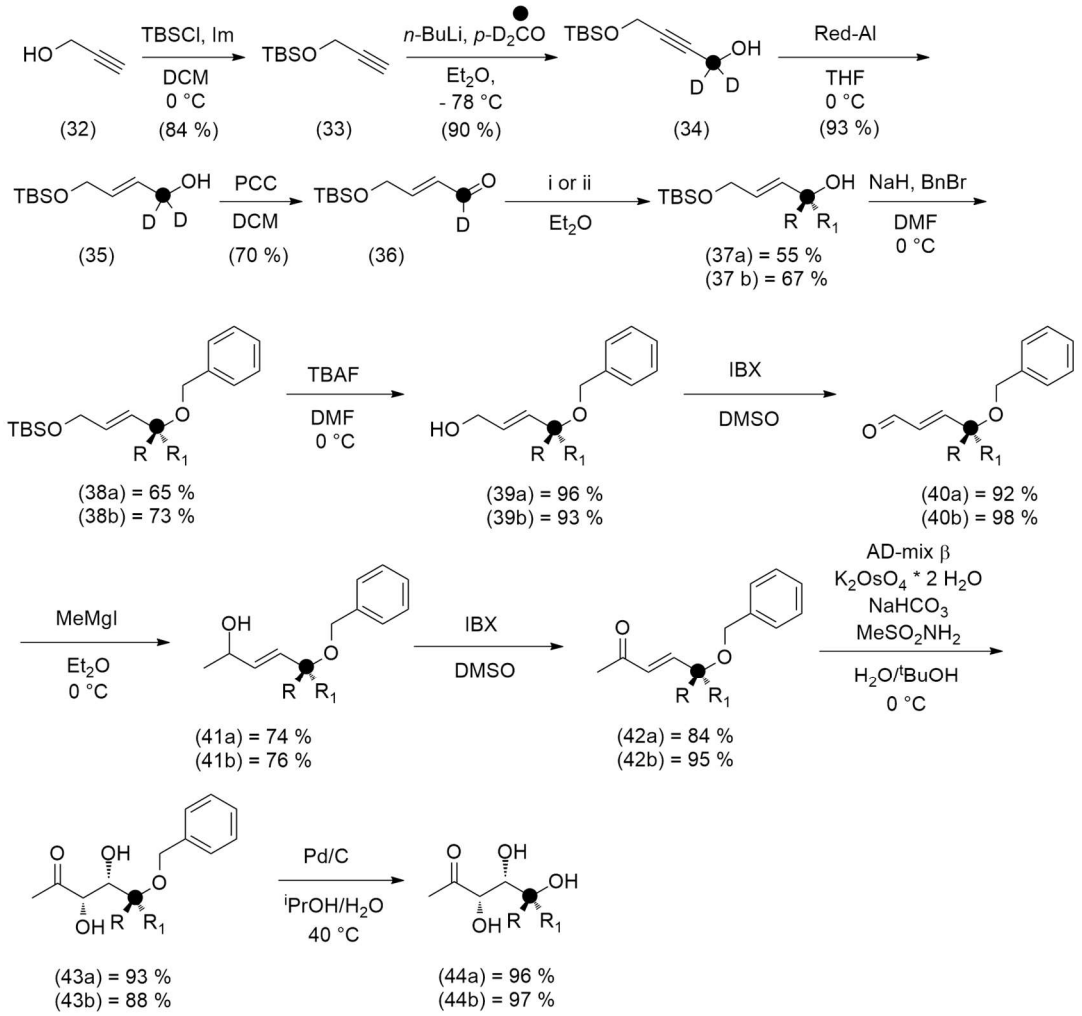


Fig. 2.9 Synthesis of (5*R*)- and (5*S*)-(5-^{13}C,5-^{2}H)-1-deoxy-1-D-xylulose (Respectively 44a and 44b). The enantioselective reduction was done with (*S*)-Alpine borane (i) for series "a" providing R = H; R_1 = D while (*R*)-Alpine borane (ii) generated series "b" providing R = D; R_1 = H

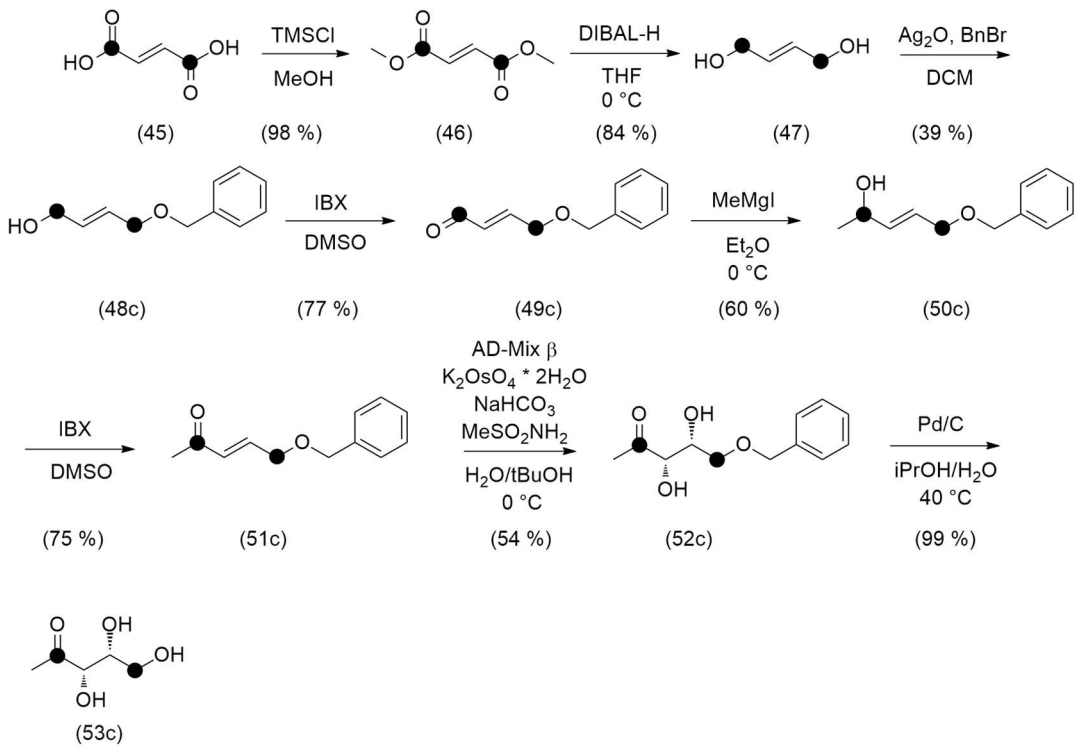


Fig. 2.10 Synthesis of (2-^{13}C, 5-^{13}C)-1-deoxy-1-D-xylulose

2.7 Labelling pattern from DXP incorporation in tigliane skeleton

The labelling at the previously described DXP isotopomers have been strategically designed to evaluate in phase of biological incorporation crucial positions to prove or not the Jakupovic proposal. In fact, synthesis of di-labelled molecule **44a** and **44b**, during stochastic chain elongation, would produce isotopomers of GGPP (19) at least labelled once at C-1 position. The C-13/C-14 connection would proceed by the loss of the 1-pro-*S* proton of GPP to produce and *E,E,E*-casbene while, the C-8/C-14 connection, derived from a previous isomerization from GGPP to GLPP, would produce a *Z,E,E*-casbene because of the loss of 1-pro-*R* proton. The stereochemical course of the reaction can be evaluated by the occurrence or not at C-8 (tigliane numbering) of deuterium. Due to intrinsic difficulties that can arise from evaluation of the occurrence of a single deuterium at GGPPs produced, an additional atom of ^{13}C was

added at the same position. Thus, it would therefore generate in the spectra of the ^{13}C-NMR a singlet, if the C-8 does not contain extra deuterium, or a triplet if comes from coupling between the two isotopes.

The isotopomer **44c**, instead, was synthesized to establish if the formation of hydroxy-cyclopropane is trigger by the insertion of carbonyl group in α at the cyclopropane as suggested by Jakupovic. If tigliane is synthesize in a topological manner a direct connection of carbon atom from different isoprene units would be detected by analysis of the coupling constant. Conversely, if the hydroxycyclopropane is formed after a rearrangement, the connection would arise from carbon atoms that come from the same DXP. (**Fig. 2.11**)

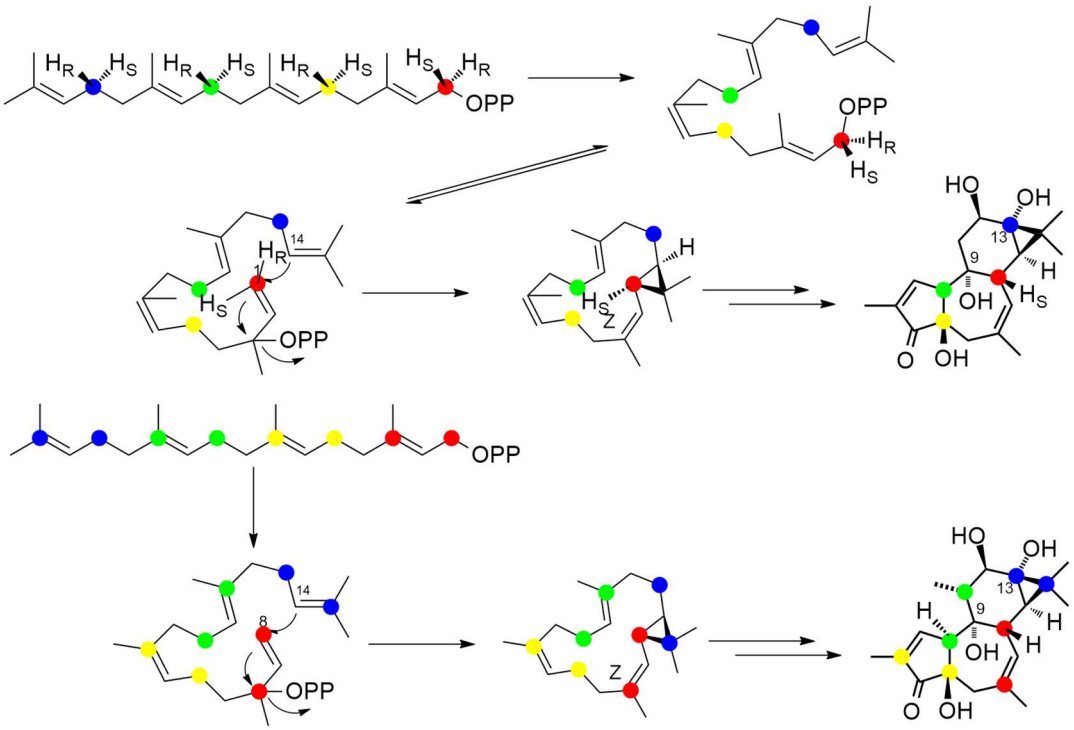


Fig. 2.11 Possible pattern generated by feeding with labelled DXP

2.8 Supporting information

Chemicals were purchased from Acros Organics (Geel, Belgium) or Sigma Aldrich Chemie (Steinheim, Germany) and used without any further purification. Solvents were purified by distillation and dried according to standard methods. All non-aqueous reactions were performed under an inert atmosphere (Ar) in flame-fired flasks. Thin-layer chromatography was performed with 0.2 mm precoated plastic sheets Polygram® Sil G/UV254 (Machery-Nagel). Column chromatography was carried out using Merck silica gel 60 (70-200 mesh). ^{1}H and ^{13}C NMR spectra were recorded with Pollux Bruker AV III Prodigy (500 MHz) and Sirius Bruker AV III HD Cryo (700 MHz) spectrometers and referenced against solvent signals (^{1}H-NMR residual proton signals: $CDCl_3$ δ=7.26, C_6D_6=7.16, D_2O δ=4.79; ^{13}C-NMR: $CDCl_3$ δ=77.16, C_6D_6=128.06). GC-MS analyses were recorded with HP 6890 gas chromatography connected to a HP 5973 inert mass detector fitted with a BPX-5 fused silica capillary column (25m, 0.25 mm i. d., 0.25 µm film). Instrumental parameters were (1) inlet pressure, 77.1 kPa, He 23.3 mL min^{-1}, (2) injection volume, 2 µL, (3) transfer line, 300 °C, and (4) electron energy 70 eV. The GC was programmed as follows: 5 min at 50 °C increasing at 10 °C min^{-1} to 320 °C and operated in split mode (10:1, 60 s valve time). The carrier gas was he at 1 mL min^{-1}. Retention indices (I) were determined from a homologous series of n-alkanes (C_8-C_{38}). Optical rotations were recorded with P8000 polarimeter (Krüss).

Synthesis of *tert*-butyldimethylsilyloxy-1-propyne (33): *tert*-butyldimethylsilylchloride (**32**, 50.72 g, 33.6 mmol, 1 eq) and imidazole (45.8 g, 67.2 mmol, 2 eq) were added to a solution of propargyl alcohol (**1**) (19.39 mL, 33.6 mmol, 1 eq) in dry DCM (600 mL) at 0 °C. After 1 h the mixture was quenched with H_2O and extracted with DCM. The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. Crude product was distillated under vacuum at 40 °C at 8 mmHg affording **2** (47.95 g, 28.15 mmol, 84%) as a colourless oil. TLC (cyclohexane/ethyl acetate 99:1): R_f= 0.2. GC (BPX-5): I= 983 .^{1}H-NMR (500 MHz, $CDCl_3$): δ = 4.31 (d, ^{4}J(H,H) = 2.4 Hz, 2H, CH_2), 2.39 (d, ^{4}J(H,H) = 2.4 Hz, 1H, CH), 0.91 (s, 9H, 3 CH_3), 0.13 (s, 6H, 2 CH_3) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ= 82.58 (C_q), 72.97 (CH), 51.66 (CH_2),

25.94 (3 CH_3), 18.43 (C_q), -5.06 (2 CH_2) ppm. MS (EI, 70 eV): m/z (%) = 171 (<1) [M^+], 146 (16), 129 (8), 99 (2), 75 (100), 57 (2), 45 (3). [**18**]

Synthesis of (1-^{13}C, 1,1-$^{2}H_2$)-4-((*tert*-butyldimethyldilyl)oxy)but-2-yn-1-ol (34): To a solution of **2** (7.73 g, 45 mmol, 1.5 eq) in dry Et_2O (165 mL), *n*-butyllithium (1.6 M in hexane, 28.12 mL, 45 mmol, 1.5 eq) was slowly added at -78 °C and stirred for 1 h at -78 °C. [^{13}C, $^{2}H_2$]-*para*-formaldehyde (1 g, 30 mmol, 1 eq) was added at -78 °C and the reaction mixture was stirred at room temperature overnight. The reaction was quenched with a saturated aqueous NH_4Cl solution and extracted with diethyl ether. The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. Purification of the crude was reached by column chromatography on silica gel (cyclohexane/ethyl acetate 8:2) affording **3** (5.49 g, 27 mmol, 90%) as a colourless oil. TLC (cyclohexane/ethyl acetate 9:1): R_f= 0.22. GC (BPX-5): *I*= 1336 .^{1}H-NMR (500 MHz, $CDCl_3$): δ = 4.35 (d, ^4J(C,H) = 1.0 Hz, 2H, CH_2), 0.91 (s, 9H, 3 CH_3), 0.12 (s, 6H, 2 CH_3) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ= 84.68-84.47 (d, ^1J(C,C) = 12.5 Hz, C_q), 84.6 (d, ^2J(C,C) = 1.6 Hz, C_q) , 51.8 (d, ^3J(C,C) = 1.3 Hz, CH_2), 50.8 (quint, ^1J(^{13}C,D) = 22.5 Hz, $^{13}CD_2$), 25.8 (3 CH_3), 18.3 (C_q), -5.2 (2 CH_2) ppm. MS (EI, 70 eV): m/z (%) = 203 (<1) [M^+], 146 (5), 129 (3), 128 (27), 127 (18), 126 (3), 85 (4), 77 (9), 76 (18), 75 (100), 73 (6), 57 (3), 45 (4), 39 (3). [**19**]

Synthesis of (1-^{13}C, 1,1-$^{2}H_2$)-4-((*tert*-butyldimethyldilyl)oxy)but-2-en-1-ol (35): To a solution of Red-Al® (17.36 mL, 53.38 mmol, 2 eq) in dry THF(135 mL) **3** (5.44 g, 26.75 mmol, 1 eq) was slowly added at -72 °C and let it warm up until 0 °C for 3 h. The reaction mixture was quenched with EtOAc (2 eq) and with an aqueous HCl solution 1 M (2 eq) at 0 °C. After 10 minutes, the mixture was concentrated under pressure and dilute with 150 mL of ethyl acetate. The organic phase was extracted with a saturated aqueous $NaHCO_3$ solution (x1) and washed with brine (x2). The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo* affording **4** (5.19 g, 25.27 mmol, 94%) as a pale-yellow oil. TLC (cyclohexane/ethyl acetate 8:2): R_f= 0.28. GC (BPX-5): *I*= 1330 .^{1}H-NMR (500 MHz, $CDCl_3$): δ = 5.92-5.73 (br m, 2 H, 2 CH), 4.18 (br d, 2 H, CH_2), 0.91 (s, 9H, 3 CH_3), 0.13 (s, 6H, 2 CH_3) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ= 131.34 (CH), 129.26-128.65 (d, ^1J(C,C) = 46.3 Hz, CH),

63.29 (CH_2), 62.65 (quint, ^1J ($^{13}C,D_2$) = 22 Hz, $^{13}C_2D_2$), 25.7 (3 CH_3), 18.34 (C_q), -5.37 (2 CH_2) ppm. MS (EI, 70 eV): m/z (%) = 205 (<1) [M^+], 148 (16), 130 (7), 129 (3), 77 (9), 76 (12), 75 (100), 73 (7), 57 (4), 45 (2). Warning: 4-((*tert*-butyldimethyldilyl)oxy)but-2-en-1-ol is unstable on silica gel. Do not perform column chromatography on this compound. [**20**]

Synthesis of (1-^{13}C, 1,^{2}H)-4-((*tert*-butyldimethyldilyl)oxy)but-2-en-1-al (36): To a solution of **4** (5.19 g, 25.27 mmol, 1 eq) in DCM (120 mL), PCC (8.17 g, 37.9 mmol, 1.5 eq) was added and the mixture was stirred at room temperature for 3h. The reaction was filtered on a silica pad with DCM and concentrated *in vacuo*. Purification of the crude was done by column chromatography on silica gel (petroleum ether/diethyl ether 8:2) affording **5** (3.6 g, 17.7 mmol, 70%) as a colourless oil. TLC (cyclohexane/ethyl acetate 8:2): R_f= 0.22. GC (BPX-5): *I*= 1282. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 6.88 (ddt, ^3J(H,H) = 16 Hz, ^3J(H,H) = 9 Hz, ^3J(C,H) = 3 Hz, 1H, CH), 6.39 (dtt, ^3J(H,H) = 16 Hz, ^3J(H,D) = 1.2 Hz, ^2J(C,H) = 3 Hz, 1H, CH), 4.44 (quint, 2H, CH_2), 0.91 (s, 9H, 3 CH_3), 0.08 (s, 6H, 2 CH_3) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 193.4 (quint ^1J(^{13}CD) = 22 Hz, C_q), 153.20-152.93 (d, ^1J(C,C) = 46 Hz, CH), 130.5 (d, ^{2}J(C,C) = 3 Hz, CH), 63.2 (CH_2), 25.8 (3 CH_3), 18.3 (C_q), -5.2 (2 CH_2) ppm. MS (EI, 70 eV): m/z (%) = 202 (12) [M^+], 146 (28), 145 (100), 131 (28), 127 (13), 115 (7), 113 (7), 101 (14), 100 (18), 99 (14), 85 (6), 77 (25), 76 (93), 75 (100), 73 (30), 59 (15), 45 (7). [**21**]

Synthesis of (1*R*)-(1-^{13}C, 1-^{2}H)-4-((*tert*-butyldimethyldilyl)oxy)but-2-en-1-ol (37a): A solution of **5** (1.8 g, 8.89 mmol, 1 eq) in 5 mL of Et_2O was added dropwise to a solution of (*S*)-Alpine borane® (0.5 M sol in THF, 21.34 mL, 10.67 mmol, 1.2 eq) in dry Et_2O (40 mL) at room temperature and stirred for 4 h .The reaction was added of acetaldehyde (30 µL $mmol^{-1}$) and stirred for 1 h, concentrated then under *vacuum* and diluted with diethyl ether (1.2 mL $mmol^{-1}$). After cooling the mixture at 0 °C, 2-aminoethanol (590.3 µL, 9.78 mmol, 1.1 eq) was added at the mixture and stirred for 1 h. The mixture was filtered with diethyl ether and extracted with water. The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. Purification of the crude was reached by column chromatography on silica gel (cyclohexane/ethyl acetate 75:25) affording **6a** (997 mg, 4.87 mmol, 55%) as a colourless oil. TLC (cyclohexane/ethyl acetate 9:1): R_f= 0.53. GC (BPX-5): *I*= 1300. ^{1}H-NMR (500 MHz,

$CDCl_3$): δ = 5.91-5.84 (br m, 1 H, CH), 5.82-5.77 (m, 1 H, CH), 4.27-3.99 (dm, $^1J(^{13}C,H)$ = 142 Hz, 1H, ^{13}CH), 4.18 (m, 2 H, CH_2), 0.91 (s, 9H, 3 CH_3), 0.08 (s, 6H, 2 CH_3) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ= 131.25 (CH), 129.14-128.9 (d, $^1J(C,C)$ = 46.3 Hz, CH), 63.20 (CH_2), 62.85 (quint, ^1J ($^{13}C,D_2$) = 22 Hz, $^{13}C_2D_2$), 25.94 (3 CH_3), 18.39 (C_q), -5.25 (2 CH_2) ppm. MS (EI, 70 eV): m/z (%) = 204 (<1) [M^+], 146 (28), 145 (100), 131 (28), 127 (13), 101 (14), 100 (18), 99 (14), 77 (25), 76 (93), 75 (100), 73 (30), 59 (15), 45 (7). [**22**]

Synthesis of (1*S*)-(1-^{13}C, 1-^{2}H)-4-((*tert*-butyldimethyldilyl)oxy)but-2-en-1-ol (37b): A solution of **5** (1.8 g, 8.89 mmol, 1 eq) in 5 mL of Et_2O was added dropwise to a solution of (*R*)-Alpine borane® (0.5 M sol in THF, 21.34 mL, 10.67 mmol, 1.2 eq) in dry Et_2O (40 mL) at room temperature and stirred for 4 h .Acetaldehyde (30 µL $mmol^{-1}$) was added at the reaction and stirred for 1 h, concentrated then under *vacuum* and diluted with diethyl ether (1.2 mL $mmol^{-1}$). After cooling the mixture at 0 °C, 2-aminoethanol (590.3 µL, 9.78 mmol, 1.1 eq) was added and let it stir for 1 h. The mixture was filtered with diethyl ether and extracted with water. The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo.* Purification of the crude was reached by column chromatography on silica gel (cyclohexane/ethyl acetate 75:25) affording **6b** (1.22 g, 5.97 mmol, 67%) as a colourless oil. TLC (cyclohexane/ethyl acetate 9:1): R_f= 0.16. GC (BPX-5): *I*= 1300. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 5.91-5.84 (br m, 1 H, CH), 5.82-5.77 (m, 1 H, CH), 4.27-3.99 (dm, $^1J(^{13}C,H)$ = 142 Hz, 1H, ^{13}CH), 4.18 (m, 2 H, CH_2), 0.91 (s, 9H, 3 CH_3), 0.08 (s, 6H, 2 CH_3) ppm.^{13}C-NMR (125 MHz, $CDCl_3$): δ= 131.25 (CH), 129.14-128.9 (d, $^1J(C,C)$ = 46.3 Hz, CH), 63.20 (CH_2), 62.85 (quint, ^1J ($^{13}C,D_2$) = 22 Hz, $^{13}C_2D_2$), 25.94 (3 CH_3), 18.39 (C_q), -5.25 (2 CH_2) ppm. MS (EI, 70 eV): m/z (%) = 204 (<1) [M^+], 146 (28), 145 (100), 131 (28), 127 (13), 101 (14), 100 (18), 99 (14), 77 (25), 76 (93), 75 (100), 73 (30), 59 (15), 45 (7). [**22**]

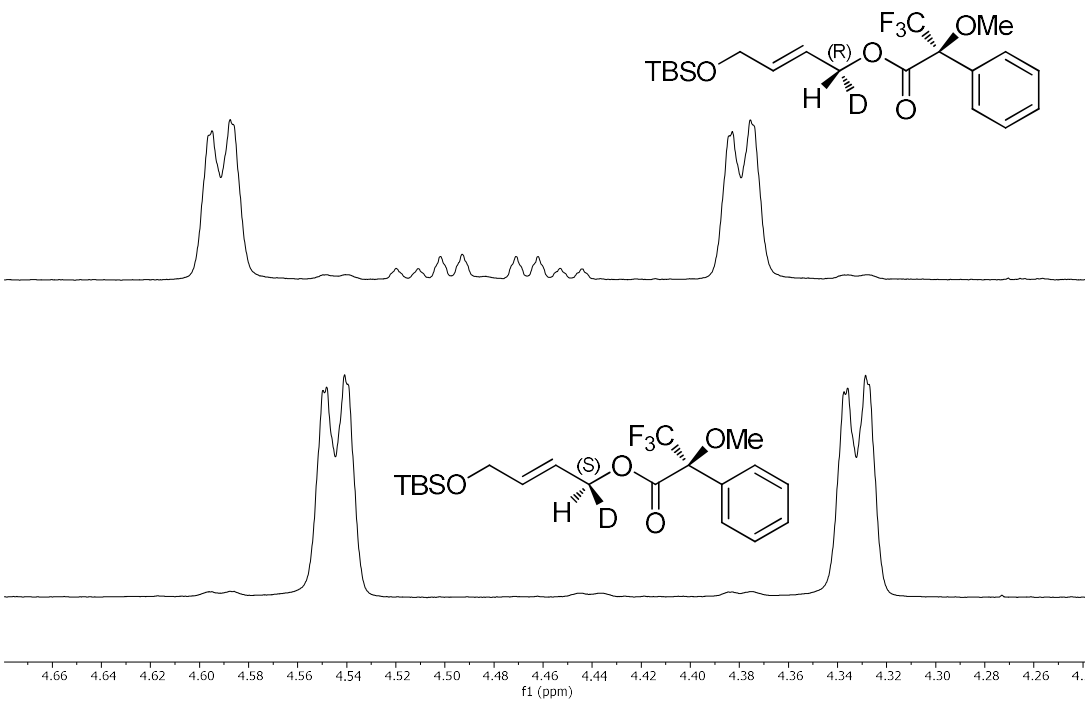


Figure 2.. The enantiomeric excess was determined by ^{1}H-NMR spectroscopy. Compounds **37a-b** (5 mg) were dissolved in dry DCM (380 µL). Dry pyridine (6 µL, 75.64 µmol, 3.1 eq) was added at room temperature and, after 30 min, (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (8.67 µL, 46.36 µmol, 1.9 eq) was added. The reaction mixture was stirred for 2 h, quenched with water and extracted with diethyl ether. The organic layers were dried over $MgSO_4$ and concentrated *in vacuo.* The analysis of the products, after purification by column chromatography (petroleum ether/diethyl ether 98:2), reveals an *ee* of 94.6% for **37a** (upper signals) and 96.2% for **37b** (lower signals).

General Procedure for the Preparation of Isotopologues of (4-^{13}C, 4-^{2}H)-4-Benzyloxy-1-((*tert*-butyldimethylsilyl)oxy)but-2-ene: To a solution of sodium hydride (60% suspension in mineral oil, 2.0 eq.) in dry THF (25 mL) **37a** (1 eq, for 7a) or **37b** (1 eq, for 7b) was added dropwise at 0 °C. Benzyl bromide (2.0 eq.) and dry DMF (2.0 eq) were added after 1h and the reaction mixture was stirred at room temperature overnight. The reaction was quenched with saturated aqueous NH_4Cl solution and extracted with diethyl ether and water. The combined

organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. Column chromatography on silica gel (petroleum ether/diethyl ether 95:5) afforded **38a-b** as a colourless oil. TLC (cyclohexane/ethyl acetate 9:1): R_f = 0.35. [**23**]

(4*R*)-(4-^{13}C, 4-^{2}H)-4-Benzyloxy-1-((*tert*-butyldimethylsilyl)oxy)but-2-ene (**38a**): Yield (927 mg, 3.14 mmol, 65%), GC (BPX-5): I = 1914. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.37-7.26 (m, 5 H, 5 CH), 5.83 (m, 2 H, 2 CH), 4.52 (d, ^{3}J(C,H) = 4 Hz, 2 H, CH_2), 4.20 (m, 2H, CH_2), 4.13-3.91 (dm, ^{1}J(CH)= 141 Hz, 1 H, ^{13}CHD), 0.92 (s, 9H, 3 CH_3), 0.08 (s, 6H, 2 CH_3) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ= 138.52 (d, ^{2}J(C,C) = 2.9 Hz, CH), 132.83 (d, ^{3}J(C,C) = 1.5 Hz, C_q), 128.51 (2 CH), 127.89 (2 CH), 127.70 (CH), 126.48-126.41 (d, ^{1}J(C,C) = 48 Hz, CH), 72.15 (d, ^{3}J(C,C) = 1.5 Hz, CH_2), 70.03 (t,^{1}J(C,D) = 21.6 Hz, ^{13}CD), 63.30 (d, ^{2}J(C,C) = 6 Hz, CH_2), 26.10 (3 CH_3), 18.56 (C_q), -5.08 (2 CH_2) ppm. MS (EI, 70 eV): m/z (%) = 294 (<1) [M^+], 237 (8), 165 (48), 135 (46), 131 (49), 101 (20), 91 (100), 75 (100), 73 (15).

(4*S*)-(4-^{13}C, 4-^{2}H)-4-Benzyloxy-1-((*tert*-butyldimethylsilyl)oxy)but-2-ene (**38b**): Yield (1.26 g, 4.28 mmol, 73%), GC (BPX-5): I = 1914. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.37-7.26 (m, 5 H, 5 CH), 5.83 (m, 2 H, 2 CH), 4.52 (d, ^{3}J(C,H) = 4 Hz, 2 H, CH_2), 4.20 (m, 2H, CH_2), 4.13-3.91 (dm, ^{1}J(CH)= 141 Hz, 1 H, ^{13}CHD), 0.92 (s, 9H, 3 CH_3), 0.08 (s, 6H, 2 CH_3) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ= 138.52 (d, ^{2}J(C,C) = 2.9 Hz, CH), 132.83 (d, ^{3}J(C,C) = 1.5 Hz, C_q), 128.51 (2 CH), 127.89 (2 CH), 127.70 (CH), 126.48-126.41 (d, ^{1}J(C,C) = 48 Hz, CH), 72.15 (d, ^{3}J(C,C) = 1.5 Hz, CH_2), 70.03 (t,^{1}J(C,D) = 21.6 Hz, ^{13}CD), 63.30 (d, ^{2}J(C,C) = 6 Hz, CH_2), 25.97 (3 CH_3), 18.47 (C_q), -5.09 (2 CH_2) ppm. MS (EI, 70 eV): m/z (%) = 294 (<1) [M^+], 237 (9), 165 (53), 135 (51), 131 (54), 101 (24), 91 (100), 75 (13), 73 (15).

General Procedure for the Preparation of Isotopologues of 4-Benzyloxybut-2-en-1-ol: To a solution of **38a** or **38b** (1.0 eq) in dry THF (20 mL) at 0 °C, TBAF (1.1 eq) was added dropwise under stirring. After 2h, the reaction mixture was 86ransformatio water and extracted with diethyl ether. The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. Column chromatography on silica gel (cyclohexane/ethyl acetate 6:4) afforded **39a-b** as a colourless oil. TLC (cyclohexane/ethyl acetate 7:3): R_f = 0.19 [**23**]

(4*R*)-(4-^{13}C, 4-^{2}H)-4-Benzyloxybut-2-en-1-ol (**39a**): Yield (536 mg, 2.97 mmol, 96 %), GC (BPX-5): I = 1569. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.37-7.27 (m, 5 H, 5 CH), 5.95-5.89 (ddtd, ^{3}J(H,H) = 16 Hz, ^{3}J(H,H) = 5.3 Hz, ^{3}J(C,H) = 6 Hz, ^{4}J(D,H) = 1.3 Hz, 1 H, CH), 5.87-5.82 (br dt, ^{3}J(H,H) = 16 Hz, ^{3}J(C,H) = 4.5 Hz, 1 H, CH), 4.53 (d, ^{3}J(C,H)= 4 Hz, 2 H, CH_2), 4.16 (dt, ^{3}J(H,H) = 5.3 Hz, ^{2}J(H,H) = 1.3 Hz, 2 H, CH_2), 4.13-3.90 (dm, ^{1}J(CH)= 141 Hz, 1 H, ^{13}CHD), 1.65 (br s, 1 H, OH) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ= 138.32 (d, ^{2}J(C,C) = 2.9 Hz, CH), 132.4 (d, ^{3}J(C,C) = 1 Hz, C_q), 128.53 (2 CH), 128.06 (2 CH), 127.88 (CH), 128.07-127.79 (d, ^{1}J(C,C) = 48 Hz, CH), 127.79 (CH), 72.4 (d, ^{2}J(C,C) = 1.75 Hz, CH_2), 69.85 (t,^{1}J(C,D) = 21.8 Hz, ^{13}CD), 63.14 (d, ^{2}J(C,C) = 6 Hz, CH_2) ppm. MS (EI, 70 eV): m/z (%) = 180 (<1) [M^+], 146 (10), 131 (6), 107 (7), 105 (7), 92 (22), 91 (100), 79 (11), 77 (12), 65 (11), 51 (5).

(4*S*)-(4-^{13}C, 4-^{2}H)-4-Benzyloxybut-2-en-1-ol (**39b**): Yield (711 mg, 3.94 mmol, 93 %), GC (BPX-5): I = 1570. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.37-7.27 (m, 5 H, 5 CH), 5.95-5.89 (ddtd, ^{3}J(H,H) = 16 Hz, ^{3}J(H,H) = 5.3 Hz, ^{3}J(C,H) = 6 Hz, ^{4}J(D,H) = 1.3 Hz, 1 H, CH), 5.87-5.82 (br dt, ^{3}J(H,H) = 16 Hz, ^{3}J(C,H) = 4.5 Hz, 1 H, CH), 4.53 (d, ^{3}J(C,H)= 4 Hz, 2 H, CH_2), 4.16 (dt, ^{3}J(H,H) = 5.3 Hz, ^{2}J(H,H) = 1.3 Hz, 2 H, CH_2), 4.13-3.91 (dm, ^{1}J(CH)= 141 Hz, 1 H, ^{13}CHD), 1.59 (br s, 1 H, OH) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ= 138.32 (d, ^{2}J(C,C) = 2.9 Hz, CH), 132.4 (d, ^{3}J(C,C) = 1 Hz, C_q), 128.53 (2 CH), 128.06 (2 CH), 127.88 (CH), 128.07-127.79 (d, ^{1}J(C,C) = 48 Hz, CH), 127.79 (CH), 72.4 (d, ^{2}J(C,C) = 1.75 Hz, CH_2), 69.85 (t,^{1}J(C,D) = 21.8 Hz, ^{13}CD), 63.14 (d, ^{2}J(C,C) = 6 Hz, CH_2) ppm. MS (EI, 70 eV): m/z (%) 180 (<1) [M^+], 146 (10), 131 (6), 107 (7), 105 (7), 92 (22), 91 (100), 79 (11), 77 (12), 65 (11), 51 (5).

General Procedure for the Preparation of Isotopologues of 4-Benzyloxy-2-butenal: To a 0.5 M solution of IBX (3.5 eq) in dry DMSO, **39a-c** (1.0 eq) were added. The reaction mixture was stirred overnight at room temperature, filtered under vacuum on a sintered funnel covered by filter paper and diluted by water. The aqueous layer was extracted with ethyl acetate (3 x). The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. Column chromatography on silica gel (petroleum ether/diethyl ether 75:25) afforded **40a-c** as a colourless oils. TLC (cyclohexane/ethyl acetate 75:25): R_f = 0.36 [**19**]

(4*R*)-(4-^{13}C, 4-^{2}H)-4-Benzyloxy-2-butenal (**40a**): Yield (488 mg, 2.74 mmol, 92 %), GC (BPX-5): I = 1530 . ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 9.59 (d, ^{3}J(H,H) = 8 Hz, 1 H, CHO), 7.39-7.29 (m, 5 H, 5 CH), 6.87-6.82 (ddd, ^{3}J(H,H) = 16 Hz, ^{2}J(C,H) = 5.4 Hz, ^{3}J(H,H) = 4.1 Hz, 1 H, CH), 6.43-6.38 (dddd, ^{3}J(H,H) = 16 Hz, ^{3}J(H,H) = 8 Hz, ^{3}J(C,H) = 5.4 Hz, ^{4}J(H,H) = 2 Hz, 1 H, CH), 4.59 (d, ^{3}J(C,H)= 4.3 Hz, 2 H, CH_2), 4.38-4.16 (dsest, ^{1}J(^{13}C,H)= 141 Hz, ^{1}J(D,H)= 2.2 Hz, 1 H, ^{13}CHD) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ= 193.4 (d, ^{3}J(C,C) = 7.3 Hz, C_q), 153.20-152.93 (d, ^{1}J(C,C) = 46 Hz, CH), 137.56 (d, ^{2}J(C,C) = 3 Hz, CH), 132.05 (C_q), 128.67 (2 CH), 128.11 (CH), 127.82 (2 CH), 73.1 (d, ^{2}J(C,C) = 1.4 Hz, CH_2), 68.35 (t,^{1}J(C,D) = 21.8 Hz, ^{13}CHD) ppm. MS (EI, 70 eV): m/z (%) = 178 (<1) [M^{+}], 146 (11), 131 (6), 107 (6), 105 (6), 92 (19), 91 (100), 79 (11), 77 (12), 65 (11), 51 (4).

(4*S*)-(4-^{13}C, 4-^{2}H)-4-Benzyloxy-2-butenal (**40b**): Yield (688 mg, 3.86 mmol, 96 %), GC (BPX-5): I = 1530. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 9.59 (d, ^{3}J(H,H) = 8 Hz, 1 H, CHO), 7.39-7.29 (m, 5 H, 5 CH), 6.87-6.82 (ddd, ^{3}J(H,H) = 16 Hz, ^{2}J(C,H) = 5.4 Hz, ^{3}J(H,H) = 4.1 Hz, 1 H, CH), 6.43-6.38 (dddd, ^{3}J(H,H) = 16 Hz, ^{3}J(H,H) = 8 Hz, ^{3}J(C,H) = 5.4 Hz, ^{4}J(H,H) = 2 Hz, 1 H, CH), 4.59 (d, ^{3}J(C,H)= 4.3 Hz, 2 H, CH_2), 4.38-4.16 (dsest, ^{1}J(^{13}C,H)= 141 Hz, ^{1}J(D,H)= 2.2 Hz, 1 H, ^{13}CHD) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ= 193.4 (d, ^{3}J(C,C) = 7.3 Hz, C_q), 153.20-152.93 (d, ^{1}J(C,C) = 46 Hz, CH), 137.56 (d, ^{2}J(C,C) = 3 Hz, CH), 132.05 (C_q), 128.67 (2 CH), 128.11 (CH), 127.82 (2 CH), 73.1 (d, ^{2}J(C,C) = 1.4 Hz, CH_2), 68.35 (t,^{1}J(C,D) = 21.8 Hz, ^{13}CHD) ppm. MS (EI, 70 eV): m/z (%) = 178 (<1) [M^{+}], 146 (11), 131 (6), 107 (6), 105 (6), 92 (19), 91 (100), 79 (11), 77 (12), 65 (11), 51 (4).

(1,4-$^{13}C_2$)-4-Benzyloxy-2-butenal (**40c**): Yield (355 mg, 1.99 mmol, 77 %), GC (BPX-5): I = 1530. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 9.59 (dd, ^{1}J(C,H) = 172.4 Hz, ^{3}J(H,H) = 8 Hz, 1 H, CHO), 7.40-7.29 (m, 5 H, 5 CH), 6.85 (dddt, ^{3}J(H,H) = 16 Hz, ^{2}J(C,H) = 9.3 Hz, ^{3}J(H,H) = 5.4 Hz, ^{3}J(C,H) = 4.1 Hz, 1 H, CH), 6.47-6.39 (m, 1 H, CH), 4.60 (d, ^{3}J(C,H)= 4.3 Hz, 2 H, CH_2), 4.43-4.15 (ddddd, ^{1}J(^{13}C,H)= 142 Hz, ^{4}J(C,H)= 4.3 Hz, ^{4}J(H,H)= 1.9 Hz, ^{2}J(H,H)= 1.5 Hz, 1 H, ^{13}CHD) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ= 193.4 (d, ^{3}J(C,C) = 7.4 Hz, C_q), 153.13 (dd, ^{1}J(C,C) = 46 Hz, , ^{2}J(C,C) = 4.4 Hz CH), 137.55 (d, ^{2}J(C,C) = 2.8 Hz, C_q), 132.19-131.75 (^{1}J(C,C) = 46 Hz, CH), 128.69 (2 CH), 128.12 (CH), 127.82 (2 CH), 73.2 (d, ^{2}J(C,C) = 1.3 Hz,

CH_2), 68.7 (d,^1J(C,D) = 7.4 Hz, ^{13}CHD) ppm. MS (EI, 70 eV): m/z (%) = 178 (<1) [M^+], 135 (4), 108 (15), 107 (14), 92 (37), 91 (100), 79 (11), 77 (10), 65 (8).

General Procedure for the Preparation of Isotopologues of 5-Benzyloxy-3-penten-2-ol: A solution of MeMgI (3.0 eq) prepared from Mg (3.0 eq) and MeI (3.0 eq) in diethyl ether (1.0 M) was added dropwise to a ice cooled solution (0.12 M) of **40a-c**. The reaction mixture was stirred for 3 h at 0 °C and quenched with water. The aqueous layer was extracted with diethyl ether, dried over $MgSO_4$ and concentrated *in vacuo*. Column 89ransformation on silica gel (petroleum ether/ethyl acetate 7:3) afforded **41a-c** as colourless oils. TLC (cyclohexane/ethyl acetate 75:25) R_f=0.13 [**19**]

(5*R*)-(5-^{13}C, 5-^{2}H)-5- Benzyloxy-3-penten-2-ol (**41a**): Yield (395 mg, 2.03 mmol, 74 %), GC (BPX-5): I = 1584. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.37-7.26 (m, 5 H, 5 CH), 5.85-5.75 (m, 2 H, 2 CH), 4.52 (d, ^2J(C,H)= 4.3 Hz, 2 H, CH_2), 4.34 (m, 1H, CH), 4.15-4.34 (dm, ^1J(C,C) = 142 Hz, 1 H, ^{13}CHD), 1.61 (br s, 1 H, OH), 1.28 (d, ^4J(C,H) = 6.4 Hz, 3 H, CH_3) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ= 138.35 (d, ^2J(C,C) = 2.8 Hz, C_q), 137.32 (CH), 128.54 (2 CH), 127.9 (CH), 127.77 (2 CH), 126.5-126.1 (^1J(C,C) = 48 Hz, CH), 72.4 (d, ^2J(C,C) = 1.2 Hz, CH_2), 69.9 (t,^1J(C,D) = 21.7 Hz, ^{13}CHD), 68.37 (^3J(C,H) = 6 Hz, CH), 23.3 (CH_3) ppm. MS (EI, 70 eV): m/z (%) = 194 (<1) [M^+], 165 (47), 145 (5), 135 (46), 131 (47), 115 (4), 101 (21), 91 (100), 75 (15), 73 (15), 65 (4).

(5*S*)-(5-^{13}C, 5-^{2}H)-5- Benzyloxy-3-penten-2-ol (**41b**): Yield (573 mg, 2.95 mmol, 76 %), GC (BPX-5): I = 1585. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.37-7.26 (m, 5 H, 5 CH), 5.85-5.74 (m, 2 H, 2 CH), 4.52 (d, ^2J(C,H)= 4.3 Hz, 2 H, CH_2), 4.34 (m, 1H, CH), 4.15-4.34 (dm, ^1J(C,C) = 142 Hz, 1 H, ^{13}CHD), 1.61 (br s, 1 H, OH), 1.28 (d, ^4J(C,H) = 6.4 Hz, 3 H, CH_3) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ= 138.35 (d, ^2J(C,C) = 2.8 Hz, C_q), 137.32 (CH), 128.54 (2 CH), 127.9 (CH), 127.77 (2 CH), 126.5-126.1 (^1J(C,C) = 48 Hz, CH), 72.4 (d, ^2J(C,C) = 1.2 Hz, CH_2), 69.9 (t,^1J(C,D) = 21.7 Hz, ^{13}CHD), 68.37 (^3J(C,H) = 6 Hz, CH), 23.3 (CH_3) ppm. MS (EI, 70 eV): m/z (%) = 194 (<1) [M^+], 165 (46), 145 (5), 135 (45), 131 (46), 115 (4), 101 (21), 91 (100), 75 (15), 73 (15), 65 (5).

[2,5-$^{13}C_2$]-5- Benzyloxy-3-penten-2-ol (**41c**): Yield (235 mg, 1.21 mmol, 60 %), GC (BPX-5): I = 1584. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.36-7.26 (m, 5 H, 5 CH), 5.85-5.76 (m, 2 H, 2 CH), 4.53 (d, ^2J(C,H)= 4.3 Hz, 2 H, CH_2), 4.46-4.21 (dm, ^2J(C,H)= 142 Hz, 1H, CH), 4.13-3.91 (dm, ^2J(C,C) = 142 Hz, 1 H, ^{13}CHD), 1.29 (dd, ^1J(C,H) = 6.4 Hz,^4J(C,H) = 4.4 Hz, 3 H, CH_3) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ= 137.38-137.09 (dd, ^1J(C,C) = 47 Hz, ^2J(C,C) = 1.4 Hz CH), 130.80 (d,^{3}J(C,C) = 3.5 Hz, C_q), 128.55 (2 CH), 127.9 (2 CH), 127.8 (CH), 126.58-126.28 (^1J(C,C) = 48 Hz, ^2J(C,C) = 1.4 Hz , CH), 72.5 (d, ^2J(C,C) = 1.5 Hz, CH_2), 70.27 (d, ^1J(C,H)= 6 Hz, ^{13}CH), 68.41 (d,^1J(C,H) = 6 Hz, ^{13}CH), 23.3 (d, ^1J(C,C)= 39 Hz,CH_3) ppm. MS (EI, 70 eV): m/z (%) = 178 (<1) [M$^+$], 161 (4), 108 (27), 107 (19), 92 (34), 91 (100), 79 (10), 77 (6), 65 (8), 44 (7).

General Procedure for the Preparation of Isotopologues of 5-Benzyloxy-3-penten-2-one:

To a 0.5 M solution of IBX (3.5 eq) in dry DMSO were added **41a-c** (1.0 eq). The reaction mixture was stirred overnight at room temperature, filtered under vacuum on a sintered funnel covered by paper and diluted by water. The aqueous layer was extracted with ethyl acetate (3 x). The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo*. Column chromatography on silica gel (cyclohexane/ethyl acetate 85:15) afforded **42a-c** as colourless oils. TLC (cyclohexane/ethyl acetate 75:25): R_f = 0.37 [**19**]

(5*R*)-(5-^{13}C, 5-^{2}H)-5- Benzyloxy-3-penten-2-one (**42a**): Yield (320 mg, 1.66 mmol, 84 %), GC (BPX-5): I = 1617. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.39-7.28 (m, 5 H, 5 CH), 6.83-6.77 (dt, ^3J(H,H) = 16 Hz, ^2J(C,H) = 5.25 Hz, ^3J(H,H) = 4.7, 1H, CH), 6.38-6.32 (ddd, ^3J(H,H) = 16 Hz, ^3J(C,H) = 6 Hz, ^4J(H,H) = 1.9 Hz, 1H, CH), 4.57 (d, ^3J(C,H) = 4.25 Hz, 2 H, CH_2), 4.35-4.02 (ses., ^2J(H,D) = 2.25 Hz, ^1J(C,H) = 141.36 Hz, 1 H, ^{13}CHD), 2.27 (s, 3 H, CH_3) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 198.35 (d, ^3J(C,C)= 6.0 Hz, C_q), 143.0 (d, ^1J(C,C)= 46.0 Hz, CH), 137.74 (^2J(C,C)= 2.8 Hz, CH), 130.6 (C_q), 128.64 (2 CH), 128.04 (CH), 127.84 (2 CH), 73.02 (d, ^2J(C,C)= 1.0 Hz, CH_2), 68.59 (t, ^1J(C,D)= 21.6 Hz, ^{13}CHD), 27.42 (CH_3) ppm. MS (EI, 70 eV): m/z (%) = 192 (<1) [M$^+$], 145 (5), 134 (7), 106 (7), 105 (8), 92 (19), 91 (100), 86 (10), 79 (6), 77 (12), 65 (7), 51 (5), 43 (11).

(5*S*)-(5-^{13}C, 5-^{2}H)-5- Benzyloxy-3-penten-2-one (**42b**): Yield (528 mg, 2.75 mmol, 95 %), GC (BPX-5): *I* = 1614. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.39-7.28 (m, 5 H, 5 CH), 6.83-6.77 (dt, ^{3}J(H,H) = 16 Hz, ^{2}J(C,H) = 5.25 Hz, ^{3}J(H,H) = 4.7, 1H, CH), 6.38-6.32 (ddd, ^{3}J(H,H) = 16 Hz, ^{3}J(C,H) = 6 Hz, ^{4}J(H,H) = 1.9 Hz, 1H, CH), 4.57 (d, ^{3}J(C,H) = 4.25 Hz, 2 H, CH_2), 4.35-4.02 (ses., ^{2}J(H,D) = 2.25 Hz, ^{1}J(C,H) = 141.36 Hz, 1 H, ^{13}CHD), 2.27 (s, 3 H, CH_3) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 198.35 (d, ^{3}J(C,C)= 6.0 Hz, C_q), 143.0 (d, ^{1}J(C,C)= 46.0 Hz, CH), 137.74 (d,^{2}J(C,C)= 2.8 Hz, CH), 130.6 (C_q), 128.64 (2 CH), 128.04 (CH), 127.84 (2 CH), 73.02 (d, ^{2}J(C,C)= 1.0 Hz, CH_2), 68.59 (t, ^{1}J(C,D)= 21.6 Hz, ^{13}CHD), 27.42 (CH_3) ppm. MS (EI, 70 eV): m/z (%) = 192 (<1) [M^+], 145 (5), 134 (7), 106 (7), 105 (8), 92 (19), 91 (100), 86 (10), 79 (6), 77 (12), 65 (7), 51 (5), 43 (11).

(2,5-$^{13}C_2$)-5- Benzyloxy-3-penten-2-one (**42c**): Yield (165 mg, 0.86 mmol, 75 %), GC (BPX-5): *I* = 1616. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.39-7.28 (m, 5 H, 5 CH), 6.85-6.76 (dddt, ^{3}J(H,H) = 16 Hz, ^{2}J(C,H) = 6.4 Hz, ^{3}J(C,H) = 5.1, ^{3}J(H,H) = 4.5, 1H, CH), 6.39-6.32 (dddt, ^{3}J(H,H) = 16 Hz, ^{2}J(C,H) = 6.4 Hz, ^{3}J(C,H) = 2.8, ^{4}J(H,H) = 1.9, 1H, CH), 4.58 (d, ^{3}J(C,H) = 4.2 Hz, 2 H, CH_2), 4.36-4.05 (dddd, ^{1}J(C,H) = 142 Hz, ^{3}J(H,H) = 4.4 Hz, ^{4}J(H,H) = 1.9 Hz, ^{2}J(H,H) = 1.2 Hz, 1 H, $^{13}CH_2$), 2.27 (d,^{1}J(C,H) = 1.2 Hz, 3 H, CH_3) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 198.36 (d, ^{1}J(C,C)= 6.0 Hz, C_q), 143.3-142.9 (dd, ^{1}J(C,C)= 46.0 Hz, ^{2}J(C,C)= 1.8 Hz, CH), 137.8 (d,^{2}J(C,C)= 2.8 Hz, C_q), 130.7-130.26 (d, ^{1}J(C,C) = 53 Hz, CH), 128.65 (2 CH), 128.04 (CH), 127.83 (2 CH), 69.62 (d, ^{2}J(C,C)= 2.0 Hz, CH_2), 68.95 (d, ^{2}J(C,C)= 6 Hz, $^{13}CH_2$), 27.4 (CH_3) ppm. MS (EI, 70 eV): m/z (%) = 192 (<1) [M^+], 189 (29), 149 (10), 148 (20), 147 (100), 133 (5), 131 (4), 117 (4), 73 (12).

General procedure for the preparation of isotopologues of (3*S*,4*R*)-5-(Benzyloxy)-3,4-dihydroxypentan-2-one: To a 1:1 mixture of distilled water and ^{t}BuOH, AD-mix β (1.6 g $mmol^{-1}$), $K_2OsO_4 \cdot 2H_2O$ (0.1 eq), $NaHCO_3$ (3.0 eq) and $MeSO_2NH_2$ (1.0 eq) were added and cooled down at 0 °C. After 30 min, compounds **42a-c** dissolved in a small amount of toluene were added dropwise to give a final concentration of 0.8 M of ketone in the reaction mixture. The reaction was stirred overnight at 0 °C and then diluted with ethyl acetate. $Na_2S_2O_5$ (8.0 eq) was added carefully in small portions and extracted with ethyl acetate (3x), dried over $MgSO_4$

and concentrated *in vacuo*. Column chromatography on silica gel (cyclohexane/ethyl acetate 65:35) afforded **43a-c** as colourless oils. TLC (cyclohexane/ethyl acetate 6:4): R_f = 0.25 [**19**]

(5-^{13}C, 5-^{2}H)-(3*S*,4*R*,5*R*)-5-(Benzyloxy)-3,4-dihydroxypentan-2-one (**43a**): Yield (337 mg, 1.49 mmol, 93 %). $[\alpha]_D^{20}$ = +35.7 (*c* 10.4 in $CHCl_3$). GC (BPX-5, MSTFA): I = 1929. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.39-7.27 (m, 5H, 5 CH), 4.57 (d, ^3J(C,H)= 4.0 Hz, 2 H, CH_2), 4.24 (br s, 1H, CH), 4.21 (br s, 1H, CH), 3.76-3.48 (dd, ^3J(H,D)= 4.6 Hz, ^1J(C,H)= 141.3 Hz, 1 H, ^{13}CH), 2.28 (s, 3 H, CH_3). ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 208.2 (d, ^3J(C,C)= 3.0 Hz, C_q), 137.75 (d, ^3J(C,C)= 2.5 Hz, C_q), 128.66 (2 CH), 128.10 (CH), 128.02 (2 CH), 77.28 (CH), 73.78 (d, ^2J(C,C)= 1.3 Hz, CH_2), 70.8 (t, ^1J(C,D)= 22.0 Hz, ^{13}CH), 25.74 (CH_3) ppm. MS (EI, 70 eV): m/z (%) = 226 (<1) [M$^+$], 218 (34), 193 (4), 179 (4), 147 (5), 91 (100), 73 (25).

(5-^{13}C, 5-^{2}H)-(3*S*,4*R*,5*S*)-5-(Benzyloxy)-3,4-dihydroxypentan-2-one (**43b**): Yield (536 mg, 2.37 mmol, 88 %). $[\alpha]_D^{20}$ = +32.9 (*c* 11.35 in $CHCl_3$). GC (BPX-5, MSTFA): I = 1929. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.38-7.29 (m, 5H, 5 CH), 4.57 (d, ^3J(C,H)= 4.0 Hz, 2 H, CH_2), 4.24 (t, 1H, CH), 4.20 (m, 1H, CH), 3.75-3.46 (dd, ^1J(H,D)= 6.5 Hz, ^1J(C,H)= 143.5 Hz, 1 H, ^{13}CHD), 2.28 (s, 3 H, CH_3). ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 208.2 (d, ^3J(C,C)= 3.0 Hz, $^{13}C_q$), 137.75 (d, ^3J(C,C)= 2.5 Hz, C_q), 128.66 (2 CH), 128.10 (CH), 128.02 (2 CH), 77.28 (CH), 73.78 (d, ^2J(C,C)= 1.3 Hz, CH_2), 70.8 (t, ^1J(C,H)= 22.0 Hz, $^{13}CH_2$), 25.76 (CH_3) ppm. MS (EI, 70 eV): m/z (%) = 226 (<1) [M$^+$], 218 (31), 193 (4), 179 (4), 147 (5), 92 (10), 91 (100), 73 (25).

(2,5-$^{13}C_2$)- (3*S*,4*R*)-5-(Benzyloxy)-3,4-dihydroxypentan-2-one (**43c**): Yield (95 mg, 0.42 mmol, 54 %). $[\alpha]_D^{20}$ = +35.1 (*c* 5.98 in $CHCl_3$). GC (BPX-5, MSTFA): I = 1929. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.39-7.28 (m, 5H, 5 CH), 4.58 (d, ^3J(C,H)= 4.0 Hz, 2 H, CH_2), 4.24 (m, 1H, CH), 4.20 (m, 1H, CH), 3.78 (dd, ^1J(C,H)= 6.1 Hz, ^2J(H,H)= 1 H, $^{13}CH_2$), 3.52-3.46 (dd, ^1J(C,H)= 6.1 Hz, ^4J(H,H)= 1.8 Hz, 1 H, $^{13}CH_2$), 2.28 (d, ^2J(C,H)= 6.1 Hz, 3 H, CH_3). ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 208.17 (d, ^3J(C,C)= 3.1 Hz, $^{13}C_q$), 137.75 (d, ^3J(C,C)= 2.5 Hz, C_q), 128.67 (2 CH), 128.11 (CH), 128.02 (2 CH), 71.16 (d, ^1J(CH) = 3.1 Hz, $^{13}CH_2$), 73.81 (d, ^2J(C,C)= 1.6 Hz, CH), 70.9 (d, ^2J(C,H)= 1.5 Hz, $^{13}CH_2$), 25.69 (d, ^1J(C,H)= 42.0 Hz, CH_3) ppm. MS (EI, 70 eV): m/z (%) = 226 (<1) [M$^+$], 224 (6), 220 (10), 219 (36), 204 (4), 193 (5), 179 (4), 147 (14), 103 (4), 92 (9), 91 (100), 73 (26).

General Procedure for the Preparation of 1-deoxy-D-xylulose isotopologues: To a 0.2 M solution of compounds **43a-c** in ^{i}PrOH and water (9:1) a catalitic amount of Pd/C on activated charcoal was added (5 wt.%, 0.1 eq). The reaction mixture was stirred in a high-pressure reactor (HR-100, Berghof, Eningen) under a hydrogen atmosphere (40 bar) at 40 °C until consumption of the starting material. The mixture was filtered through a short pad of celite with ^{i}PrOH yielding 13a-c as colourless oils. TLC (ethyl acetate): R_f = 0.14. Warning: 1-deoxy-D-xylulose derivatives decompose during column chromatography. [**19**]

(5*R*)-(5-^{13}C, 5-^{2}H)-1-deoxy-D-xylulose (**44a**): Yield (182 mg, 1.49 mmol, 96 %). $[\alpha]_D^{20}$ = + 2.8 (*c* 15.5a in MeOH). ^{1}H-NMR (500 MHz, D_2O): δ = 4.43 (t, ^{3}J(H,H)= 2.0 Hz, 1 H, CH), 4.20 (dt, ^{2}J(C,H) = 5.7, ^{3}J(H,H) = 5.2 Hz, ^{3}J(H,H) = 2.0 Hz, 1H, CH), 3.86-3.57 (dd, ^{3}J(H,H)= 5.2 Hz, ^{1}J(^{13}C,H)= 141.3 Hz, 1 H, ^{13}CHD), 2.31 (s, 3 H, CH_3). ^{13}C-NMR (125 MHz, D_2O): δ = 213.2 (d, ^{3}J(C,C)= 3.0 Hz, C_q), 71.16 (t, ^{2}J(C,C)= 23 Hz, CH), 69.2 (t, ^{1}J(C,C)= 23 Hz, CH), 61.86 (t, ^{1}J(C,C)= 23 Hz, ^{13}CHD), 25.70 (CH_3) ppm. MS (ESI) m/z [M+Na]+calcd. 159.05, obsd. 159.058

(5*S*)-(5-^{13}C, 5-^{2}H)- 1-deoxy-D-xylulose (**44b**): Yield (303 mg, 2.37 mmol, 96 %). $[\alpha]_D^{20}$ = + 2.6 (*c* 13.86 in MeOH). ^{1}H-NMR (500 MHz, D_2O): δ = 4.43 (t, ^{3}J(H,H)= 2.0 Hz, 1 H, CH), 4.20 (dt, ^{2}J(C,H) = 5.7 Hz, ^{3}J(H,H) = 5.2 Hz, ^{3}J(H,H) = 2.0 Hz, 1H, CH), 3.86-3.57 (dd, ^{3}J(H,H)= 5.2 Hz, ^{1}J(^{13}C,H)= 141.3 Hz, 1 H, ^{13}CHD), 2.31 (s, 3 H, CH_3). ^{13}C-NMR (125 MHz, D_2O): δ = 213.03 (d, ^{3}J(C,C)= 3.0 Hz, C_q), 71.10 (t, ^{2}J(C,C)= 23 Hz, CH), 69.13 (t, ^{1}J(C,C)= 23 Hz, CH), 61.85 (t, ^{1}J(C,C)= 23 Hz, ^{13}CHD), 25.70 (CH_3) ppm. MS (ESI) m/z [M+Na]+calcd. 159.05, obsd. 159.057

(2,5-$^{13}C_2$)- 1-deoxy-D-xylulose (**44c**): Yield (54 mg, 0.42 mmol, 99 %). $[\alpha]_D^{20}$ = + 2.8 (*c* 15.5a in MeOH). ^{1}H-NMR (500 MHz, D_2O): δ = 4.43 (dt, ^{3}J(C,H)= 3.7 Hz ,^{3}J(H,H)= 2.0 Hz, 1 H, CH), 4.21 (dddd, ^{2}J(C,H) = 7.4 Hz, ^{3}J(H,H) = 5.6 Hz, ^{3}J(C,H) = 3.9 Hz, ^{3}J(H,H) = 1.9 Hz, 1H, CH), 3.82-3.50 (dm, ^{1}J(^{13}C,H)= 141 Hz, 1 H, $^{13}CH_2$), 2.30 (d, ^{2}J(C,H) = 6 Hz, 3 H, CH_3). ^{13}C-NMR (125 MHz, D_2O): δ = 213.01 (d, ^{1}J(C,C)= 3.1 Hz, C_q), 70.46 (d, ^{1}J(C,C)= 245.7 Hz, CH), 62.93 (dd, ^{1}J(C,C)= 21.2 Hz, ^{2}J(C,C)= 2.3 Hz, CH), 62.8 (d, ^{1}J(C,H)= 3.2 Hz, $^{13}CH_2$), 25.70 (d, ^{1}J(C,C)= 41 Hz, CH_3) ppm. MS (ESI) m/z [M+Na]+calcd. 159.05, obsd. 159.055

Synthesis of (1,4-$^{13}C_2$) dimethylfumarate (45): To a solution of [1,4-$^{13}C_2$] fumaric acid (**15**) in dry MeOH (40 mL), trimethylsilylchloride was added (2.17 mL, 16.94 mmol, 2 eq). The reaction mixture was stirred overnight at room temperature and then quenched with water. After concentration, the residue was extracted with diethyl ether and brine. The organic layers were dried over $MgSO_4$ and concentrated *in vacuo* affording **15** (1.21 g, 8.28 mmol, 98%) a white powder. Caution: The compound is highly volatile. Do not concentrated with high vacuum. TLC (cyclohexane/ethyl acetate 8:2): R_f= 0.65.GC (BPX-5): *I*= 1024. ^{1}H-NMR (500 MHz, $CDCl_3$): δ= 6.86 (t, ^2J(C,H)= 4.7 Hz, 2 H, 2 CH), 3.81 (d, ^3J(C,H)= 3.8 Hz, 6 H, 2 CH_3) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): 165.5 ($^{13}C_q$), 133.86-133.26 (d, ^1J(C,C)= 75.4 Hz, 2 CH), 52.48 (t, ^2J(C,C)= 1.0 Hz, CH_3) ppm. MS (EI, 70 eV): m/z (%) = 146 (2) [M^+], 116 (20), 115 (100), 86 (35), 60 (10). [**24**]

Synthesis of (1,4-$^{13}C_2$)-l-but-2-en-1,4-diol (46): To a solution of [1,4-$^{13}C_2$] dimethylfumarate in dry THF (40 mL) at 0 °C, DIBAL-H (1.0 M sol in hexane, 33.02 mL, 5.5 eq) was added dropwise. After 3h, the reaction was quenched with ethyl acetate (5.5 eq) and aqueous 1.0 M solution of hydrochloric acid (5.5 eq). The reaction mixture was concentrated *in vacuo* and extracted several times with ethyl acetate. The organic layers were dried over $MgSO_4$ and concentrated *in vacuo* affording **17** (622 mg, 6.90 mmol, 84%) as a pale-yellow oil. TLC (ethyl acetate): R_f= 0.24. GC (BPX-5, MSTFA): *I*=1475 . ^{1}H-NMR (500 MHz, MeOD): δ= 5.83 (m, 2 H, 2 CH), 4.22 (m, 2 H, CH_2), 3.94 (m, 2 H, CH_2) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ 167.2 (d, J(C,C)= 6.9 Hz, CH), 131.3 (d, J(C,C)= 47.78 Hz, CH), 63.07 (s, 2 $^{13}CH_2$) ppm. [**25**]

Synthesis of (1,4-$^{13}C_2$)-4-Benzyloxybut-2-en-1-ol (**48**): To a solution of **17** (591 mg, 6.56 mmol, 1.0 eq) in dry DCM (33 mL), Ag_2O (2.28 g, 9.84 mmol, 1.5 eq) was added under stirring at room temperature. After 30 min, Benzyl bromide (863.1 µL, 7.22 mmol, 1.1 eq) was added. The reaction mixture proceeds overnight at room temperature. The crude was filtered on silica pad and concentrated *in vacuo*. Column chromatography on silica gel (cyclohexane/ethyl acetate 6:4) afforded **8c** (467 mg, 2.59 mmol, 39%) as a colourless oil. TLC (cyclohexane/ethyl acetate 7:3): R_f = 0.19. GC (BPX-5): *I*=1568. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.39-7.26 (m, 5 H, 5 CH), 5.97-5.81 (m, 2 H, 2 CH), 4.53 (d, ^3J(C,H)= 4 Hz, 2 H, CH_2),

4.32-4.01 (dddd, ^{1}J(C,H) = 142 Hz, ^{3}J(H,H) = 5.4 Hz, ^{4}J(H,H) = 1.9 Hz, ^{2}J(H,H) = 0.9 Hz, 2 H, $^{13}CH_2$), 4.20-3.89 (ddp, ^{1}J(C,H) = 142 Hz, ^{3}J(H,H) = 5.4 Hz, ^{2}J(H,H) = 1.0 Hz, 2 H, $^{13}CH_2$) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ= 138.34 (d, ^{3}J(C,C) = 3 Hz, C_q), 132.48-132.10 (d, ^{1}J(C,C) = 46 Hz, CH), 128.55 (2 CH), 128.24-127.85 (d, ^{1}J(C,C) = 46 Hz, CH), 127.89 (2 CH), 127.79 (CH), 70.2 (d, ^{1}J(C,H) = 6.3 Hz, $^{13}CH_2$), 63.19 (d,^{1}J(C,H) = 6.3 Hz, $^{13}CH_2$), 61.98 (dd, ^{2}J(C,C) = 6.3 Hz, ^{5}J(C,C) = 1.2 Hz, CH_2) ppm. MS (EI, 70 eV): m/z (%) = 180 (<1) [M^+], 151 (2.5), 135 (5), 108 (16), 107 (14), 92 (39), 91 (100), 79 (10), 77 (9), 65 (8). [**26**]

N.B.: Compound **17** was turned into **40c** applying the same reaction sequence used for the 95ransformation of **40a-b** into **44a-b**

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3. Feeding experiments on *Fontainea picrosperma* C.T.White

Feeding exogenous precursors in leaving organism represent since the first application in the first half of the XX century a challenge for chemists interested into elucidation of biosynthetic pathway. So far, different established protocols occur in literature providing insight into the administration of labelled compounds. Quite often, these protocols rely on leaves or roots cell cultures where physiological and growth conditions can be controlled very precisely. Unfortunately, an optimized method to produce a cell culture from *F.picrosperma*, the chosen candidate for investigating tiglianes production, was not established yet. Just recently, plant domestication was achieved in Queensland to enhance the production of the experimental drug, EBC-46. [**1**]

Direct application of ^{14}C-amino acids was successfully performed in several experiments to elucidate fatty acids or alkaloids biosynthesis but, even though the low rate of incorporation, the emitted radioactivity has been efficiently used in decomposition study to establish the relative pathway by retro-synthetical analysis. [**2**]

In order to achieve preliminary data about the biosynthesis of tigliane skeleton in the Euphorbiaceuous candidate, *Fontainea picrosperma* C.T. White, a series of different approaches are presented concurrently with relative issued encountered.

3.1 Feeding experiment with $^{13}CO_2$

Exposing plant tissues to $^{13}CO_2$ is reported to be an efficient method to study the biosynthesis of primary and secondary metabolites and can provide, as well, insight about the tissue localization of the pathway itself tracing CO_2-fixation. Heintze and co-workers proved that CO_2 flow takes place more efficiently at the leaf base of seedlings because of active developing chloroplasts with incomplete thylakoid stacking compared to leaf tips, where the thylakoids showed a maximal synthetical photosynthetic activity. [**3**]

Feeding experiments on diatoms pointed out how CO_2-fixation is strictly associated with MEP pathway for isoprenoid by preferential incorporation of carbon dioxide into phytol, the acyclic isoprenoid side chain of chlorophyll. Conversely, feeding with acetate resulted in better incorporation in triterpenoids produced in cytosol, possibly by enzymes involved in fatty acid metabolism. [**4**]

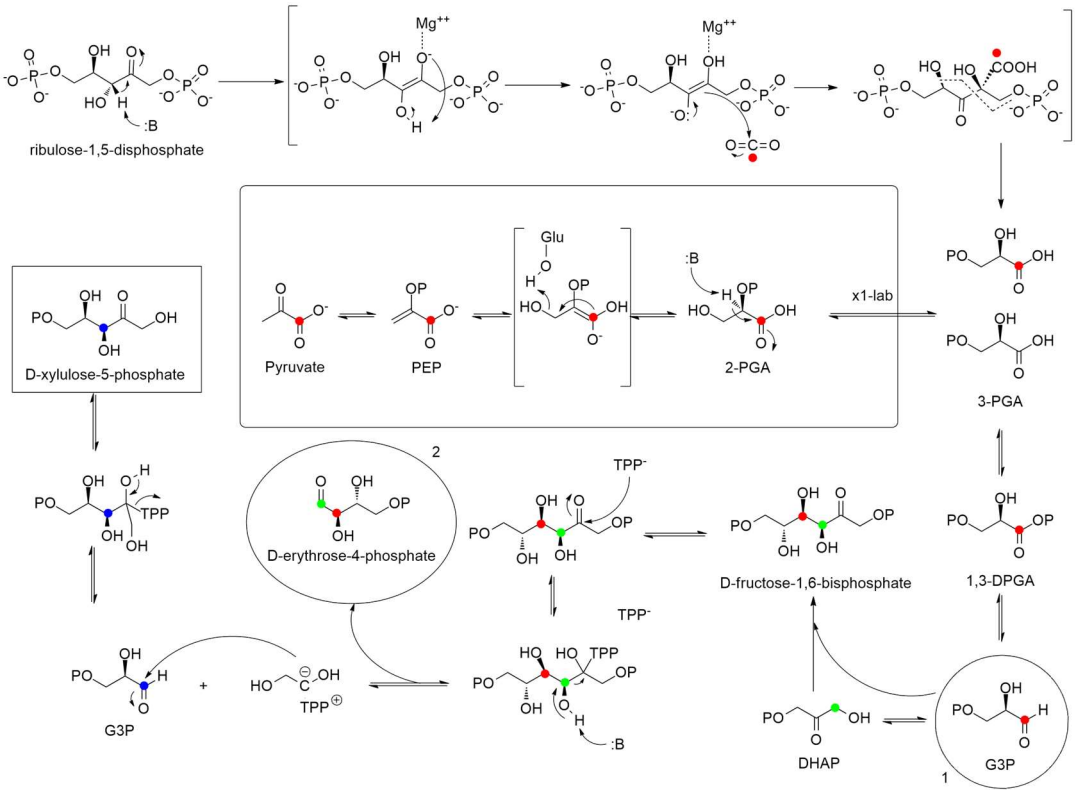

Fig. 3.1 First sequence of reactions aimed to fix carbon dioxide in chloroplasts.

Mono-fixation can be traced by the reaction of CO_2 with ribulose-1,5-disphosphate and the downstream process can be followed in light of Calvin cycle. The production of glyceraldehyde-3-phosphate (G3P) represents the key to have access to the synthesis of DXP and later on IPP/DMAP. (**Fig. 3.1**)

Moreover, the process is aimed to recycle the primary phosphorylated sugar that, subsequentially, can be used to fix another molecule of carbon dioxide. In an atmospheric environment saturated with $^{13}CO_2$, the iterative incorporation would continue until achieving a steady-state condition. A second incorporation of labelled $^{13}CO_2$ is shown in **Fig. 3.2** where complexity arises in terms of labelled positions at the precursors.

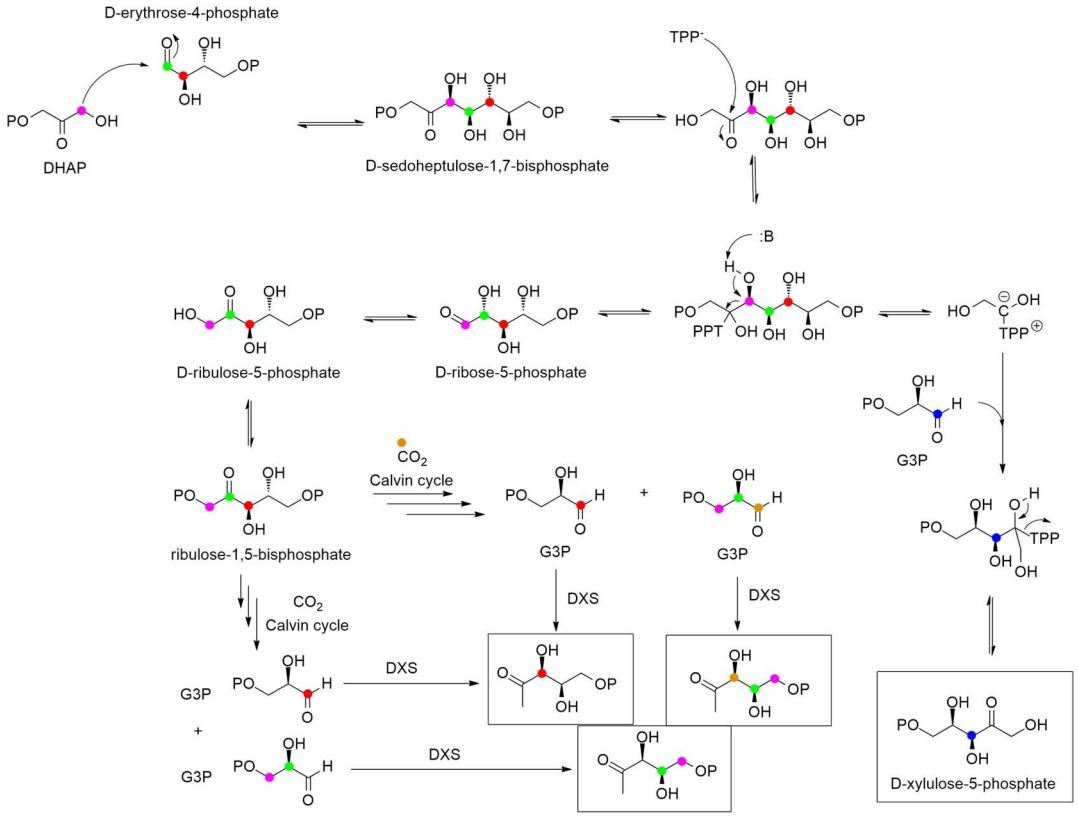

Fig. 3.2 Second sequence of reactions in Calvin cycle increase complex labelling pattern in DXP

A summary of the labelled pattern is presented in **Table 1** to provide a complete series of possibility at any repetition of the biological process. As previously discussed, phorbol esters

occur in Euphorbiaceae and Thymelaceae families as a mixture of di- or tri-esters. In the case of tigilanol tiglate (EBC-46), tiglic and α-methylbutyric acids represent the acyl decorations at C-12 and 13. Plants biosynthesize these compounds starting from L-isoleucine that, in turn, is produced by pyruvic acid and α-ketoglutarate. [**5**] Thus, eventual labelling should be evaluated for the substituents as well.

Noteworthy is C_4-fixation pathway operated by plants in response of low level of atmospheric CO_2 aimed to increase its concentration in bundle sheath cells, eliminating oxygenase activity and hence photorespiration.[**6**]

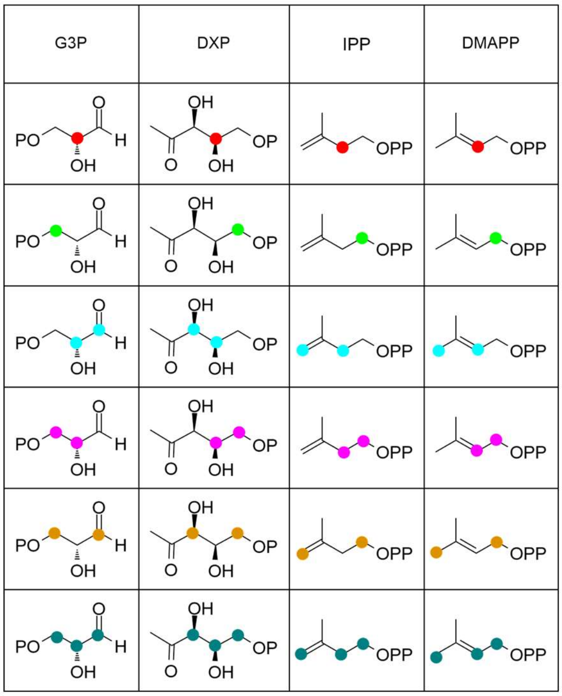

Table. 1 Possible patterns arising from $^{13}CO_2$ fixation in C-3 cycle

3.1.1 Results and discussion

Four specimens of *Fontainea picrosperma* were used by analogy with the experiment designed by Bromand and co-workers applying $^{13}CO_2$ at regular interval to enhance the distribution of fixed carbon in the entire plants. [**7**] All the plants were exposed twice a week at an aliquot of $^{13}CO_2$, produced by injection in a water sealed Perspex box of a solution of H_2SO_4 1 M inside a becher containing $Na_2{}^{13}CO_3$. Every 2/3 days, the box was opened to allow the sampling and plants remained outside the box for 24 h prior repetition of the exposure. (**Fig. 3.3**)

Fig. 3.3 $^{13}CO_2$ feeding experiment in *Fontainea picrosperma*

Among the period of 30 days, leaves and roots were sampled and extracted with methanol and analysed by HRMS-QToF to establish the isotopic ratio of EBC-46, EBC-47 and EBC-83 occurring in a significant amount in *F. picrosperma*. Any consideration of the change in weight of the extract cannot be done because of the variability of the collected sample.

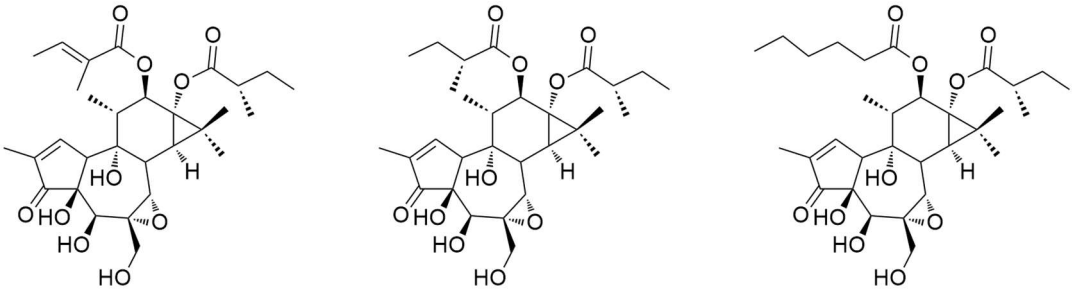

Fig. 3.4 EBC-46 (1), EBC-47 (2), EBC-83 (3)

Analysis of the average values suggests that, aside from an initial increase of the isotopic ratio, the incorporation of labelled $^{13}CO_2$ proceeds in a fluctuating way towards a maximum value reached more than 10 days after the last pulse. The ratio comes back to the initial values after few weeks (60 days from time 0). The relative isotopic ratio between leaves and roots are quite comparable, with generally higher values in leaves. According to the shown trends for EBC-46, EBC-47 and EBC-83 it is not possible to draw the tissue localization of tiglianes production univocally. Leaves are more exposed to the close environment created for the experiments, as proven by the higher value. Conversely, it is known the carbon dioxide can efficiently flow through the soil and exchange gas with the radical apparatus.

Two possibilities are, in conclusion, designed to explain the obtained result. As first, biosynthesis occurs only in one tissue and thanks to phloem/xylem system is delocalized in other sites. Secondly, biosynthesis occurs in both tissues with higher expression in leaves where the biosynthetic machinery affords the primary production.

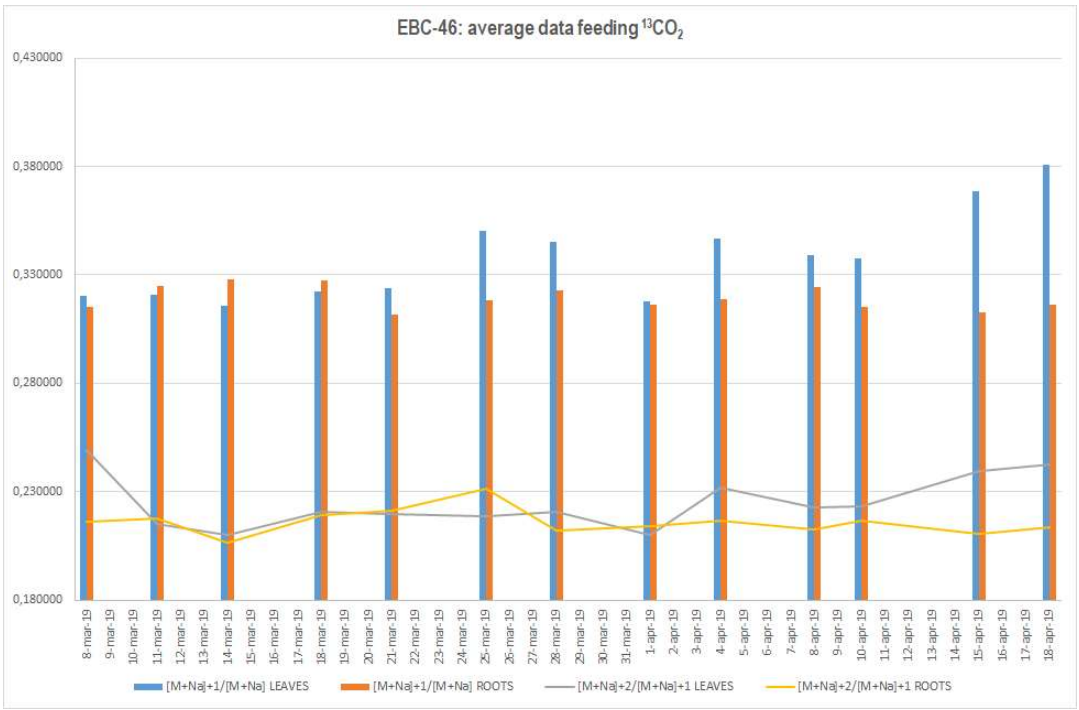

Fig. 3.5 Average values for EBC-46 isotopic ratio in *Fontainea picrosperma*

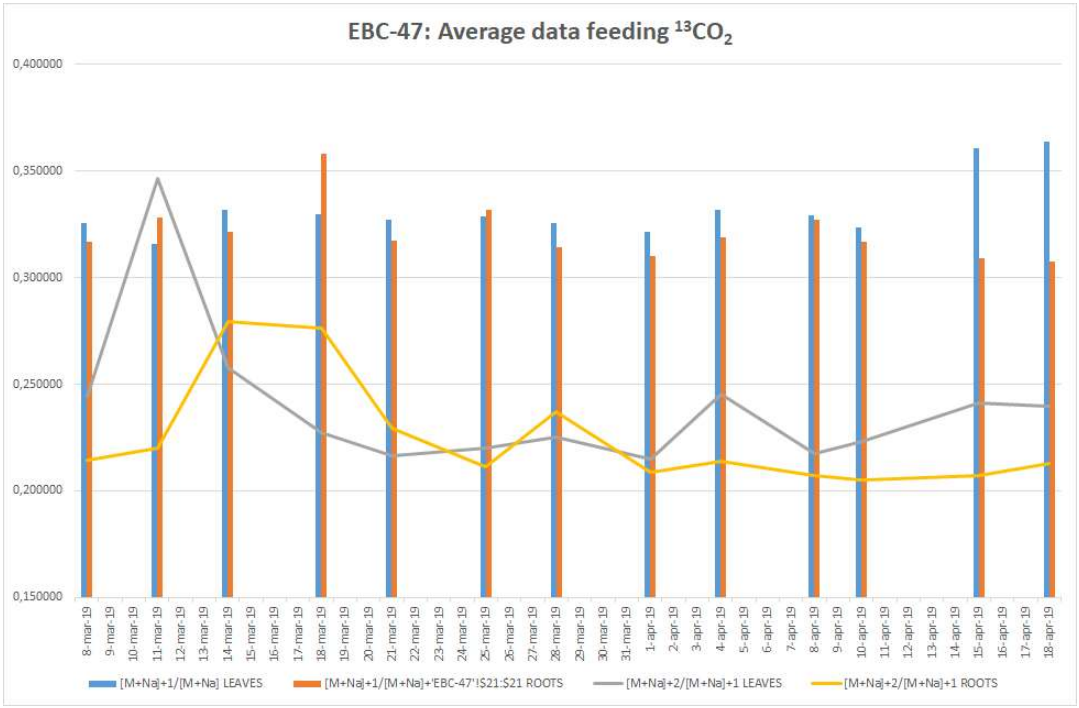

Fig. 3.6 Average values for EBC-47 isotopic ratio in *Fontainea picrosperma*

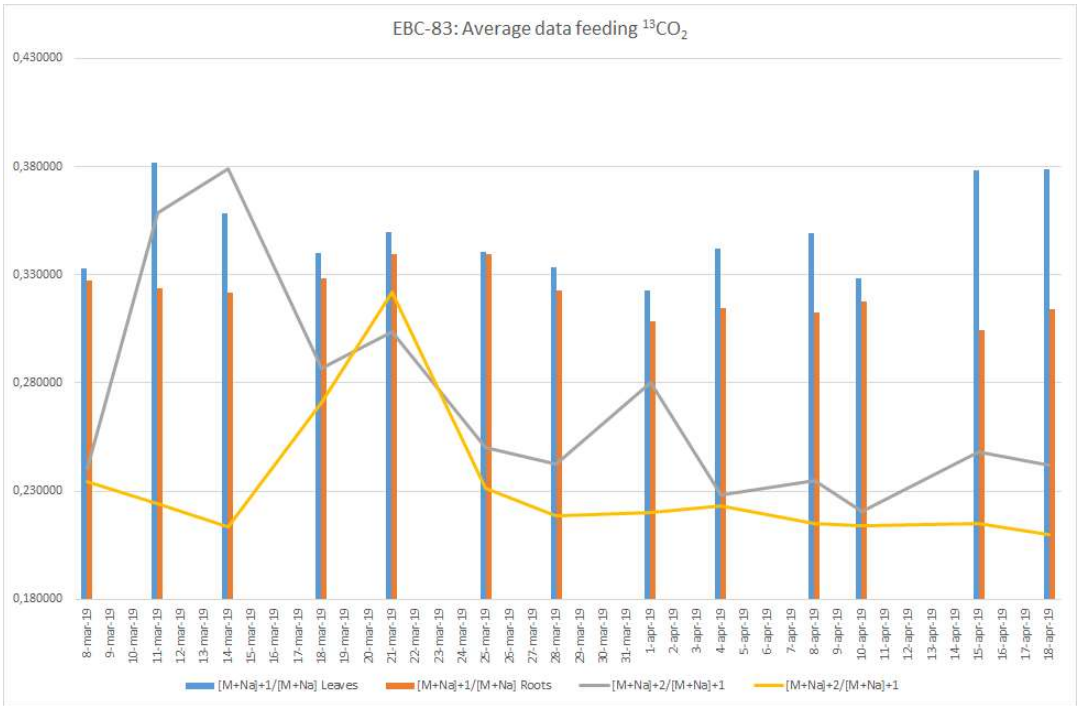

Fig. 3.7 Average values for EBC-83 isotopic ratio in *Fontainea picrosperma*

3.2 Feeding experiments with 1-D-^{13}C-glucose and DXP isotopomers

The use of exogenous glucose in feeding experiments is a well-established method to predict in light of glycolytic pathway, glycogenesis, and the pentose phosphate pathway labelled pattern for isoprenoid precursors (acetyl-coA and pyruvate for MVA pathway or DXP for MEP pathway). In fact, following the downstream glycolytic process it would result in acetyl-coA labelled at C-2 which in MVA pathway could potentially bring at three labelled positions in IPP. (**Fig.3.8A**) Conversely, by condensation of glycerldehyde-3-phosphate and pyruvic acid (PA) in MEP pathway, a different labelling can be predicted for IPP, that could incorporate ^{13}C at C-1 and C-5. (**Fig. 3.8B**) [**8**]

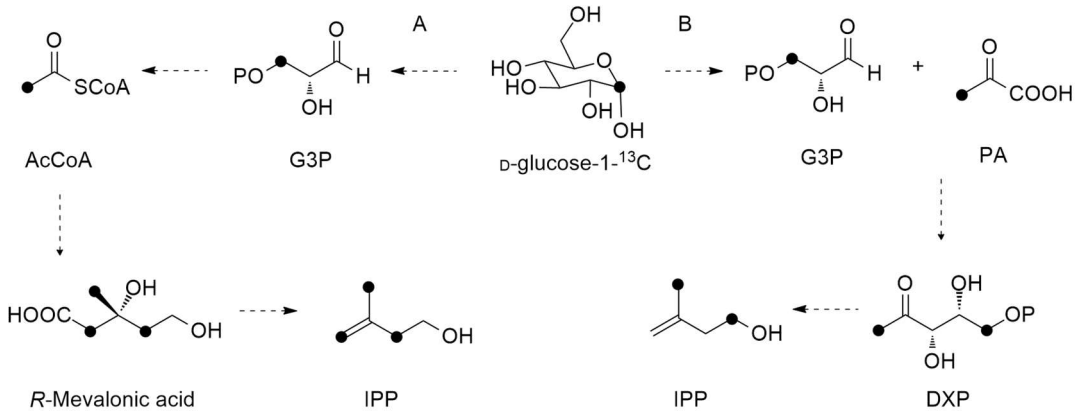

Fig. 3.8 (A) Labelling generated by MVA pathway (B) Labelling generated by MEP pathway.

3.2.1 Results and discussion

During the investigation of the most suitable technique for direct feeding, we have considered germination of *F.picrosperma* seeds in agar medium under controlled temperature and humidity. For this purpose, two seeds were cracked and sterilized using sterile water, EtOH 70 % and bleach 1 %. Seeds were, after drying on sterile paper, laid down on a dish with half-strength Murashige-Skoog basal medium. Unfortunately, after 3 days, fungal contamination starts to spread from the seed coat and cover the entire medium. Seeds collection, re-sterilizing process and inoculation on a new media did not prevent the spreading of the fungi. For this reason and because of the intrinsic difficulties with handling *Fontainea* seeds (ca. 2 cm long), the protocol was discharged.

A group of four specimens, 1 seedling and 3 cuttings at different stages, were used to evaluate the performances of hydroponic system, the technique adopted for growing plant without soil by a water-based mineral nutrient solution. Oxygenation of the water tank was achieved by the use of a fish tank air pump laid directly into the system. Although all the plants look vital and healthy after 15 days, issues regarding the volume of the solution (at least 1.5 L) in which the labelled starting precursors should have been dispersed, led to the decision to change the technique again.

Fig. 3.8 Hydroponic experiment with Fontainea picrosperma

Plant injection is a dispensing mean developed to administrate drugs (against pest or other diseases) directly inside the trunk of big trees. The concentrated fluid is usually vehiculate through under pressure devices and require one or two visits, before completion.

In order to try the feasibility of the method, to one shooting and one cutting with 2.5 cm of the root apparatus, a solution of D-glucose (0.2 mM) in ethanol 10 % aq. was injected inside the stem using an insulin syringe. Stems were scratched and manual pressure was applied to promote the uptake among the day, while an air-filled balloon was connected during the night to maintain constant the administration. After one week the injection came to the end and 48 h more were left to foster the processing of the sugar. Vegetal materials were sampled and finely chopped, stored in silica bags for 48 h to afford a complete drying and extracted several times with methanol. The extract was partitioned to achieve a fraction enriched with phorbol esters and simplify as much as possible the crude extract. Partitioning occured by diluting the crude with a 90 % methanol solution and washing the polar phase with *n*-hexane. Pure methanol brought to the formation of an emulsion with the apolar solvent in which was slightly soluble; after several considerations, we've found that adding a modest percentage of water (10 %) prevents the mixing of the two phases. The apolar fraction was collected separately and analyze it as well, while the polar was diluted with water (ratio 1:1) and re-extracted with dichloromethane. The organic phases were dried and analyze it with UHPLC-QToF to detect the occurrence of EBC-46, EBC-47 and EBC83.

Analysis of the lipophilic and polar fraction of the cutting revealed a complete lack of phorbol esters in the polar phase interestingly, ruling out the possibility to use cuttings as specimens in further experiments. (**Fig. 3.9a and b**) Seedling, otherwise, shown the presence of EBC-46 in the polar fraction as expected and declaring that the newly germinated seeds contain the desired product. (**Fig. 3.10a and b**)

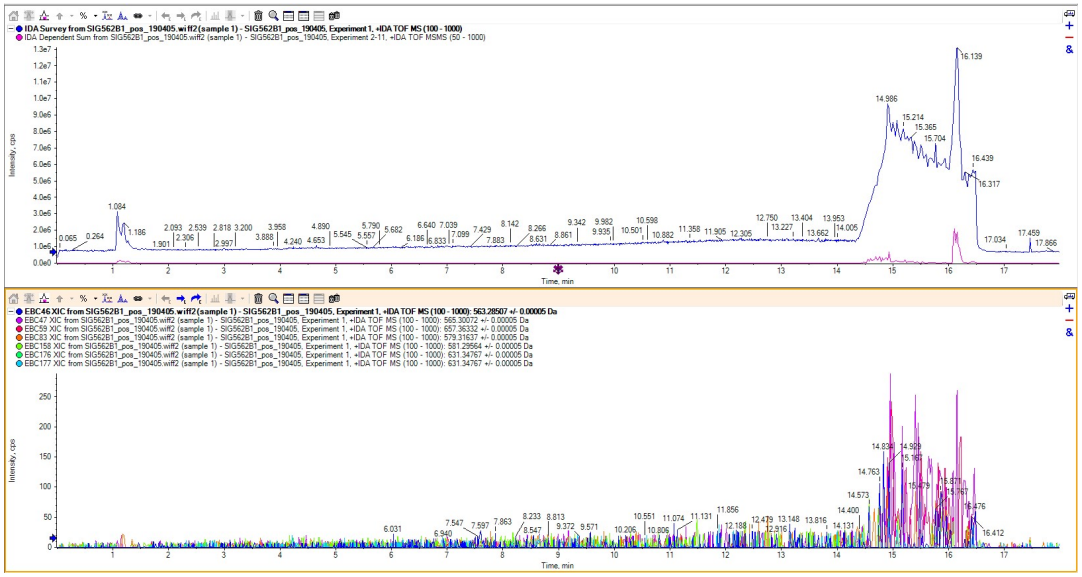

Fig. 3.9a Lipophilic fraction of *F.picrosperma* cutting

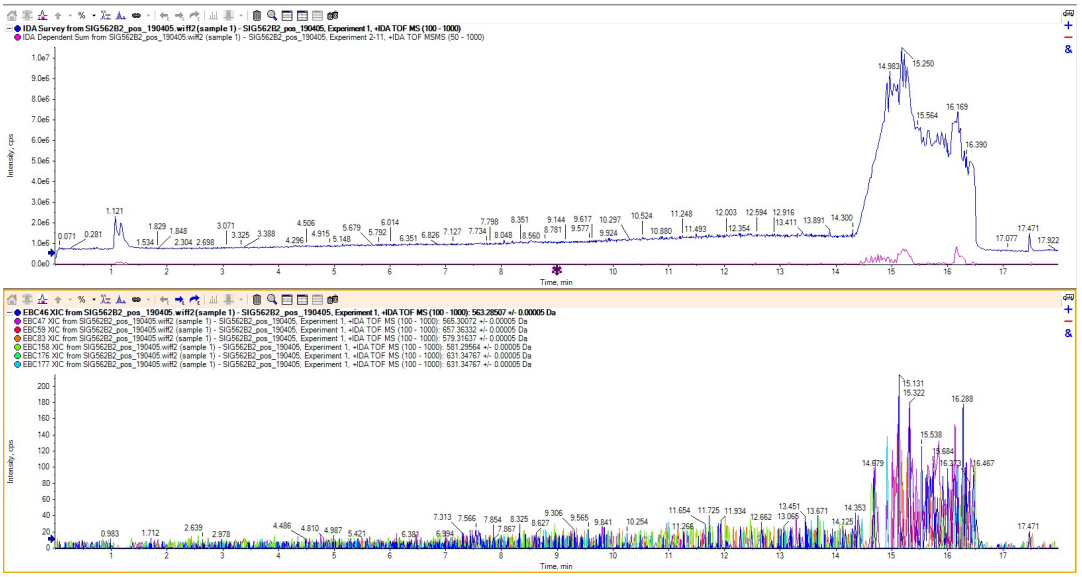

Fig. 3.9b Polar fraction of *F.picrosperma* cutting

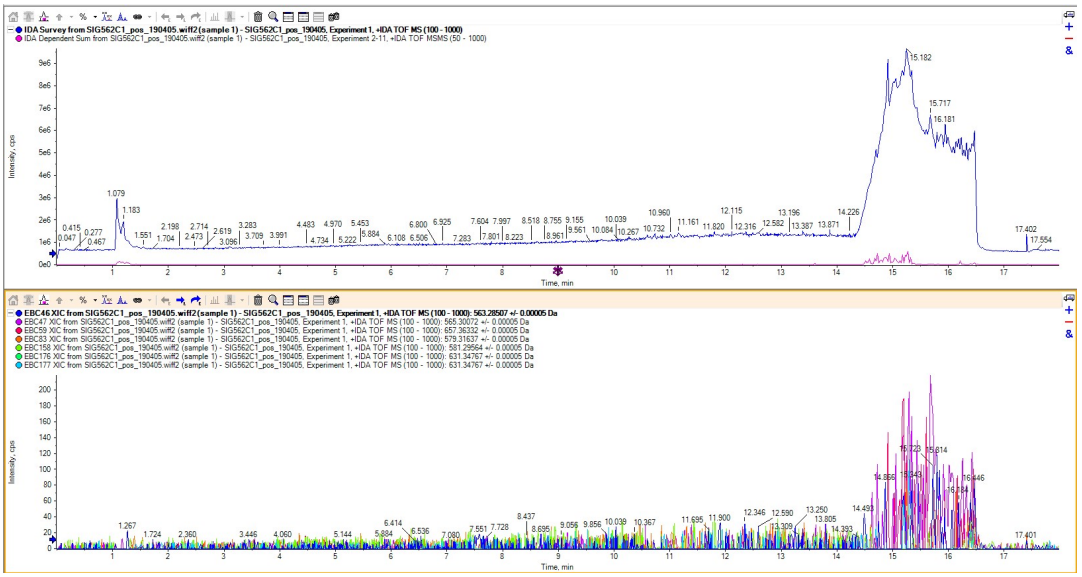

Fig. 3.10 Lipophilic fraction of *F.picrosperma* shooting

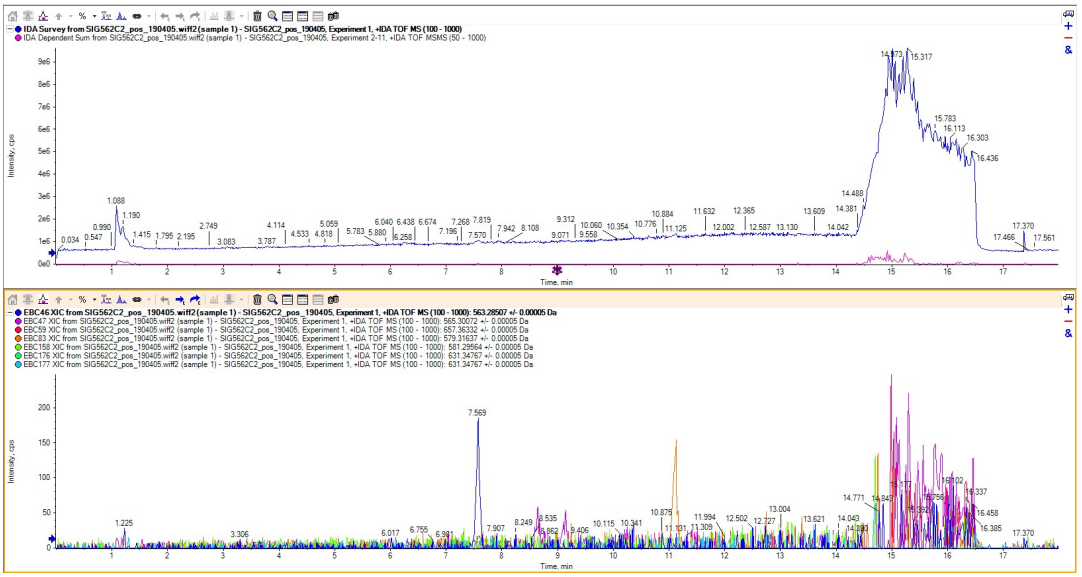

Fig. 3.10b Polar fraction of *F.picrosperma* shooting

A batch of 20 plants 5-months old were injected among a period of 3 weeks (2 weeks for injection, 1 week for setting) with a solution 0.4 mM (36.2 mg in 0.5 mL) of commercially available D-1-^{13}C-glucose under controlled temperature and humidity conditions. The injections were performed by stabbing the stem of the plant and applying manual pressure. Vegetal tissues oppose resistance at the administration but using a pump or applying extra pressure at the piston translate in leaking from the site or injection or damaging the stem itself. Thus, direct injections into the leaves mid-ribs was evaluated to promote the maximum distribution of products in all the different tissues. All the vegetal material was processed as described above in order to obtain an enriched fraction of phorbol esters. The vast plethora of phorbol esters were reduced to one singular molecular entity as described in **Supporting Information 1.2.2.2.** providing 8.5 mg of 5β-hydroxy-6,7α-epoxy phorbol 5,20-acetonide (**SIG 609**).

Due to limited time to perform the experiments and optimizing methods, the DXP isotopomers synthesize in paragraph **2.6** were delivered in three plants each in the same way of glucose experiment.

Labelled DXP	**Mark**	**Quantity**
(5*R*)-(5-^{13}C,5-^{2}H)-1-deoxy-1-D-xylulose	SIG606A	3.1 mg
(5*R*)-(5-^{13}C,5-^{2}H)-1-deoxy-1-D-xylulose	SIG607A	3.4 mg
(2-^{13}C,5-^{13}C)-1-deoxy-1-D-xylulose	SIG608A	0.7 mg

The spectroscopical analysis of 5β-hydroxy-6,7α-epoxy phorbol 5,20-acetonide performed at the University of the Sunshine Coast (Sippy Downs,Australia) with a 400 MHz NMR and at the University of Queensland (Brisbane, Australia) with 900 MHz did not provide any insight in terms of labelled position at the final skeleton, showing a complete overlapping of the signals of the unlabeled material in ^{1}H-spectra (**Fig. 3.11.1**) while weak satellites signals were detected in products obtained with labelling with D-1-^{13}C-glucose and (2-^{13}C,5-^{13}C)-1-deoxy-1-D-xylulose, whose spectra were accumulated longer compared to the others. (**Fig. 3.11.2)**

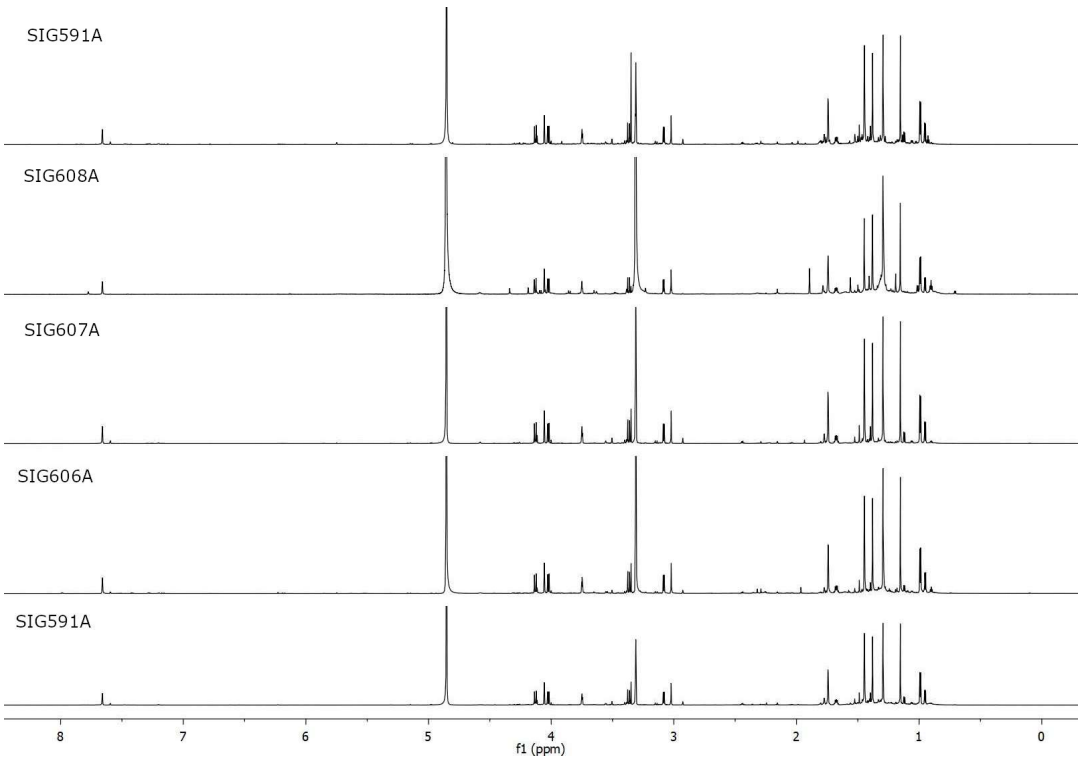

Fig. 3.11.1 ^{1}H-NMRs of 5β-hydroxy-6,7α-epoxy phorbol 5,20-acetonide in MeOD-d_4

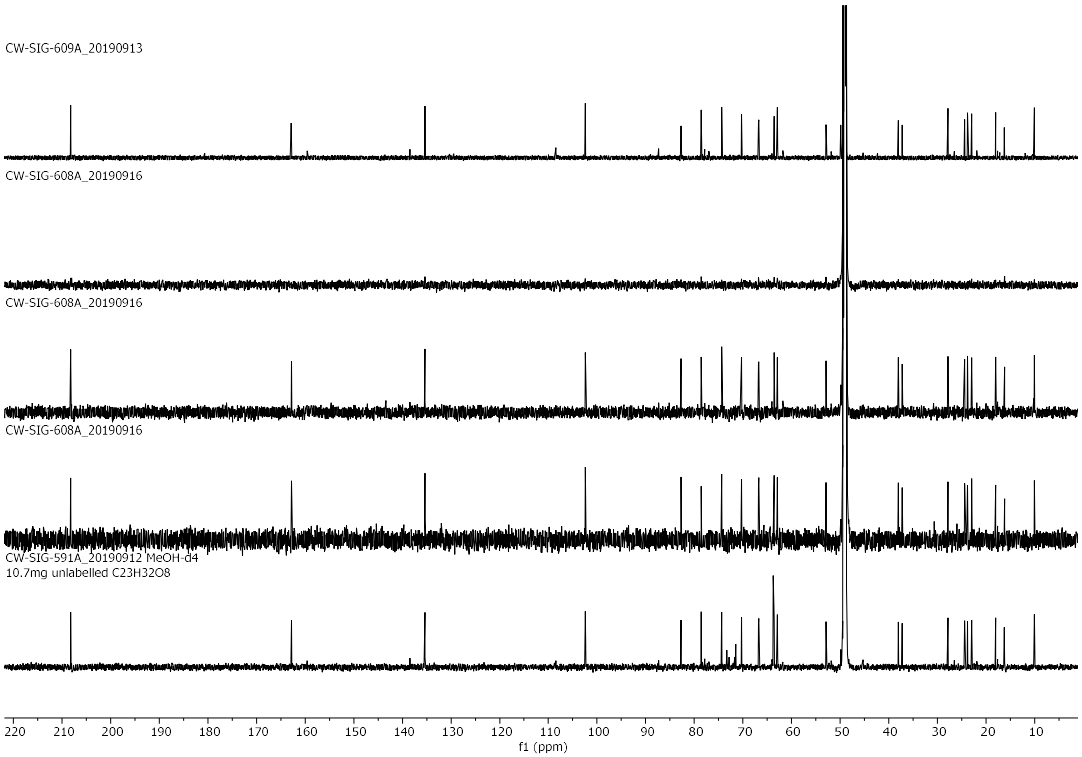

Fig. 3.11.2 Detailed ^{13}C-NMR of SIG 609A and SIG608A in MeOD-d_4

The low enrichment was detected by UHPLC analysis, by the way, analysis of the peak of the product and further MS-MS spectra did not afford a clear pattern of incorporation. By calculation of the isotopic mean between the sodium salt of 5β-hydroxy-6,7α-epoxy phorbol 5,20-acetonide plus one and the sodium salt, an increase of less than 5 % was detected in SIG 609 while a mean of almost 2 % in other DXP isotopomers. Given the natural abundance of ^{13}C in nature, the obtained result confirms the issue in the spectroscopic analysis. (**Fig.**

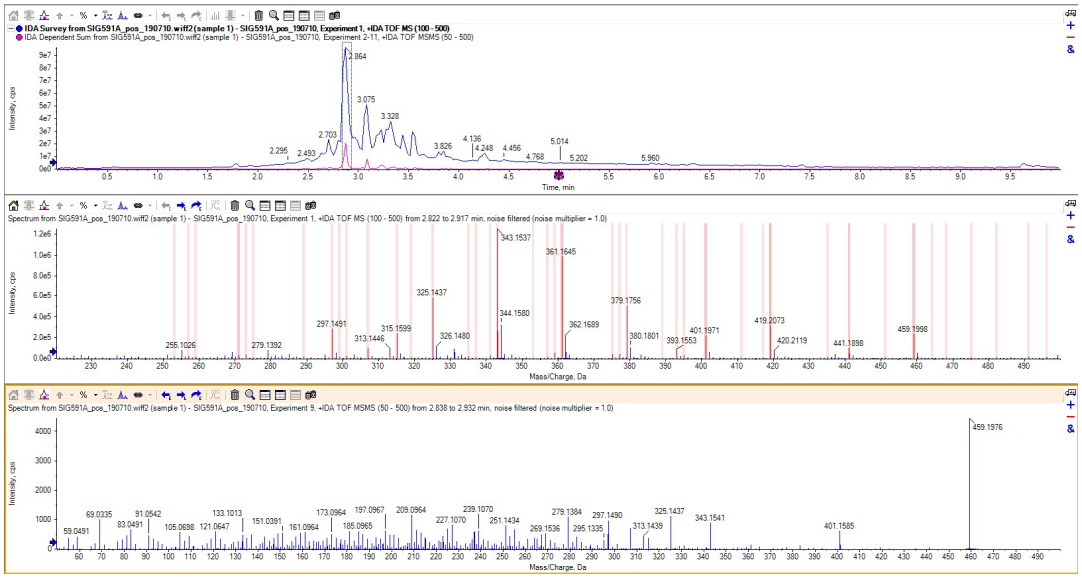

Fig. 3.12.1 HPLC-MS spectra of SIG591A (unlabeled)

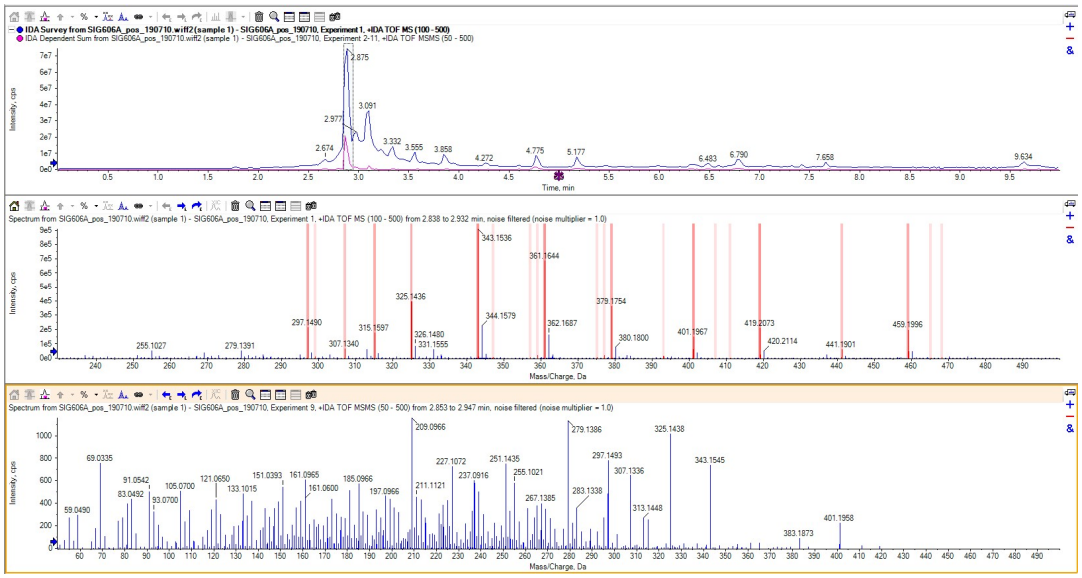

Fig. 3.12.2 HPLC-MS spectra of SIG606A

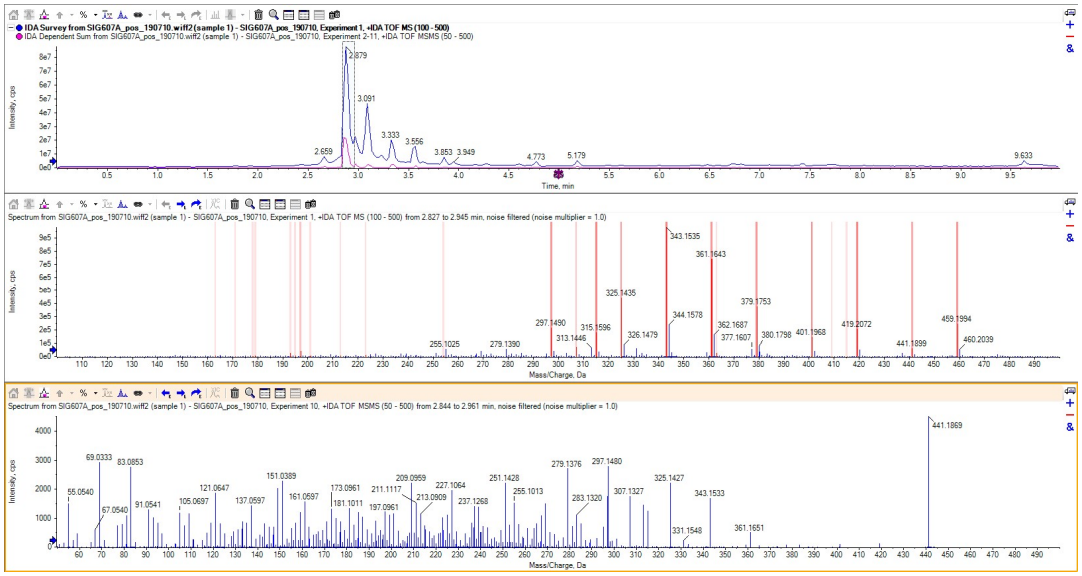

Fig. 3.12.3 HPLC-MS spectra of SIG607A

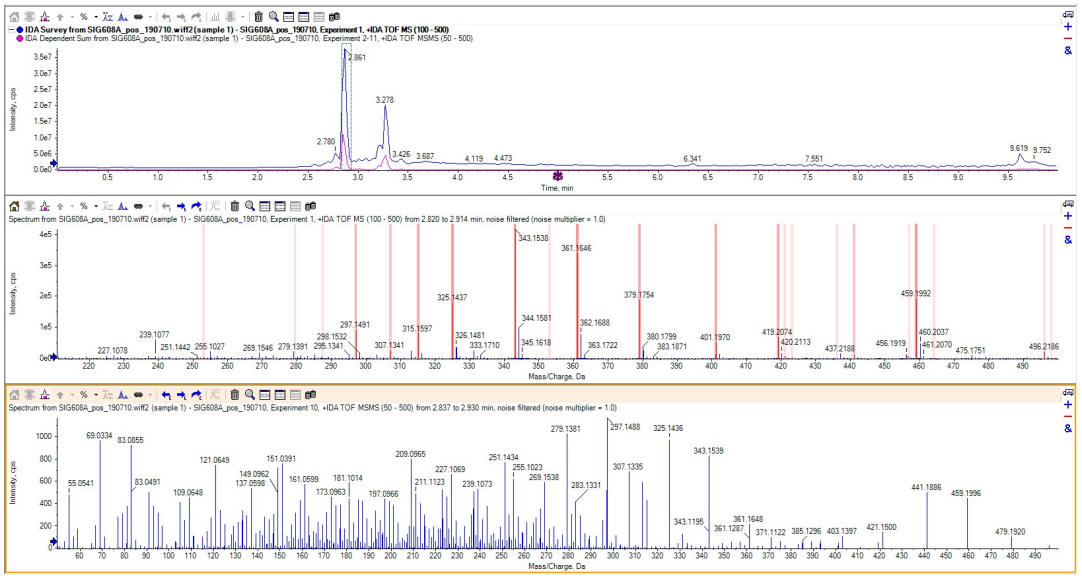

Fig. 3.12.4 HPLC-MS spectra of SIG608A

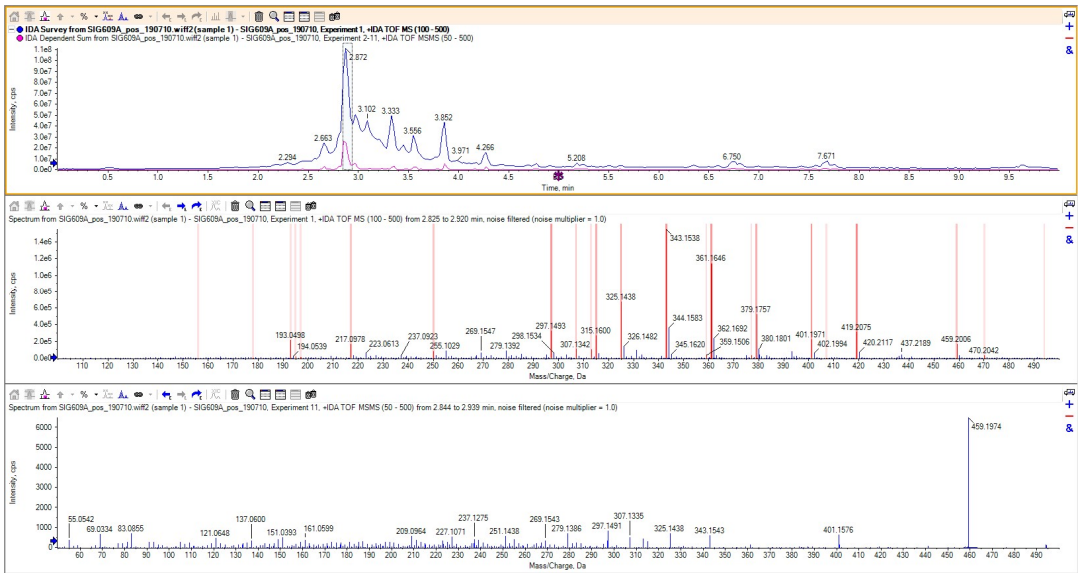

Fig. 3.12.5 HPLC-MS spectra of SIG609A

Interestingly, injection of glucose solution did not seem to interfere with the inner homeostasis of plants (**Fig. 3.13**) while all the experiments performed with labelled DXP produced necrosis of the tissues since 24 h from the first application. The evolution of the necrosis was followed day-by-day during the experiment fearing for the death of the specimens but newly differentiated leaves at the top and the growing axillary buds led us to the conclusion the plants were not under an irreversible condition. (**Fig. 3.14)**

Fig. 3.13 Feeding experiment with D-1-^{13}C-glucose

Fig. 3.14 Example of feeding experiment with DXP

In conclusion, the negligible incorporation of the prospected precursors in tigliane skeleton pointed a series of hypothesis about the production of these compounds. As first, compartmentation effects prevent the access of biosynthetic precursors to biological sites (in this case, plastids). The second hypothesis is related to the production of tigliane diterpenoids at the early stages. Due to the complete lack of diterpenoids in cutting sample analyzed, it has been suggested that plants activate the biosynthetic machinery only later in life (possibly after blossoming or fruiting) in order to selectively delocalize the vesicles containing these secondary metabolites in seeds to protect them by predators or dispersers. After germination, plant distribute these compounds across all the vegetal tissue to ensure protection by herbivores. As third, the established method was not correct for these purposes and no labelled molecule was actually absorbed by the plant; however, extensive tissue necrosis would not be so marked.

3.3 Supporting information

Chemicals and organic solvents were purchased from Cambridge Isotope Laboratories, Inc. (Massachusetts, USA) and Merck (Germany) and used without any further purification. All the samples were analysed by UHPLC, ExionLC (SCIEX, Boston, MA, USA) using a column Halo® RP-Amide (4.6 x 150 mm, 2.7 µm). A SCIEX X500 QTOF System operating in positive ionisation mode was used to analyse the eluted compounds. Formic acid (0.01%) was added to both mobile phases, water (A) and acetonitrile (B). The gradient elution condition was as follow: 58% B (0-13.0 min), 58-95% B (13.0-13.5 min), 95% B (13.5-15 min), 95-45% B (15-15.1 min), 45% B (15.10-18 min). Column oven temperature and autosampler temperature were set at 40 ˚C and 15 ˚C, respectively. Sample injection volume was 2.0 µL with a flow rate of 1.0 mL/min. The MS parameters were as follow: ion source temperature, 650 ˚C; ion spray voltage, 5500 V; curtain gas, 40 psi; ion source gas 1, 50 psi; ion source gas 2, 60 psi; declustering potential (DP), 100 V; collision energy, 10 V; collision energy spread, 0 v; mass range for MS scan, m/z 100-1000. MS/MS experiments began with the system default minum value, namely m/z 50-100; DP, 100; collision energy 35 V; collision energy spread, 15.

3.3.1 Feeding experiment with $^{13}CO_2$

3.3.1.1 Plant material and feeding experiment

Specimens of *F. picrosperma* (5-7 years old, 43-70 cm height) were cultivated in the greenhouse of USC, Sippy Downs, Queensland, Australia. Feeding experiments were performed in a period of 30 days on 4 plants. All the plants were arranged in a Perspex box with a total volume of 90 L and kept under controlled humidity and temperature in a glasshouse. According to the last measurement of atmospheric CO_2 (455 ppm, 08/03/19), all the specimens were treated with an extra equivalent of $^{13}CO_2$ by applying 1 M aq. sol. to $Na_2{}^{13}CO_3$ (181.97 mg). Every pulsing lasted for 36 h and, after opening the Perspex box, plants were left for 24 h in a free environment. Leaves and roots were collected and dried separately on silica for at least 48 h, before extracting with MeOH at room temperature over-night. After complete removal of the solvent under *vacuo*, leaves and roots extracts were re-dissolvent in MeOH with a concentration, respectively, of 10 mg mL^{-1} and 4.2 mg mL^{-1}.

3.3.1.2 Clone 123

EBC-46

	Leaves			*Roots*		
Date	**Yield(mg)**	**M+1/M**	**M+2/M+1**	**Yield(mg)**	**M+1/M**	**M+2/M+1**
8-mar-19	**3.3**	0.320451	0.249001	**0.7**	0.315089	0.216405
11-mar-19	**37.1**	0.342440	0.218931	**2.2**	0.325973	0.211861
14-mar-19	**34.3**	0.310939	0.215290	**4.7**	0.325648	0.207307
18-mar-19	**47.9**	0.321519	0.220108	**5.6**	0.310481	0.199837
21-mar-19	**35.3**	0.331584	0.199099	**4.5**	0.292752	0.216195
25-mar-19	**57.5**	0.347871	0.227146	**10.4**	0.314132	0.235566
28-mar-19	**46.7**	0.357909	0.203626	**3.5**	0.303937	0.216780
1-apr-19	**45.9**	0.340595	0.216416	**12.9**	0.320046	0.198581
4-apr-19	**62.5**	0.360585	0.258004	**6.1**	0.326757	0.218994
8-apr-19	**29.7**	0.388521	0.250349	**10.5**	0.299284	0.200506
10-apr-19	**37.6**	0.373776	0.234011	**13.5**	0.324797	0.210392
15-apr-19	**12.2**	0.381759	0.235607	**6.6**	0.304359	0.207582
18-apr-19	**25.8**	0.375308	0.251025	**8.4**	0.323057	0.211149

Table 1. Summary of the isotopic ratio in leaves and roots of EBC-46 in clone 123 among the days

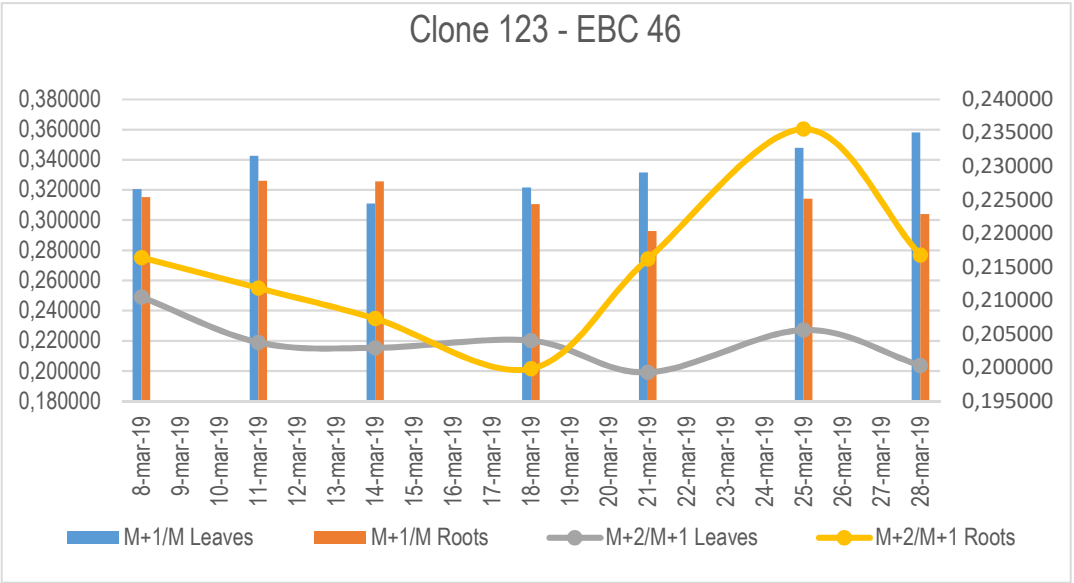

Fig. 1. Graphical scheme related to isotopic ratio distribution of EBC-46 in leaves and roots among the established period

EBC-47

	Leaves			*Roots*		
Date	**Yield(mg)**	**M+1/M**	**M+2/M+1**	**Yield(mg)**	**M+1/M**	**M+2/M+1**
8-mar-19	**3.30**	0.330920	0.239489	**0.70**	0.318487	0.212206
11-mar-19	**37.10**	0.283189	0.422041	**2.20**	0.328599	0.224023
14-mar-19	**34.30**	0.333380	0.278687	**4.70**	0.326253	0.212897
18-mar-19	**47.90**	0.330823	0.212081	**5.60**	0.303233	0.198045
21-mar-19	**35.30**	0.324686	0.197888	**4.50**	0.326008	0.196037
25-mar-19	**57.50**	0.333852	0.223354	**10.40**	0.328568	0.218379
28-mar-19	**46.70**	0.322600	0.232836	**3.50**	0.299744	0.219363
1-apr-19	**45.9**	0.316780	0.214181	**12.9**	0.293394	0.200744
4-apr-19	**62.5**	0.352288	0.234824	**6.1**	0.320833	0.199789
8-apr-19	**29.7**	0.364322	0.227218	**10.5**	0.305893	0.211451
10-apr-19	**37.6**	0.351456	0.242813	**13.5**	0.317910	0.220990
15-apr-19	**12.2**	0.382542	0.278613	**6.6**	0.311026	0.207477
18-apr-19	**25.8**	0.364337	0.225526	**8.4**	0.308770	0.214526

Table 2. Summary of the isotopic ratio in leaves and roots of EBC-47 in clone 123

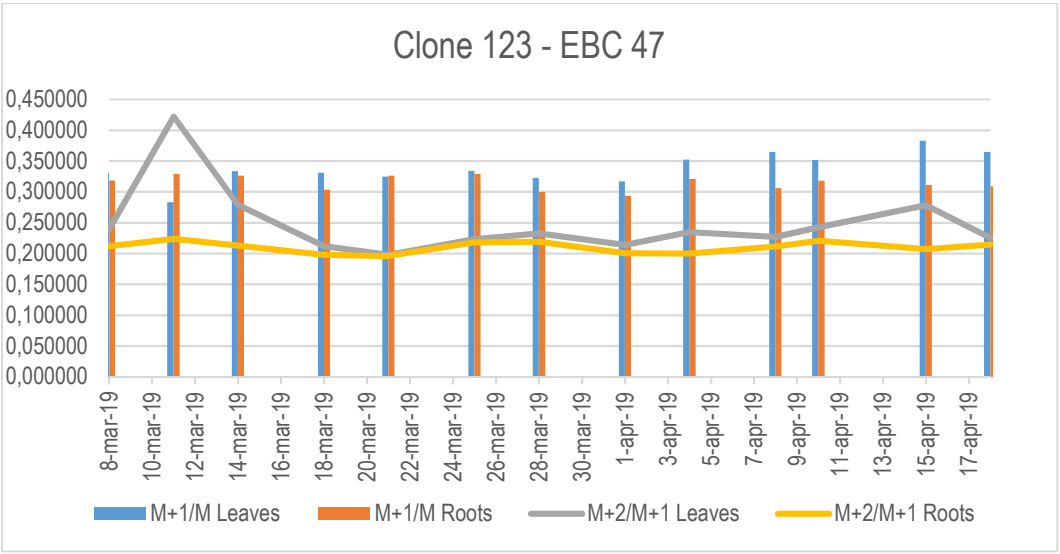

Fig. 2. Graphical scheme related to isotopic ratio distribution of EBC-47 in leaves and roots among the established period

EBC-83

	Leaves			*Roots*		
Date	**Yield(mg)**	**M+1/M**	**M+2/M+1**	**Yield(mg)**	**M+1/M**	**M+2/M+1**
8-mar-19	**3.30**	0.345697	0.231997	**0.70**	0.339295	0.252389
11-mar-19	**37.10**	0.350170	0.315819	**2.20**	0.330901	0.225930
14-mar-19	**34.30**	0.328205	0.351726	**4.70**	0.330612	0.205041
18-mar-19	**47.90**	0.317843	0.352889	**5.60**	0.312593	0.226028
21-mar-19	**35.30**	0.370180	0.416098	**4.50**	0.298166	0.232307
25-mar-19	**57.50**	0.342211	0.223680	**10.40**	0.309621	0.232309
28-mar-19	**46.70**	0.318726	0.238159	**3.50**	0.324922	0.233717
1-apr-19	**45.9**	0.319768	0.219893	**12.9**	0.300576	0.219633
4-apr-19	**62.5**	0.354538	0.242564	**6.1**	0.302379	0.210528
8-apr-19	**29.7**	0.352858	0.224662	**10.5**	0.300365	0.209399
10-apr-19	**37.6**	0.362491	0.227417	**13.5**	0.306821	0.210159
15-apr-19	**12.2**	0.400904	0.264194	**6.6**	0.311427	0.211719
18-apr-19	**25.8**	0.366814	0.236975	**8.4**	0.319675	0.193712

Table 3. Summary of the isotopic ratio in leaves and roots of EBC-83 in clone 123

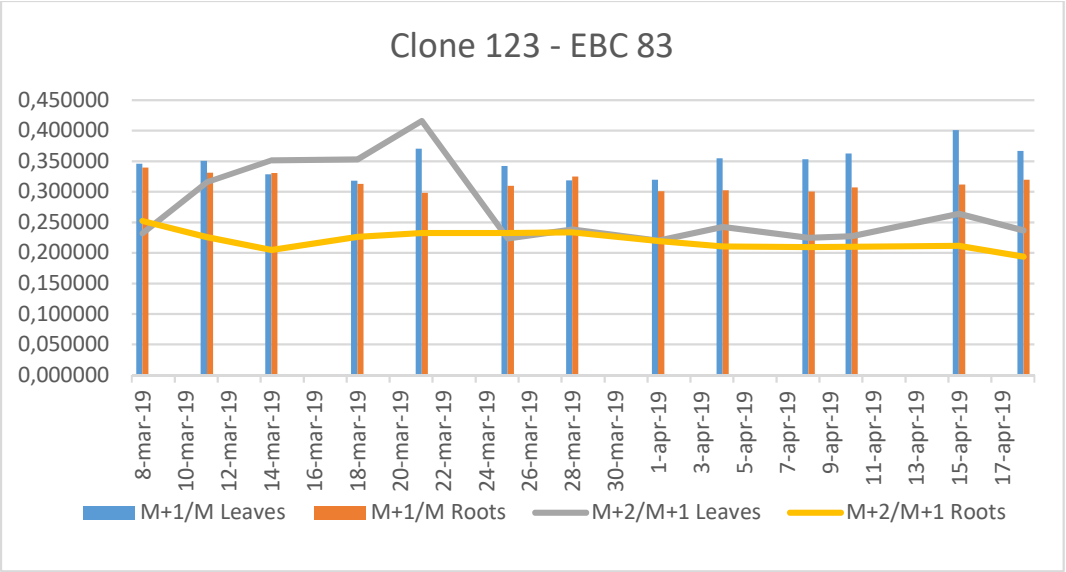

Fig. 3. Graphical scheme related to isotopic ratio distribution of EBC-83 in leaves and roots among the established period

3.3.1.3 Clone 234

EBC-46

	Leaves			*Roots*		
Date	**Yield(mg)**	**M+1/M**	**M+2/M+1**	**Yield(mg)**	**M+1/M**	**M+2/M+1**
8-mar-19	**4.3**	0.320451	0.249001	**1.2**	0.315089	0.216405
11-mar-19	**51.9**	0.315889	0.222192	**6.4**	0.327742	0.219431
14-mar-19	**49.1**	0.316379	0.197403	**5.8**	0.335813	0.205336
18-mar-19	**56.9**	0.314789	0.207172	**10.5**	0.324489	0.207730
21-mar-19	**125.3**	0.316729	0.225565	**5.5**	0.309401	0.219097
25-mar-19	**55.3**	0.335580	0.209750	**13.5**	0.337158	0.220784
28-mar-19	**75.4**	0.352690	0.228117	**18.1**	0.331391	0.213570
1-apr-19	**146.3**	0.313406	0.187916	**14.9**	0.320652	0.217249
4-apr-19	**43.6**	0.335476	0.234620	**14**	0.310971	0.204902
8-apr-19	**49.2**	0.311635	0.204889	**21.1**	0.328348	0.204249
10-apr-19	**36**	0.318858	0.219139	**11.5**	0.313385	0.222415
15-apr-19	**18.8**	0.385029	0.242721	**10.2**	0.311189	0.216570
18-apr-19	**17.5**	0.387078	0.228981	**16**	0.310913	0.207212

Table 4. Summary of the isotopic ratio in leaves and roots of EBC-46 in clone 234

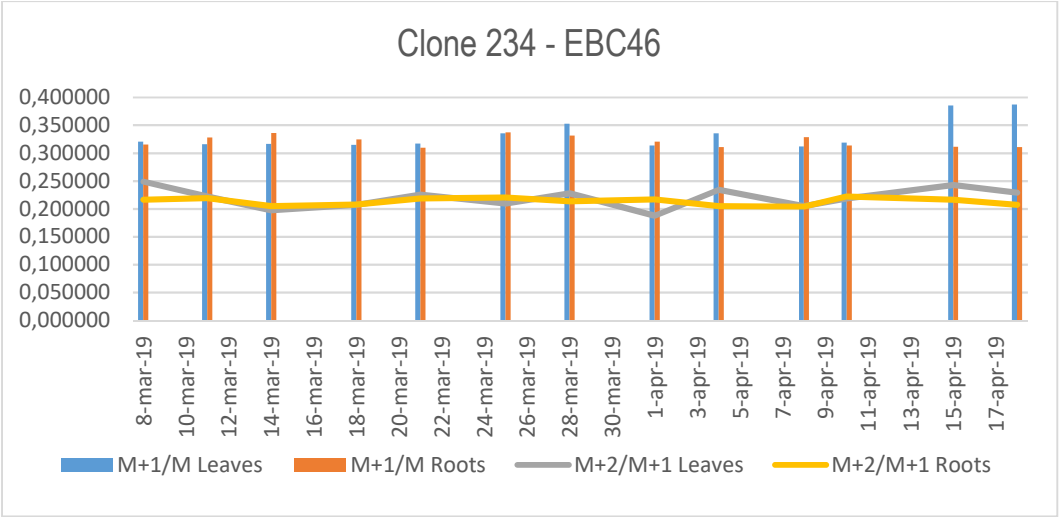

Fig. 4. Graphical scheme related to isotopic ratio distribution of EBC-46 in leaves and roots among the established period

EBC-47

	Leaves			***Roots***		
Date	**Yield(mg)**	**M+1/M**	**M+2/M+1**	**Yield(mg)**	**M+1/M**	**M+2/M+1**
8-mar-19	**4.3**	0.330920	0.239489	**1.2**	0.318487	0.212206
11-mar-19	**51.9**	0.350355	0.427246	**6.4**	0.314500	0.221440
14-mar-19	**49.1**	0.348673	0.311236	**5.8**	0.316725	0.238504
18-mar-19	**56.9**	0.307639	0.249206	**10.5**	0.318299	0.217887
21-mar-19	**125.3**	0.340323	0.230539	**5.5**	0.307823	0.218202
25-mar-19	**55.3**	0.331139	0.228063	**13.5**	0.344877	0.195427
28-mar-19	**75.4**	0.330131	0.213896	**18.1**	0.324529	0.215703
1-apr-19	**146.3**	0.340734	0.228421	**14.9**	0.312503	0.223259
4-apr-19	**43.6**	0.328636	0.204615	**14**	0.323914	0.197295
8-apr-19	**49.2**	0.329295	0.213121	**21.1**	0.321406	0.208438
10-apr-19	**36**	0.319617	0.211018	**11.5**	0.314876	0.197125
15-apr-19	**18.8**	0.379006	0.245023	**10.2**	0.313701	0.190474
18-apr-19	**17.5**	0.369876	0.239879	**16**	0.295888	0.213960

Table 5. Summary of the isotopic ratio in leaves and roots of EBC-47 in clone 234

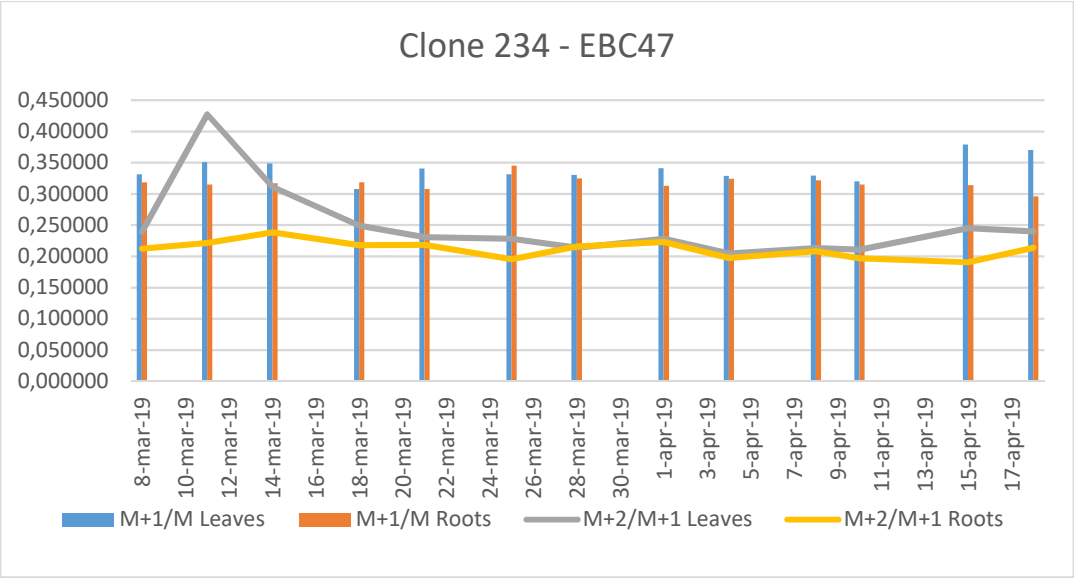

Fig. 5. Graphical scheme related to isotopic ratio distribution of EBC-47 in leaves and roots among the established period

EBC-83

	Leaves			*Roots*		
Date	**Yield(mg)**	**M+1/M**	**M+2/M+1**	**Yield(mg)**	**M+1/M**	**M+2/M+1**
8-mar-19	**4.3**	0.345697	0.231997	**1.2**	0.339295	0.252389
11-mar-19	**51.9**	0.416747	0.337734	**6.4**	0.308692	0.226049
14-mar-19	**49.1**	0.390137	0.558026	**5.8**	0.318285	0.227930
18-mar-19	**56.9**	0.331423	0.277685	**10.5**	0.328337	0.212412
21-mar-19	**125.3**	0.391059	0.325338	**5.5**	0.317988	0.194929
25-mar-19	**55.3**	0.351800	0.283425	**13.5**	0.328120	0.228109
28-mar-19	**75.4**	0.357524	0.237116	**18.1**	0.330673	0.234315
1-apr-19	**146.3**	0.331264	0.448675	**14.9**	0.307863	0.229897
4-apr-19	**43.6**	0.327806	0.217774	**14**	0.309813	0.208660
8-apr-19	**49.2**	0.405826	0.267421	**21.1**	0.312061	0.210917
10-apr-19	**36**	0.300588	0.212280	**11.5**	0.320984	0.211009
15-apr-19	**18.8**	0.411787	0.258464	**10.2**	0.309444	0.215736
18-apr-19	**17.5**	0.389142	0.242736	**16**	0.316825	0.225008

Table 6. Summary of the isotopic ratio in leaves and roots of EBC-83 in clone 234

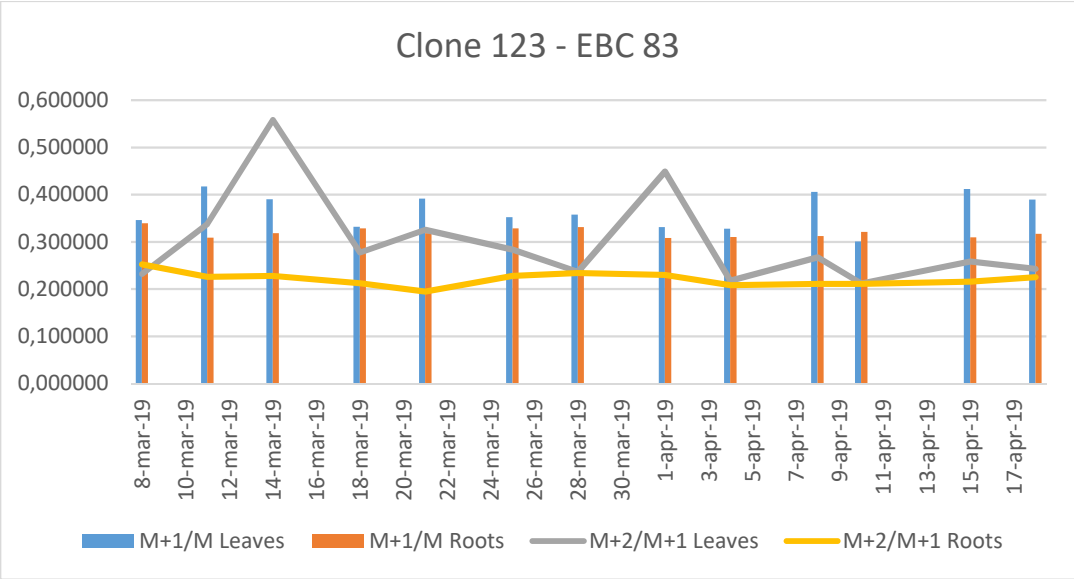

Fig. 6. Graphical scheme related to isotopic ratio distribution of EBC-83 in leaves and roots among the established period

3.3.1.4 Clone 591

EBC-46

	Leaves			*Roots*		
Date	**Yield(mg)**	**M+1/M**	**M+2/M+1**	**Yield(mg)**	**M+1/M**	**M+2/M+1**
8-mar-19	**15.1**	0.320451	0.249001	**3.8**	0.315089	0.216405
11-mar-19	**35.8**	0.317348	0.194489	**2.1**	0.329729	0.219496
14-mar-19	**31.6**	0.313335	0.223431	**3.7**	0.333511	0.205905
18-mar-19	**27.8**	0.343412	0.243235	**5.1**	0.345570	0.230859
21-mar-19	**44.5**	0.337389	0.226536	**5.6**	0.324695	0.227529
25-mar-19	**34.1**	0.363900	0.220709	**8.6**	0.319861	0.240233
28-mar-19	**44.7**	0.333188	0.234040	**12.6**	0.314049	0.223009
1-apr-19	**32.5**	0.310465	0.236561	**9.4**	0.319660	0.222404
4-apr-19	**35.5**	0.337838	0.211217	**10.8**	0.318662	0.212925
8-apr-19	**30.9**	0.329260	0.209219	**14.2**	0.345427	0.226374
10-apr-19	**26.8**	0.342144	0.237677	**11.9**	0.317437	0.207408
15-apr-19	**23.4**	0.365923	0.247769	**3.5**	0.314049	0.194763
18-apr-19	**23.8**	0.357170	0.236860	**8.3**	0.318316	0.217117

Table 7. Summary of the isotopic ratio in leaves and roots of EBC-46 in clone 591

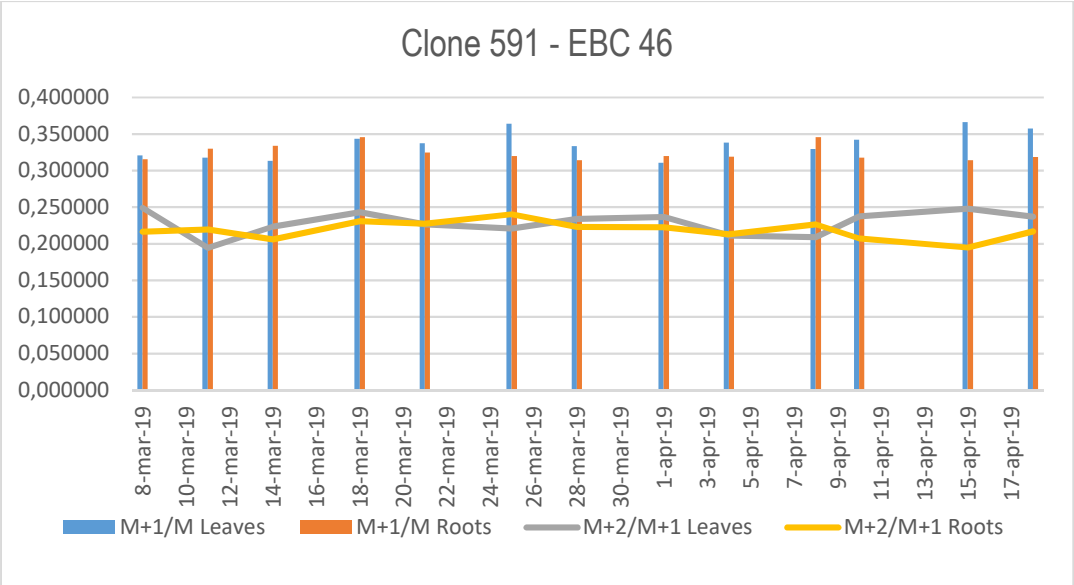

Fig. 7. Graphical scheme related to isotopic ratio distribution of EBC-46 in leaves and roots among the established period

EBC-47

	Leaves			*Roots*		
Date	**Yield(mg)**	**M+1/M**	**M+2/M+1**	**Yield(mg)**	**M+1/M**	**M+2/M+1**
8-mar-19	**15.1**	0.320451	0.249001	**3.8**	0.315089	0.216405
11-mar-19	**35.8**	0.296871	0.244227	**2.1**	0.338507	0.215312
14-mar-19	**31.6**	0.318345	0.228526	**3.7**	0.327353	0.212199
18-mar-19	**27.8**	0.344329	0.226325	**5.1**	0.408811	0.391635
21-mar-19	**44.5**	0.333464	0.219953	**5.6**	0.309324	0.269489
25-mar-19	**34.1**	0.326623	0.215483	**8.6**	0.311741	0.215552
28-mar-19	**44.7**	0.319783	0.211259	**12.6**	0.296233	0.220976
1-apr-19	**32.5**	0.322004	0.209455	**9.4**	0.317665	0.203731
4-apr-19	**35.5**	0.303806	0.312539	**10.8**	0.301881	0.235651
8-apr-19	**30.9**	0.301978	0.220980	**14.2**	0.352408	0.200764
10-apr-19	**26.8**	0.317915	0.213685	**11.9**	0.313955	0.197992
15-apr-19	**23.4**	0.355071	0.221616	**3.5**	0.292728	0.215938
18-apr-19	**23.8**	0.343237	0.243397	**8.3**	0.315868	0.210640

Table 8. Summary of the isotopic ratio in leaves and roots of EBC-47 in clone 591

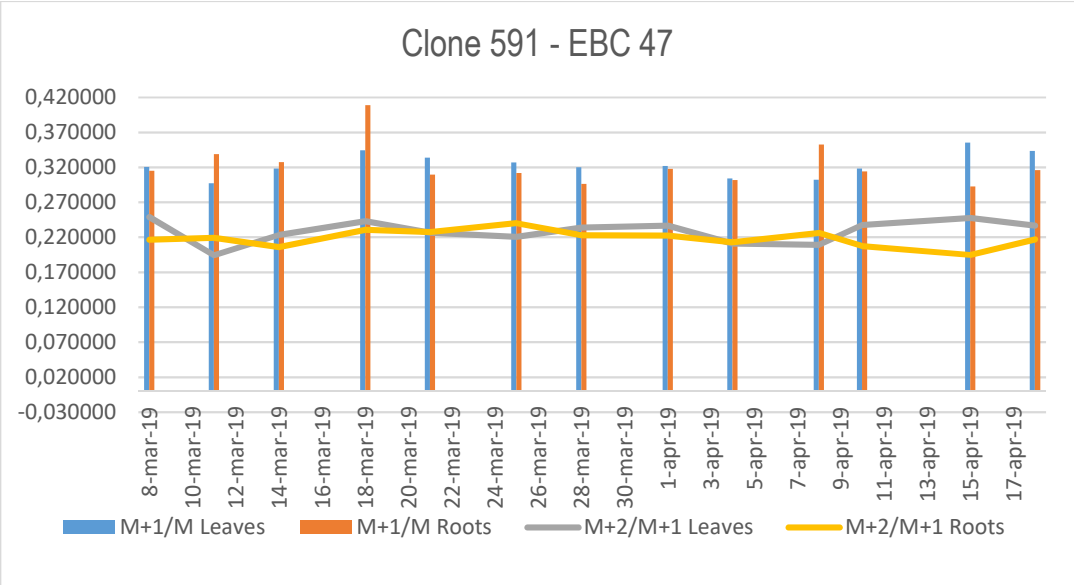

Fig. 8. Graphical scheme related to isotopic ratio distribution of EBC-47 in leaves and roots among the established period

EBC-83

	Leaves			*Roots*		
Date	**Yield(mg)**	**M+1/M**	**M+2/M+1**	**Yield(mg)**	**M+1/M**	**M+2/M+1**
8-mar-19	**15.1**	0.320451	0.249001	**3.8**	0.315089	0.216405
11-mar-19	**35.8**	0.379004	0.501309	**2.1**	0.330847	0.220238
14-mar-19	**31.6**	0.363694	0.272587	**3.7**	0.321922	0.211519
18-mar-19	**27.8**	0.347546	0.225435	**5.1**	0.307153	0.211656
21-mar-19	**44.5**	0.332879	0.233678	**5.6**	0.365096	0.582947
25-mar-19	**34.1**	0.340783	0.226099	**8.6**	0.328648	0.207812
28-mar-19	**44.7**	0.334489	0.225676	**12.6**	0.330995	0.197035
1-apr-19	**32.5**	0.318296	0.223376	**9.4**	0.318975	0.218567
4-apr-19	**35.5**	0.321551	0.213100	**10.8**	0.307737	0.230368
8-apr-19	**30.9**	0.331951	0.206268	**14.2**	0.323272	0.234059
10-apr-19	**26.8**	0.335751	0.220172	**11.9**	0.310519	0.215102
15-apr-19	**23.4**	0.366164	0.241970	**3.5**	0.296614	0.213204
18-apr-19	**23.8**	0.347188	0.234047	**8.3**	0.305804	0.217489

Table 9. Summary of the isotopic ratio in leaves and roots of EBC-83 in clone 591

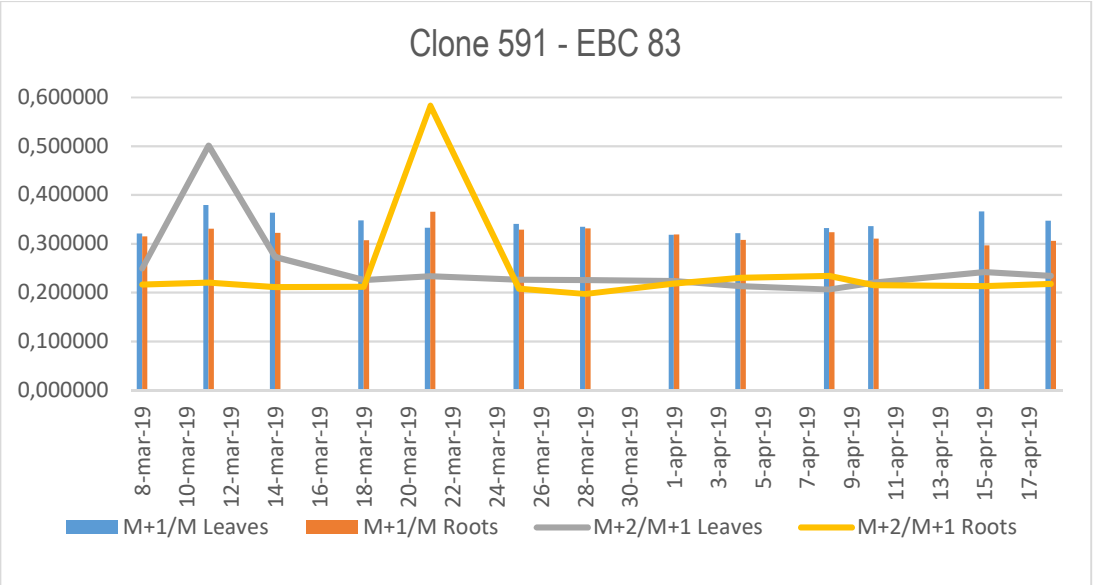

Fig. 9. Graphical scheme related to isotopic ratio distribution of EBC-83 in leaves and roots among the established period

3.3.1.5 Clone 593

EBC-46

	Leaves			*Roots*		
Date	**Yield(mg)**	**M+1/M**	**M+2/M+1**	**Yield(mg)**	**M+1/M**	**M+2/M+1**
8-mar-19	**7.2**	0.320451	0.249001	**0.9**	0.315089	0.216405
11-mar-19	**33**	0.306714	0.225440	**3.4**	0.316414	0.220487
14-mar-19	**27.8**	0.323241	0.203469	**3.4**	0.317020	0.207177
18-mar-19	**47.4**	0.309805	0.212533	**5.8**	0.329882	0.238132
21-mar-19	**41.1**	0.309158	0.227720	**2.3**	0.320438	0.223110
25-mar-19	**33**	0.353869	0.216820	**5.4**	0.301863	0.228347
28-mar-19	**43.8**	0.336519	0.216419	**4.1**	0.341631	0.196126
1-apr-19	**31.7**	0.307288	0.200347	**9.8**	0.305384	0.217611
4-apr-19	**32.1**	0.352773	0.223185	**2.8**	0.318349	0.229101
8-apr-19	**39.3**	0.326524	0.226765	**12.5**	0.324026	0.218413
10-apr-19	**30.1**	0.314588	0.202449	**12.2**	0.304722	0.225791
15-apr-19	**17.7**	0.341458	0.232977	**4.9**	0.321555	0.223608
18-apr-19	**26.2**	0.403993	0.253899	**5.3**	0.312022	0.219495

Table 10. Summary of the isotopic ratio in leaves and roots of EBC-46 in clone 593

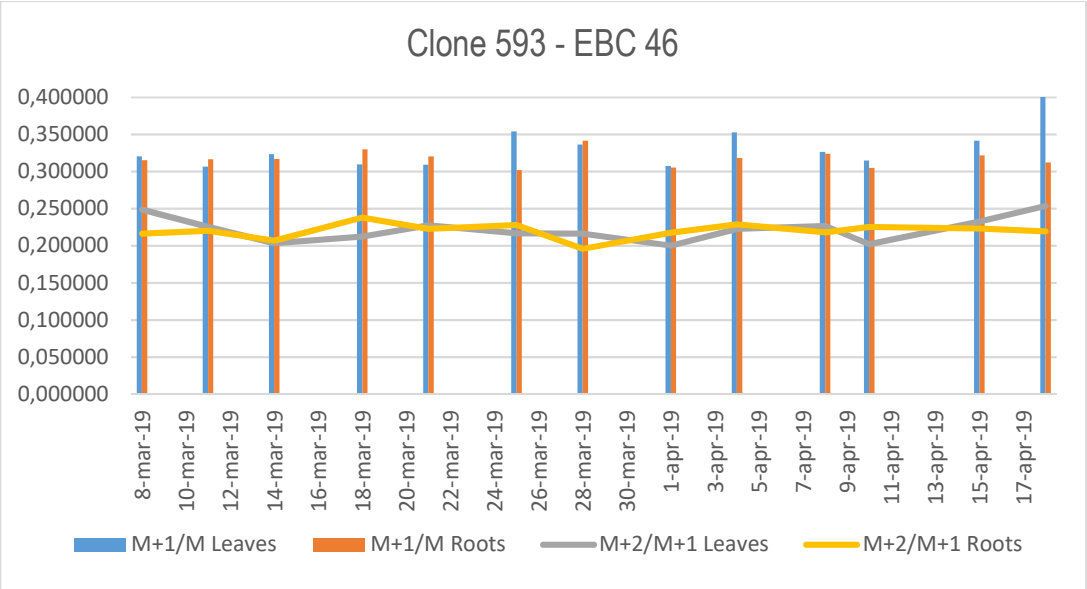

Fig. 10. Graphical scheme related to isotopic ratio distribution of EBC-46 in leaves and roots among the established period

EBC-47

	Leaves			***Roots***		
Date	**Yield(mg)**	**M+1/M**	**M+2/M+1**	**Yield(mg)**	**M+1/M**	**M+2/M+1**
8-mar-19	**7.2**	0.320451	0.249001	**0.9**	0.315089	0.216405
11-mar-19	**33**	0.332322	0.293575	**3.4**	0.332198	0.219794
14-mar-19	**27.8**	0.326776	0.213446	**3.4**	0.316226	0.454295
18-mar-19	**47.4**	0.335804	0.221817	**5.8**	0.402525	0.298190
21-mar-19	**41.1**	0.309508	0.216962	**2.3**	0.327009	0.234304
25-mar-19	**33**	0.324059	0.213673	**5.4**	0.342257	0.215073
28-mar-19	**43.8**	0.329526	0.243566	**4.1**	0.336629	0.292597
1-apr-19	**31.7**	0.305882	0.206694	**9.8**	0.317442	0.206544
4-apr-19	**32.1**	0.342892	0.228847	**2.8**	0.328156	0.222223
8-apr-19	**39.3**	0.321211	0.209429	**12.5**	0.328468	0.207897
10-apr-19	**30.1**	0.305889	0.224801	**12.2**	0.320527	0.203973
15-apr-19	**17.7**	0.325915	0.219058	**4.9**	0.319560	0.214775
18-apr-19	**26.2**	0.377371	0.249454	**5.3**	0.310715	0.211446

Table 11. Summary of the isotopic ratio in leaves and roots of EBC-47 in clone 593

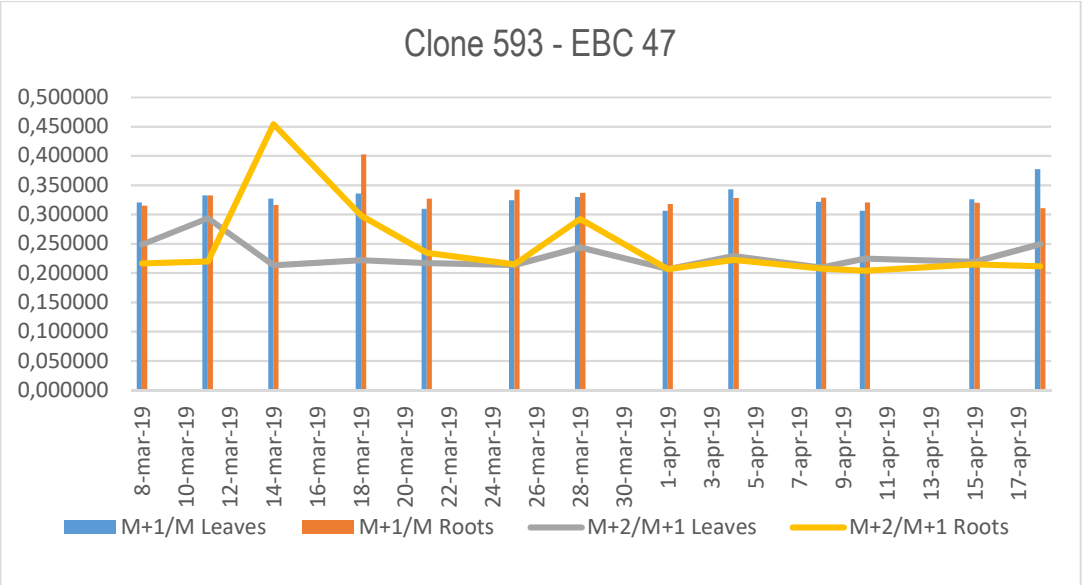

Fig. 11. Graphical scheme related to isotopic ratio distribution of EBC-46 in leaves and roots among the established period

EBC-83

	Leaves			*Roots*		
Date	**Yield(mg)**	**M+1/M**	**M+2/M+1**	**Yield(mg)**	**M+1/M**	**M+2/M+1**
8-mar-19	**7.2**	0.320451	0.249001	**0.9**	0.315089	0.216405
11-mar-19	**33**	0.381355	0.278943	**3.4**	0.325126	0.224195
14-mar-19	**27.8**	0.352089	0.332968	**3.4**	0.316455	0.209334
18-mar-19	**47.4**	0.364053	0.292279	**5.8**	0.364609	0.434709
21-mar-19	**41.1**	0.305477	0.239666	**2.3**	0.377731	0.277108
25-mar-19	**33**	0.327933	0.267329	**5.4**	0.391163	0.256521
28-mar-19	**43.8**	0.323077	0.269365	**4.1**	0.304428	0.209229
1-apr-19	**31.7**	0.321871	0.229057	**9.8**	0.306033	0.212468
4-apr-19	**32.1**	0.363928	0.239297	**2.8**	0.339319	0.243207
8-apr-19	**39.3**	0.305923	0.241541	**12.5**	0.314724	0.205176
10-apr-19	**30.1**	0.314139	0.222694	**12.2**	0.333149	0.219946
15-apr-19	**17.7**	0.334576	0.227444	**4.9**	0.300410	0.219901
18-apr-19	**26.2**	0.411879	0.254365	**5.3**	0.314889	0.204007

Table 12. Summary of the isotopic ratio in leaves and roots of EBC-83 in clone 593

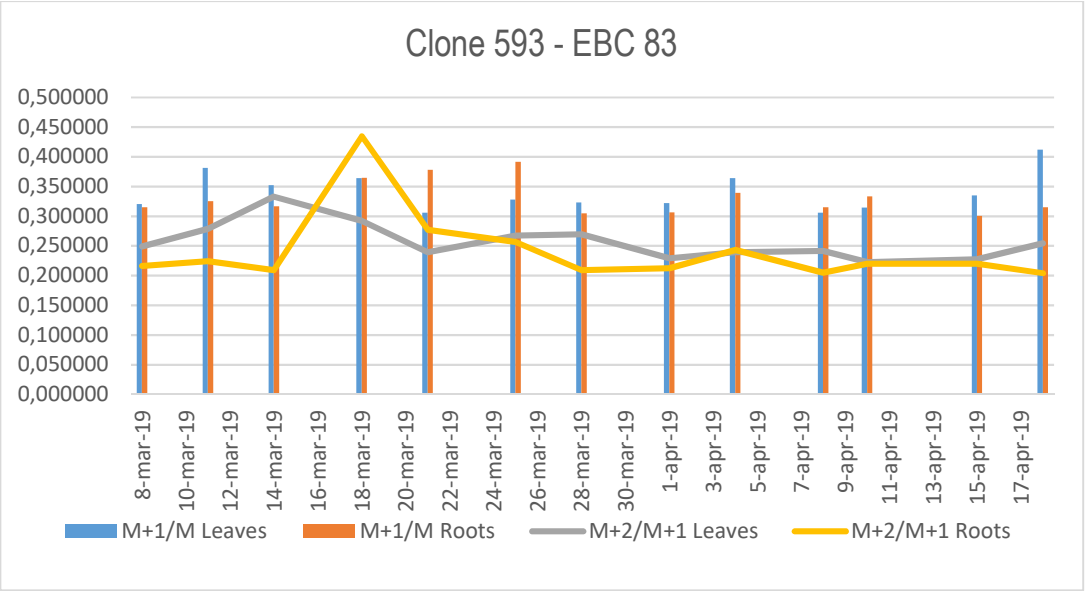

Fig. 12. Graphical scheme related to isotopic ratio distribution of EBC-83 in leaves and roots among the established period

3.3.2 Feeding experiment with 1-D-^{13}C-glucose and DXP isotopomers

General method for plant injections: Specimens of *F. picrosperma* (5 months old, 15-17 cm height) were cultivated in the greenhouse of USC, Sippy Downs, Queensland, Australia. Labelled precursors solution (0.4 mM in 10 % EtOH aq. sol) were fed twice via direct stem and leaves injections with insulin syringes in a period of 10 days under controlled temperature (20 °C). All the plants were let them rest for 7 days prior to sampling and drying in a silica bag for 48 h. Dried vegetal material was extracted twice with MeOH, filtered and dried before re-dissolving in 90 % aq. sol of MeOH. The methanolic phase was extracted three times with *n*-hexane, diluted with water (ratio 1:1) and re-extracted several times with dichloromethane. The collected organic phases were dried over Na_2SO_4 and concentrated *in vacuo*. Protection at C-5, C-20 with the acetonide and transesterification of the acyl moiety to get 5β-6,7α-epoxyphorbol-5,20-acetonide have been done as described in paragraph **1.2.2.2**

Samples	Plant extract	DCM phase	Acetonide	Transesterification
SIG591A	10 mL (oil)	60.1 mg	45 mg	10.7 mg
SIG606A	1.686 g	129 mg	144.6 mg	3.1 mg
SIG607A	2.177 g	152 mg	196.4 mg	3.4 mg
SIG608A	1.205 g	53.3 mg	40.5 mg	0.7 mg
SIG609A	3.040 g	181.4 mg	180 mg	8.5 mg

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Final conclusions

Many plants of the Euphorbiaceae family produce strongly irritant and toxic latexes and oils, whose active constituents are known as "*phorboids*". Natural phorboids like tiglianes are esters of the parent polyol phorbol. The optimized method to recover phorbol and hydroxy-epoxyphorbol, here reported, provides a way to have access at the base skeleton for investigation of its chemistry and medicinal application, disregarding by complicated total synthetical approach. The peculiar activity exerted by phorbol esters addressed these compounds as lead molecules in drug development. Accessing tigliane skeleton or functionalize it for SAR studies represent a challenge due to the undisciplined reactivity that in some cases produce serendipitous discovery in the chemistry of this class of compounds. A contribute to investigating the chemical space of phorbol esters has been discussed on the chance of setting the basis for further studies aimed at the modification of ring B, possibly involved in the biological selectivity exploited by the experimental drug EBC-46 vs TPA. Moreover, preliminary studies on tissue localization and stereochemical course of the biosynthetic pathway have been performed in light of the elegant hypothesis proposed by prof. Jasmin Jakupovic. The obtained results suggest a semi-equal distribution of hydroxy-epoxyphorbol esters in leaves and roots, with a slight tendency towards leaves. A protocol for plant injection was established by the administration of labelled glucose and DXP. If the first carbohydrate does not seem to interfere with the normal homeostasis, the second one produces significant necrosis of the tissue. Incorporation of "heavy" atom is not significant in the final tigliane skeleton invoking the suspect the biosynthetic machinery is not expressed at the early stages of plant life and that the occurrence of tiglianes in sprouts and shootings arise from what was already stored in seeds.

Future investigations of this project will pay attention to the role of roots in older specimens to determine the exact localization of the biosynthesis. Other feeding experiments on mature plants are going to be evaluated concomitantly with a transcriptomic analysis of *F.picrosperma* to establish the enzyme involved in the pathway.

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This long and incredible journey pushed myself against my limits allowing me to grow both as a scientist and as a person. Facing everyday problems in extraordinary situations made me the person that I am now and, for this reason, several people must be thanked.

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My sincere thanks go to Prof. Jeroen S. Dickschat for accepting to host me in his laboratory and introducing me at the biosynthetic pathways in natural products chemistry. My experience in Bonn would not be the same without the direct supervision of Dr. Lena Barra, an honest friend to enlighten with my Italian soul during my period in Germany.

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I want to thank all of my new friends around the world, from Germany to Australia, and of course all of my local friends who tolerate my job soliloquy without understanding barely anything.

Last but not the least, I would like to thank my family: my parents Giancarlo and Nadia for believing in me no matter what or where and bear all of my crazy moments in this long trip. My sister Sonia, a partner in crime in countless binges, to remind me that take a break from work is healthy as well as a diet after a huge fast-food meal.

Curriculum vitae

Simone Gaeta was born in 1991 in Gattinara (VC, Italy). After graduating at Liceo Scientifico "A. Avogadro" – detached section of Cossato (BI), he enrolled at the University of Eastern Piedmont in 2010 in the degree course of Medicinal and Pharmaceutical Chemistry. He graduated in March 2016 with a thesis on "Non-psychotropic phytocannabinoids from Cannabis sativa" in the laboratories of Prof. Giovanni Appendino. In the same year, he won a research fellowship in the same laboratory where, from November 2016 to November 2019, he focused his education in chemistry of bioactive natural products as part of the PhD program in "Chemistry & Biology".

The activity is documented in 2 publications and a book chapter (to press), about the phytochemistry of Apiaceae:

1. Pagani, A., Gaeta, S., Savchenko, A. I., Williams, C. M., & Appendino, G. (2017). *Beilstein journal of organic chemistry*, *13*(1), 1361-1367.

2. Pollastro, F., Talmon, M., Gaeta, S., Rossi, S., Lopatriello, A., Fresu, L. G. (2018). *Fitoterapia*, *127*, 252-256.