Novel protocols for the *in vitro* production of highly valuable sesquiterpenoids

A thesis submitted to Cardiff University for the degree of

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Abstract

The sesquiterpenes (+)-germacrene A and (-)-germacrene D are natural semiochemicals currently employed in developing alternative and more sustainable crops. *In planta* (or in Nature), these relatively complex cyclodecadienes C15 hydrocarbons are biosynthesised from farnesyl diphosphate (FDP) by the action of sesquiterpene synthases GAS and GDS. On the expectation that modifications of their chemical structure could lead to compounds with enhanced biological properties, a small library of alkyl- and fluorine-modified germacrene A and D analogues were synthesised upon exposure of the corresponding FDP analogues to (+)-germacrene A or (-)-germacrene D synthases from *Solidago canadensis*. Two novel incubation procedures were developed to secure the production of these valuable germacrenes in sufficient amounts for NMR characterisation as well as for their *in vivo* evaluation against aphids. While the use of deuterated extracting solvents in batch experiments was essential to characterise the enzymatic products and monitor their relative stability, the production of these novel bioactive semiochemicals was substantially boosted through the use of modern continuous flow extraction techniques. In addition, the synthesis and enzymatic evaluation of two hydroxylated FDP analogues as mechanistic probes provided evidence in support of a 1,10-cyclisation reaction under (+)-δ-cadinene synthase catalysis.

Results obtained from the *in vivo* evaluation of novel analogues of (+)-germacrene A or (-)-germacrene D can be use in the future for designing agrochemicals with enhance repellent activity for pest control. Further optimisation of flow chemoenzymatic preparation of sesquiterpenes could be performed, perhaps by including immobilised enzymes with improved stability in the design of the flow reactor. The potential of hydroxyl FDP analogues for exploring terpene synthases mechanism is yet to be evaluated.

Abbreviations

ºC	Degrees Celsius
9-BBN	9-Borabicyclo[3.3.1]nonane
APCI	Atmospheric pressure chemical ionisation
AS	Aristolochene synthase
bd	Broad doublet
Bn	Benzyl
bs	Broad singlet
Bu	Butyl
t-BuOOH	tert-Butyl hydroperoxide
CI	Chemical ionisation
cDNA	Cyclic/Complementary deoxyribonucleic acid
mCPBA	meta-Chloroperbenzoic acid
CMP	Cytidine monophophate
CPM	Counts per minute
CV	Column volume
DCM	Dichloromethane
DCS	(+)-δ-Cadinene synthase
DDT	Dichlorodiphenyltrichloroethane
DHP	Dihydropyran
DIBAL-H	Diisobutyl aluminium hydride
DMADP	Dimethylallyl diphosphate
DMF	*N,N*-Dimethylformamide
DNA	2'-Deoxyribonucleic acid
DPM	Decays per minute

EDTA	Ethylenediaminetetraacetic acid
EI$^+$	Electron impact
ES$^+$	Electrospray ionisation
Et	Ethyl
FDP	(*2E,6E*)-Farnesyl diphosphate
FTase	Farnesyl transferase
FTIR	Fourier transform infrared spectroscopy
g	Grams
GAS	Germacrene A synthase
GC	Gas chromatography
GC-FID	Gas chromatography –flame ionisation detector
GC-MS	Gas chromatography – mass spectrometry
GDP	Geranyl diphosphate
GDS	Germacrene D synthase
h	Hours
iPP	Inorganic diphosphate
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HMPA	Hexamethylphosphoramide
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
Hz	Herz
IDP	Isopentenyl diphosphate
iPr	Isopropyl
iPrOH	Isopropanol
IR	Infra red
J	Coupling constant
JH	Juvenile hormone

LDA	Lithium diisopropylamide
μM	Micro molar
M	Molar
Me	Methyl
MHz	Mega hertz
min	Minutes
mg	Milligrams
mM	Millimolar
mm	Millimetres
mmol	Millimol
mL	Millilitre
MsCl	Methanesulfonyl chloride
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
NDP	Nerolidyl diphosphate
NMR	Nuclear magnetic resonance
NOE	Nuclear overhauser effect
OPP	Diphosphate
PDC	Pyridinium dichromate
Ph	Phenyl
ppm	Parts per million
PPTs	Pyridinium para-toluenesulfonate
PR-AS	*Penicillium roqueforti* – aristolochene synthase
q	Quartet
RNA	Ribonucleic acid
rpm	Revolutions per minute
s	Singlet

st	Stretching
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
t	Triplet
TBDPS	*tert*-Butyldiphenylsilyl
TEAS	Tobacco 5-*epi*-aristolochene synthase
Tf	Triflate
THF	Tetrahydrofuran
THP	Tetrahydropyran
TMSCl	Trimethylsilyl chloride
TLC	Thin layer chromatography
TPP	Triphosphate
TsCl	p-Toluenesulfonyl chloride
UV	Ultra violet
WHO	World Health Organisation

Chapter 1: Introduction

Chapter 2: Chemical expansion of the terpenome: synthesis of farnesyl diphosphate analogues

Chapter 3: Innovative methodologies for the enzymatic production of valuable semiochemicals

Chapter 4: Enzymatic production and NMR characterisation of unnatural sesquiterpenoids

Chapter 5: General conclusions

Chapter 6: Detailed experimental procedures

APENDIX I:

APENDIX II:

CHAPTER 1

INTRODUCTION

1. Introduction to terpenes

1.1 General background

Terpenoids are a large and diverse class of natural organic compounds found in all kind of organisms. With at least 30,000 different naturally occurring terpenoids, this family represents the largest and most structurally diverse group of natural products.[1] Indeed, terpenoids play many functional roles in terrestrial and marine plants, bacteria and fungi, mostly as secondary metabolites. Although the function of many terpenoids is still unknown, they are mostly not essential for viability of the organism, although there is a small group of terpenoids that are primary metabolites, such as carotenes and sterols.[2] Terpenoids act as secondary metabolites, for instance as communication or defence chemicals.[3] Additionally, terpenoids have found many applications in medicine as potent drugs against cancer,[4] heart disease,[5] malaria[6] or fungi.[7]

Terpenes can be classified according to the number of carbons included in the structure. Monoterpenes contain 10 carbon atoms, sesquiterpenes contain 15, diterpenes 20, sesterterpenes 25, triterpenes 30 and so on.[8]

1.2 Examples of relevant terpenoids

1.2.1 Monoterpenoids

Monoterpenoids are often found in essential oils from plants. They are responsible for the characteristic odour of many plants and their organoleptic properties. Because of these properties and their high volatility, they are valuable chemicals for the flavouring and aroma industry.[9] In respect to citrus aromas, for instance (*R*)-(+)-limonene (**1**), is responsible for the orange-like odour, whereas the enantiomer (*S*)-(-)-limonene (**2**) has a lemon-like smell (Scheme 1).[10] Certain monoterpenoids may also act as pheromones and are used by some insects for communication. For instance, (+)-verbenone (**3**) is produced by mountain pine beetles, *Dendroctonus ponderosae*, as a dispersant pheromone.[11] Camphor (**4**) has been reported as an antimicrobial agent.[12]

(R)-(+)-limonene (S)-(-)-limonene (+)-verbenone camphor
1   2   3   4
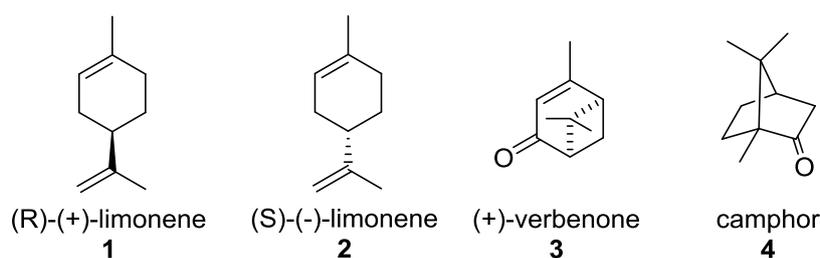
Figure 1. Examples of monoterpenoid bioactive compounds.

1.2.2 Sesquiterpenoids

Sesquiterpenoids occur in nature as hydrocarbons, or in oxygenated forms, but all of them are prepared from a precursor containing 15 carbon atoms. They are constituents of essential oils isolated from higher plants and some invertebrates.[13] Additionally, sesquiterpenes have major importance in the food industry, being responsible for many flavours and odours.[14]

For instance, α-humulene (**5**, Figure 2) was first isolated from the essential oil of the hop plant (*Humulus lupulus*).[15] *E*-β-farnesene (**6**, Figure 2) was also originally isolated from the essential oil of *Humulus lupulus* and is known to be secreted by aphids acting as an alarm pheromone.[16] Presumably, some plants have evolved to produce it in order to reduce the attack density of these harmful insects. This repellent property makes it a good chemical to be used as a pest control agent.[17] These two compounds are found in hops, and hence are of paramount importance in the brewing industry as flavouring compounds. α-Humulene has been associated with delicate and refined beer flavour often described as elegant by brewers. It accounts for 40-45% of total oil in "noble-type" hops.[18] In addition, α-humulene has been found to have anti-inflammatory properties.[19]

α-humelene   E-β-farnesine
5   6

Figure 2. Chemical structures of α-humulene (**5**), *E*-β-farnesene (**6**).

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Sesquiterpenes are also important chemicals that have many medical applications with many different compounds of this family under medical study.[20]

An example of an important sesquiterpenoid use in medicine is artemisinin (**8**, Scheme 1), first isolated from *Artemisia annua* by Dovek.[21] Artemisinin (**8**) is a potent anti-malarial chemical. The World Health Organisation (WHO) has recommended this sesquiterpenoid, in combination with other therapies, as a preferred treatment for malaria.[22] The parasite that causes malaria feeds from haemoglobin causing oxidative stress to the host.[23] The most accepted theory proposes that artemisinin (**8**) acts through the reaction of its endoperoxide group with the haem group of haemoglobin, to generate reactive oxygen radicals that damage the parasite.[24] Despite the fact that the endoperoxide appears to be crucial for its anti-malaria activity,[25] the precise mechanism of action is still an object of discussion.[26] Artemisinin was synthetically prepared for the first time from (-)-isopulegol (**7**, Scheme 1) in a 12-step synthesis process developed by Hofheinz *et al.*[27] This total synthesis was recently improved by Cook *et al.*[28] who successfully used a 5-step synthetic scheme from cyclohexenone. Despite these improvements, the total synthesis of artemisinin remains too laborious and costly process to be considered a viable alternative for supplying the market. Alternatively, semisynthesis has been developed from artemisinic acid (**9**, Scheme 1) and is produced in engineered yeast (*Saccharomyces cerevisiae*) which can produce as much as 25 g / L of artemisinic acid.[29]

Scheme 1. Artemisinin (**8**) can be prepared from (-)-isopulegol (**7**) or artemisinic acid (**9**).

Additionally the semisynthesis of artemisinin (**8**) has been implemented in a continuous flow reactor that converts dihydroartemisinic acid (**10**) to artemisinin (**8**) in a scalable process that does not require purification of intermediates. Artemisinic acid (**9**) is first reduced in batch to

dihydroartemisinic acid (**10**) with the remaining steps being performed in a flow reactor. The reaction of dihydroartemisinic acid (**10**) with singlet oxygen produces the tertiary allylic hydroperoxide **11** which undergoes Hock reaction when trifluoroacetic is added to give reactive enol **12** that then reacts with triplet oxygen to generate tertiary peroxide **13**. Tertiary peroxide **13** produces artemisinin (**8**) through a cascade of condensations. Optimisation of these steps in a flow reactor gave artemisinin (**13**) from dihydroartemisinic acid with 39% yield (Scheme 2).[30]
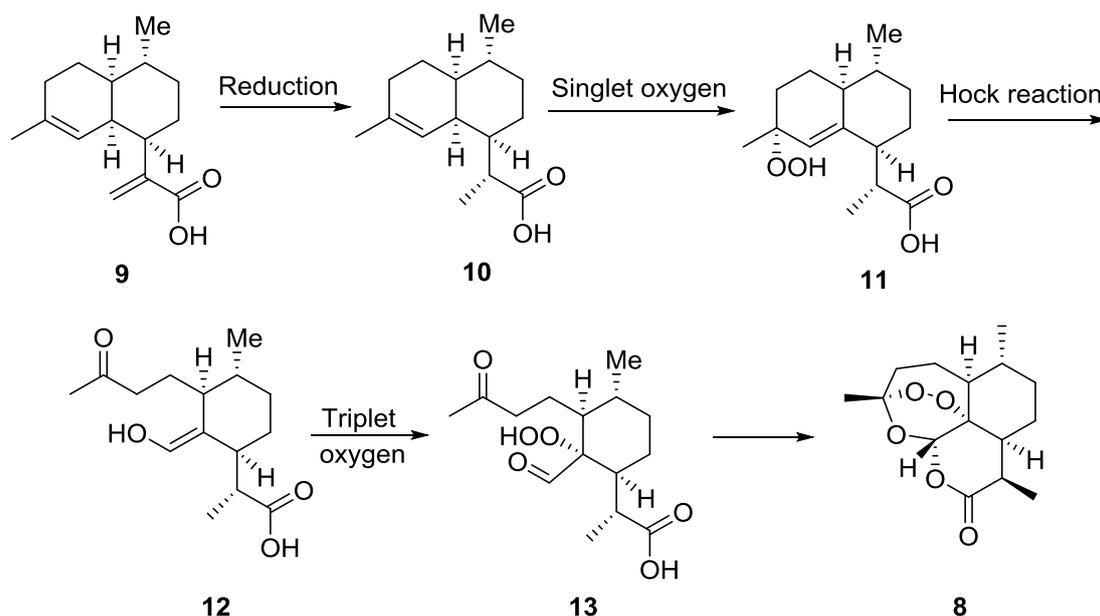
Scheme 2. Synthetic scheme for production of artemisinin (**8**) in a flow reactor.

Another example of a sesquiterpenoid used in medicine, is the phytoalexin capsidiol (**14**, Figure 3), which is produced in tobacco (*Nicotiana tabacum*) and chilli pepper (*Capsicum annuum*)[31] in response to fungal infection.[32] Capsidiol (**14**) may be obtained on a small scale by inoculating whole bell peppers with aqueous cellulase. Capsidiol (**14**) is extracted after 24 h incubation. This procedure was developed by Whitehead *et al.*[33] and successfully applied by Coates *et al.*[34] in a study of the reactivity of capsidiol (**14**) and its derivatives. Recently, capsidiol (**14**) has been reported to be a potent inhibitor of the growth of *Helicobacter pylori*, a pathogen that causes gastrointestinal ulcers.[35]
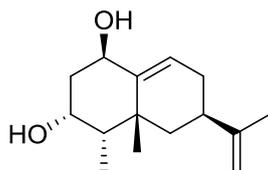
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Figure 3. Structure of capsidiol (**14**).

1.2.3 Diterpenoids and triterpenoids

Diterpenoids and triterpenoids have great importance in the pharmaceutical industry not only for their pharmaceutical properties, but also as targets for drugs given their major physiological function and role in many diseases.[36]

Paclitaxel (**15**, Figure 4) is a diterpenoid that was first isolated from the bark of the Pacific yew tree, *Taxus brevifolia*[37] and its structure was elucidated by Wall in 1971.[38] Paclitaxel (**15**) is one of the most remarkable anti-cancer drugs, which has shown excellent results for breast, non-small cell lung and ovarian cancer.[39] In the case of ovarian cancer, statistics show that the use of paclitaxel (**15**) doubles the survival expectancy of patients.[40] The cytotoxic effect of paclitaxel (**15)** is attributed to the stabilisation of the microtubule assembly of cancer cells that is required to be rapidly changing for the fast cell division characteristic of cancer cells.[41] *T. brevifolia* produces low quantity of paclitaxel that is usually present in the bark in concentrations between 0.01 and 0.05%. This extremely low concentration and the relatively large amount need for one treatment (2.5-3 g), which would require eight mature yew trees per patient, makes the harvest from nature an option that is not viable.[42] The groups of Holton *et al.* and Nicolaou *et al.* published in 1994 the first total synthesis of paclitaxel using two different routes.[43] Even though many different syntheses have been published since then, the high number of steps and the low overall yields of all synthetic routes have set back a successful commercial synthetic methodology.[44] A semi-synthetic process is currently employed commercially, which relies on the extraction of baccatin III (**16**, Figure 4) as an intermediate for the synthesis of paclitaxel.[45] Some steps of the paclitaxel biosynthetic pathway have been transferred into heterologous expression systems, including *Escherichia coli*,[46] *Saccharomyces cerevisiae*[47] and plants.[48] The best results were obtained with plant cell cultures, which can produce as much as 102 mg/kg of paclitaxel (**15**).[49]

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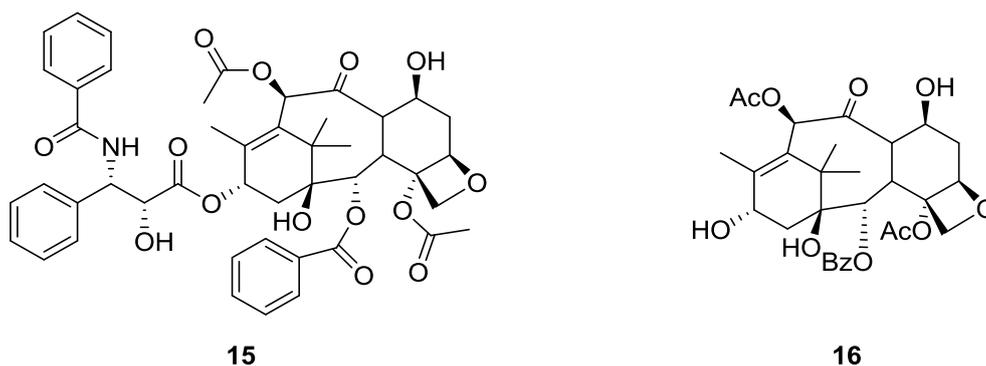
Figure 4. Structures of paclitaxel (**5**) and baccatin III (**6**).

Cholesterol (**21**) is a steroid and perhaps the most famous terpene derivative given the correlation of high plasma blood levels and risk of heart disease.[50] From a physiological point of view, cholesterol is not only used to regulate membrane fluidity in cells, but it can be the starting material to synthesise sexual hormones, bile acids and vitamin D.[51] Cholesterol is biosynthesised from the condensation of two molecules of farnesyl diphosphate (**17**) to produce the triterpene squalene (**18**) and the elimination of two molecules of diphosphate. This reaction is catalysed by squalene synthase. Squalene is oxidised by squalene synthase/monooxygenase to generate epoxide **19**. Epoxide **19** undergoes a cyclisation cascade catalysed by lanosterol synthase to yield triterpenoid lanosterol (**20**). Cholesterol (**21**) is finally obtained through a metabolic pathway involving 19 different steps (Scheme 3).[52]
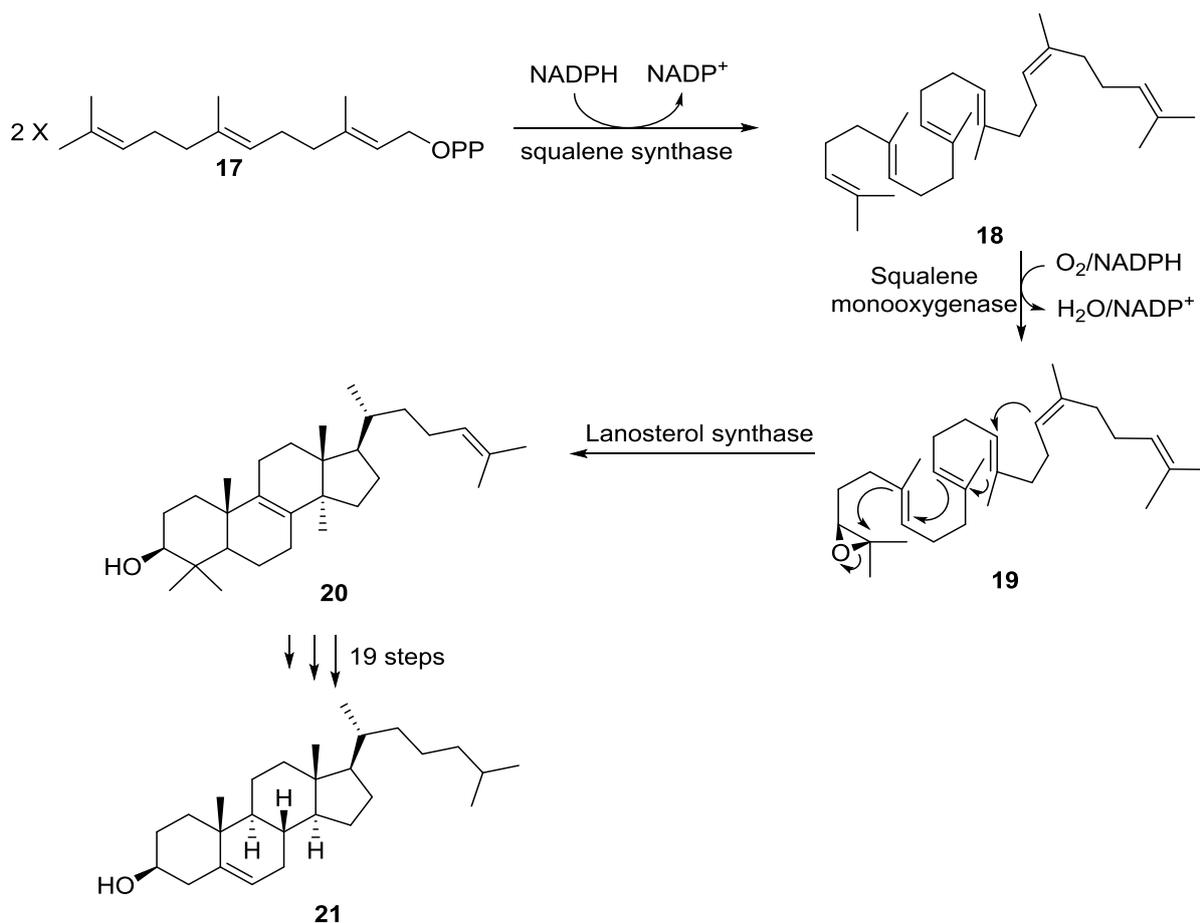
Scheme 3. Overview of the cholesterol (**21**) biosynthetic pathway from two FDP (**17**) molecules.

1.3 Biosynthesis of terpenes

1.3.1 The isoprene rule

In 1887, O. Wallach recognized for the first that all terpenoid structures were formally comprised of series of isoprene (**22**, Scheme 4) units bound together.[53] Later in 1953, L. Ružička postulated that all terpenes were biosynthesised from a common precussor. Indeed, the carbon skeletons of most terpenes are composed of isoprene units arranged in a regular (head to tail, **23**) or irregular pattern (tail to tail **24** or head to head **25**) (Scheme 4). This "isoprene rule" does not postulate the exact nature of the bio-precussor and only assumes the isoprene nature of the structure.[54]
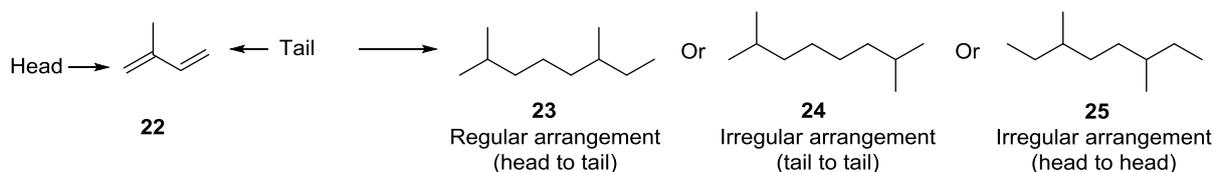
Scheme 4. Possible arrangements of isoprene units in terpenes

Terpenes are a class of molecules composed of isoprene building blocks that undergo modifications, including cyclisations and further oxidations of the carbon skeleton accounting for the enormous diversity of terpenes.

1.3.2 Biosynthesis of IDP (33) and DMADP (34)

Although isoprene units can often be seen in the final terpene structure, the isoprene itself is not the biosynthetic precursor. Instead, two activated equivalents, which are the isopentenyl diphosphate (IDP, **33**) and the dimethylallyl diphosphate (DMADP, **34**), are the precursors of terpenes.[55]

There are two metabolic routes leading to IDP (**33**) and DMADP (**34**) (Schemes 5 and 6): the mevalonate and the non-mevalonate pathways. Although they are not mutually exclusive, mammals, fungi, higher plants and bacteria often use the mevalonate pathway,[56] which takes place in the cytosol and is primarily responsible for the formation of sesquiterpenes and triterpenes. On the other hand, the non-mevalonate pathway (also known as the MEP or DOXP pathway) often takes place in plastids and chloroplasts of algae, cyanobacteria, eubacteria, apicomplexa and plants.[57] This pathway is responsible for the synthesis of monoterpenes, diterpenes and tetraterpenes.

The first committed step of the mevalonate pathway (Scheme 5) is the condensation of two molecules of acetyl coenzyme (acetyl-CoA, **26**) catalysed by acetoacetyl-CoA thiolase to produce acetoacetyl-CoA (**27**), which then condenses with a third molecule of acetyl-CoA to yield the six-carbon compound intermediate 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA,

28). These two reactions are catalysed by HMG-CoA synthase. NADPH-dependent reduction of HMG-CoA is carried out in two steps by HMG-CoA reductase to afford (3R)-mevalonic acid (**29**). The next steps feature the ATP dependent transfer of three phosphate groups, the first diphosphorylate to the primary alcohol of **30** to afford diphosphate **31** and subsequent monophosphorylation of the tertiary alcohol to afford the intermediate 3-phospho-5-diphosphomevalonate (**32**). The intermediate **32** undergoes a decarboxylation with concomitant elimination of monophosphate to afford the final products IDP (**33**) and DMADP (**34**) (Scheme 5).[58]
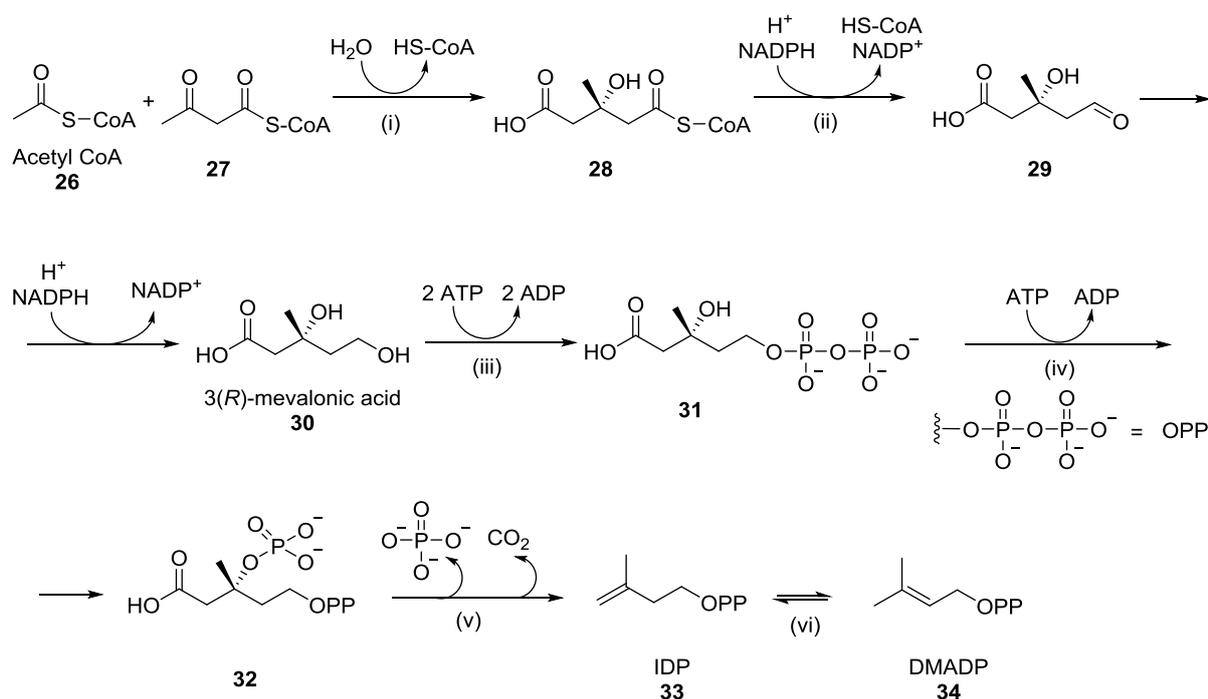
Enzymes: (i) HMG-CoA synthase, (ii) HMG-CoA reductase, (iii) mevalonate-5-kinase and phosphomevalonate kinase, (iv) mevalonate-5-diphosphate-3-kinase, (v) mevalonate-5-diphosphate decarboxylase, (vi) isopentenyl diphosphate isomerase.

Scheme 5. The mevalonate biosynthetic pathway to IDP (**33**) and DMADP (**34**).

The non-mevalonate (Scheme 6) pathway starts with the TPP dependent decarboxylation of pyruvate (**35**). The resulting enamine reacts with glyceraldehyde-3-phosphate (**36**) to produce 1-deoxy-D-xylulose 5-phosphate (DOXP, **37**). This reaction is catalysed by DOXP synthase and is followed by a pinacol-like rearrangement and reduction with NADPH of **37** to afford 2-C-methylerythritol 4-diphosphate (MEP, **38**) catalysed by DOXP reductase. Therefore, the

subsequent reaction of MEP (**38**) with cytidine triphosphate produces **39**, which is phosphorylated by one molecule of ATP to achieve 2-phospho-4-cytidinediphosphate-2-C-methylerythritol **40**. This then undergoes an internal cyclisation that eliminates cytidine diphosphate to afford the cyclic intermediate **41**. The elimination of two molecules of water assisted by two molecules of NADPH affords IDP (**33**) and DMADP (**34**) (Scheme 6).[59]
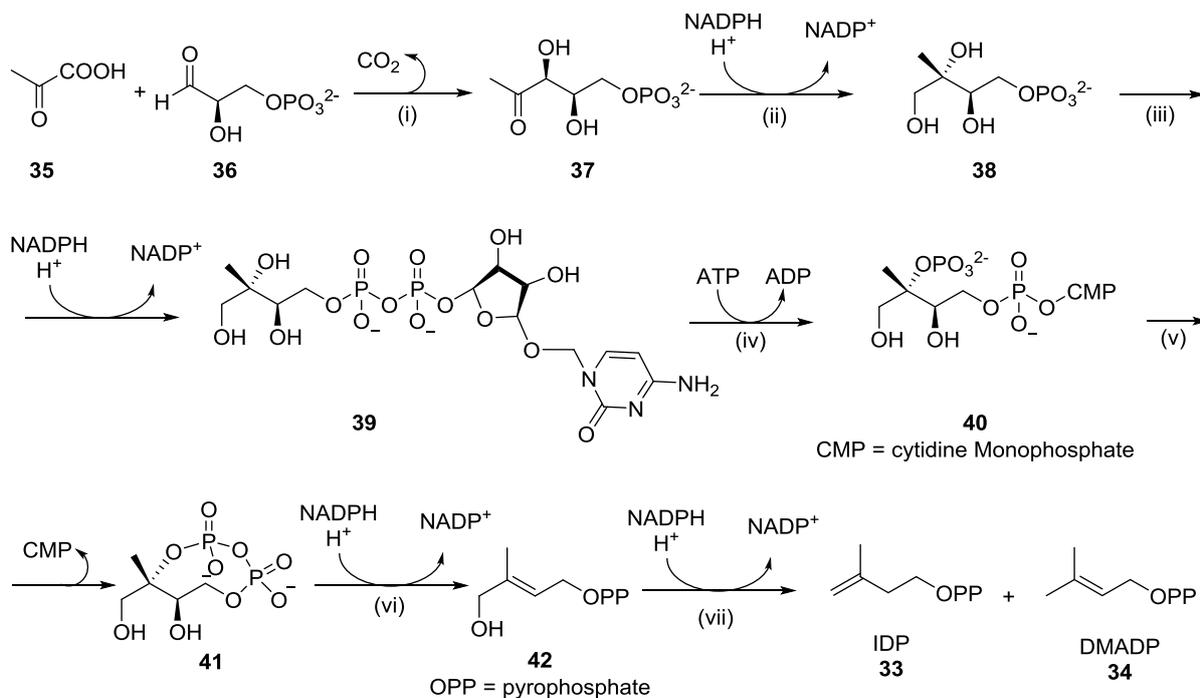
Enzymes: (i) DOXP synthase, (ii) DOXP reductase, (iii) 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, (iv) 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (v) 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, (vi) 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase, (vii) 4-hydroxy-3-methylbut-2-en-1-yl diphosphate resuctase.

Scheme 6. The non-mevalonate biosynthetic pathway for production of isoprenyl diphosphates.

1.3.3 Biosynthetic precursor of terpenes

IDP (**33**) and DMADP (**34**) are the bioprecursors of all terpenes from isoprene (C5) to carotene (C40). The universal linear prenyl diphosphate substrates of terpene synthases are biosynthesised from DMADP by iterative condensation of IDP (**33**) molecules (Scheme 20).[60]
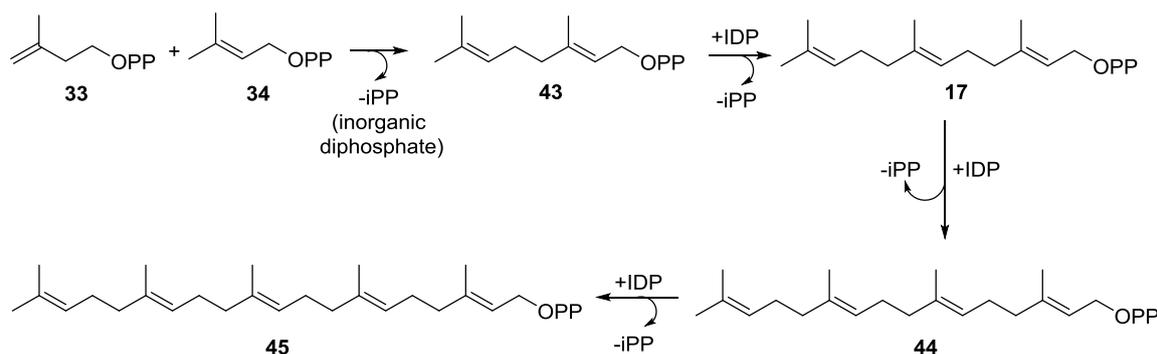
Scheme 7. Biosynthesis of isoprenyl diphosphates from IDP (**33**) and DMADP (**34**).

All diphosphates are biosynthesised by chain elongation of DMADP (**34**) and IDP (**33**) by isoprenyl diphosphate synthases. The mechanism proposed for this transformation starts with the ionisation of diphosphate group of DMADP (**34**) to generate the allylic carbocation **46**. Furthermore, the addition of the double bond of IDP affords carbocation **47**, and elimination of a proton affords GDP (**43**, Scheme 8).[61]
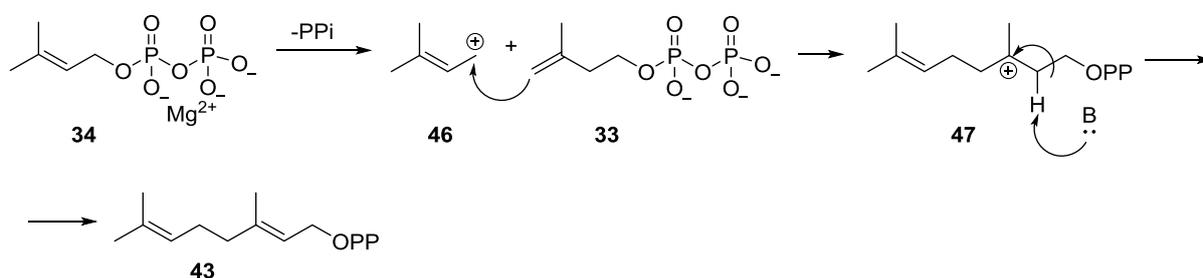
Scheme 8. Chain elongation reaction of DMADP (**34**) to geranyl diphosphates GDP (**43**).

1.4   Terpene synthases

All diphosphates generated by elongation of IDP (**33**) and DMADP (**34**) are converted into the final, often cyclic terpene products by terpene synthases. These enzymes catalyse the conversion of their substrates into numerous acyclic and carbocyclic structures. These transformations are performed with high region- and stereoselectivity discriminating different faces of prochiral centres.[62]

Terpene synthases can be found in any living organism from animals to plants, fungi, bacteria or eukariotes. Terpene synthases from fungi and bacteria bear few similarities regarding their

amino acid sequences, (usually ranging between 25-35%).[63] On the other hand, terpene synthases from plants bear greater similarity mostly ranging from 53% up to 83%.[64]

Despite the differences between amino acid sequences, terpene synthases display some common structural features. The active site of terpene synthases is surrounded by 5 α-helices connected by loops[65] as part of an α-barrel composed of 12 α-helices arranged in anti-parallel fashion.[66] The contour of the active site is different in each enzyme and it serves as a template for the flexible substrate, giving characteristic reactivity to each enzyme. The landscape of the active side (contour) is composed mainly of hydrophobic residues, which helps locating the hydrophobic chain of the substrate in the active site.[67]

According to the tertiary structure and general reaction mechanism, terpene syntheses can be classified as class I or class II. The enzymatic structure of class I terpene synthases is predominantly helical. A characteristic structural feature of class I enzymes is the presence of two binding motifs that coordinate three Mg^{2+} ions. The first specific metal binding motif is composed of the amino acid sequence DDXX(D/E) located placed at the entrance of the active site, and another binding motif is at the opposite side of the active site with the amino acid sequence (D/N)DXX(S/T)XXXE. The metal ions bind to these motifs and coordinate to the diphosphate group of the substrate, triggering ionisation of the substrate. On the other hand, class II terpene synthases only contain one DXDD metal binding motif. Class II terpene synthases initiate the enzymatic reaction with protonation of the distal C-C double bond or the corresponding epoxide.[68]

The amino acid sequence that fits with these characteristic motifs helps to find the specific DNA sequences that encode for terpene synthases. These DNA fragments encoding for terpene synthases are copied and introduced in *E.coli* to express the corresponding enzymes *in vitro*.[69]

Terpene synthases can be classified according to the number of carbons contained in the substrate used by the enzyme. Monoterpene synthases use GDP (C10) for the transformation; sesquiterpene synthases use FDP (C15); diterpene synthases use geranylgeranyl diphosphate (GGDP, C20) and so on.

1.4.1 Sesquiterpene synthases

Sesquiterpene synthases catalyse the Mg^{2+}-dependant conversion of FDP (**17**) into more than 300 hydrocarbon skeletons.[60] Most sesquiterpene synthases generate a single hydrocarbon product with remarkable region- and stereochemical fidelity. The high diversity of sesquiterpenes in nature is achieved not only by the large number of different sesquiterpene synthases, but also by promiscuity of some terpene synthases like γ-humulene synthase that can produce at least 52 different products.[70]

The high regio- and stereochemical selective conversions catalysed by sesquiterpene synthases are achieved through a complex cascade of reactions. On average, these transformations involve changes in to two-thirds of the carbon atoms present in the hydrocarbon chain of FDP such as changes in bonding, hybridisation or configuration.[68] Reaction starts with ionisation of the diphosphate to give a carbocation often followed by electrophilic attack to the central or distal double bond to produce cyclic products. Subsequent reactions involve cyclisations, rearrangements and deprotonation of highly reactive carbocationic intermediates and final proton loss to quench the positive charge.[71] This theory is now widely accepted due to its success explaining experimental results using substrate analogues, inhibitors, and intermediate analogues.[72]

Four different sesquiterpene synthases were chosen for this project due to the relevant agrochemical and medical properties of their enzymatic products. (+)-Germacrene A synthase (GAS), (-)-germacrene D synthase (GDS) and (+)-δ-cadinene synthase (DCS) were isolated from plants, thus the structures of these enzymes have two domains. GAS and GDS have well-established reaction mechanism, whereas the mechanism of DCS is still under discussion. Aristolochene synthase (AS) was isolated from fungi, thus the structure of this enzyme has one domain. Its mechanism is also well established and has been object of previous studies.[73] Whereas AS and DCS have been crystalised and their structures are known in detail,[74] GDS and GAS have not been crystalised. Therefore, study of the residues involve in the catalytic cycle of GDS and GAS, is performed using a homology model.[75]

1.4.2 Germacrene A synthase (GAS) from *Solidago canadensis*

The complete cDNA for a functional germacrene A synthase was first isolated from *Solidago canadensis*. The 80 kDa sesquiterpene synthase was overexpressed in *E. coli*, incubation of FDP (**17**) with the recombinant protein gave a product profile that revealed the presence of (+)-germacrene A (98%) and α-humulene (2%) (**5**, Figure 2).[76]

Studies with deuterium labelled FDP analogues suggested the mechanism outlined in Scheme 9. The enzymatic conversion of FDP (**17**) starts with ionisation of the diphosphate (**48**) and the attack of the double bond on C10 to C1 with displacement of the diphosphate to generate the intermediate germacradienyl cation (**49**). Subsequent deprotonation at C12 affords (+)-germacrene A (**50**).[77, 78]
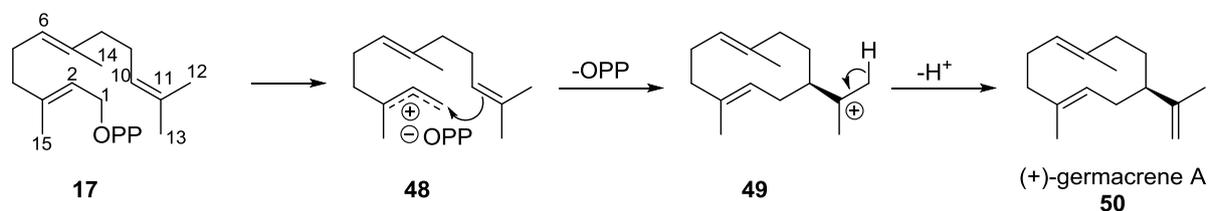
Scheme 9. Catalytic mechanism of GAS.

Germacrene A was isolated for the first time from the *E. mammosa* flower by Weinheimer as the (-)-enantiomer.[79] Then, it was also isolated from different organisms such as the aphid *T. maculala*,[80] a soft coral of the genus *Lobophytum*,[81] or the termites (*Reticulitermes lucifugus*).[82] (+)-Germacrene A (**50**) was isolated from caraway herb and root,[83] fresh cactus roots from *S. lappa* [84] and *Solidago canadensis*.[85] Whereas the (-)-enantiomer of **50** is found in animals and insects, the (+)-enantiomer of germacrene A seems to be more common of higher plants.[86] The tendency of germacrene A (**50**) to undergo thermal Cope rearrangement to β-elemene (**51**, Scheme 10) in a stereospecific reaction was used to determine its absolute configuration by comparison with an authentic sample of β-elemene using chiral GC.[87]
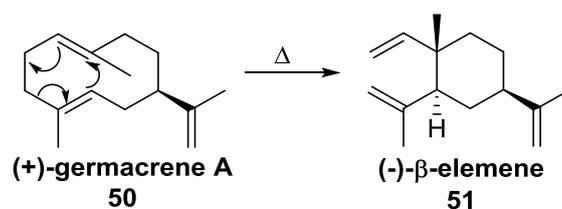
Scheme 10. Thermally induced Cope rearrangement of (+)-germacrene A (**50**).

Germacrene A (**52**) has been proposed as an intermediate in the biosynthesis of many natural phytoalexins, such as capsidiol (**14**),[88] costunolide (**57**)[89] or rishitin (**54**).[90] It has also been proposed as an intermediate in the enzymatic formation of some terpenes, like patchoulol (**55**),[91] 5-*epi*-aristolochene (**53**)[92] or aristolochene (**56**, Scheme 11).
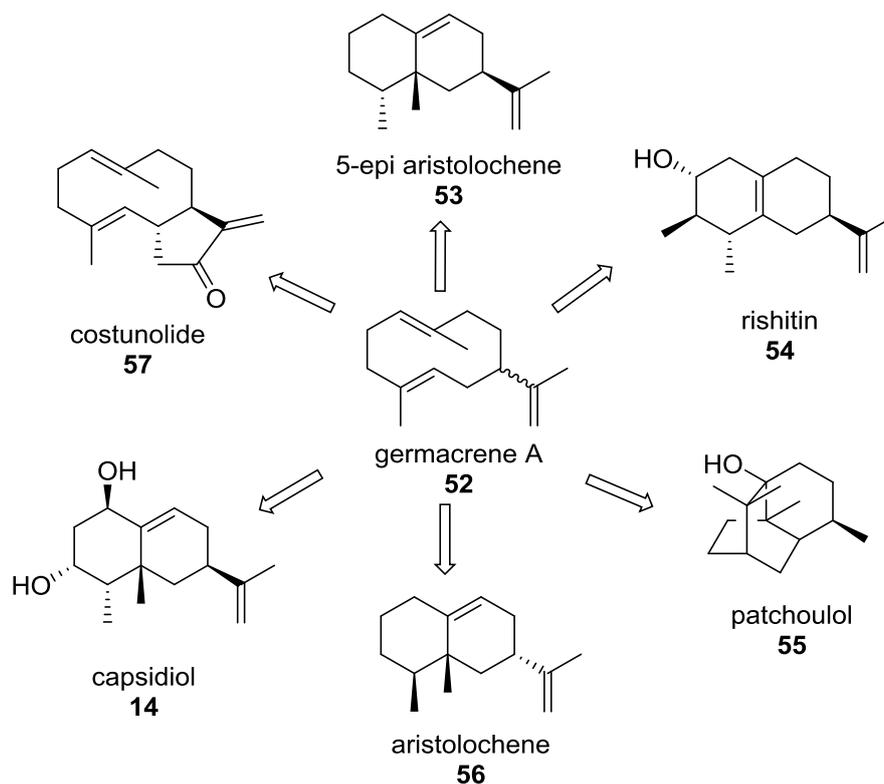
Scheme 11. Some compounds that are biosynthesised from germacrene A (**52**).

Bowers has reported the importance of (+)-germacrene A (**50**) as a potential chemical agent for pest control.[93] Crops are affected by different species of aphids, for instance, faba bean crops usually suffer damages by the black bean aphid. These insects feed from this plant

25

resulting in impairment of plant growth and yield of production.[94] In addition, these insects are vectors transmitting viruses to other plants and causing further damage.[95] Small initial colonies may result in a large infestation in a short period due to the high rates of reproduction of these insects.[96] A study of the chemical language of aphids found that (+)-germacrene A (**50**) acts as repellent for the alfalfa aphid. The economic impact that this insect has on crops makes (+)-germacrene A (**50**) an attractive target to be modified and hence, modulate its properties.

Additionally, β-elemene has been reported as a potential chemical against leukaemia and carcinomas of the brain, breast, liver and lungs. These medical properties make the production of (+)-germacrene A (**50**) derivatives very attractive compounds to be easily transformed into β-elemene (**51**) derivatives for testing in medicine.[97]

The synthesis of (+)-germacrene A (**50**) presents a formidable challenge for organic chemist due to the decomposition resulting from heat, acid and light, thus creating complications with regard to purification and product analysis.[98] Synthetic efforts to prepare the unstable cyclodecadiene hydrocarbon core of germacrene A have suffered from low yield, racemic mixtures and poor step economy.[99] Recently Baran *et al.*[100] developed an efficient synthesis of (+)-acoragermacrone (**62**) on gram scale. This work established an efficient synthetic methodology for preparing the hydrocarbon core of germacrene A from farnesol (**58**). Firstly, a regio- and stereoselective epoxidation of farnesol is carried out using a Sharpless epoxidation and then, alcohol is oxidised to aldehyde **59**. Regioselective chlorination with transposition of the distal double bond to obtain **60** was performed according to a protocol developed by Tunge *et al.*[101] The key step of the synthesis of **62** is the ring closure of **60** through an umpolung allylation with subsequent reduction of epoxide **61** and oxidation of the resulting alcohol to afford (+)-acoragermacrone (**62**, Scheme 12). Alternatively, (+)-germacrene A (**50**) has been produced in high yield and purity using genetically modified yeast.[102]

farnesol
58

59

60

61

(+)-acoragermacrone
62
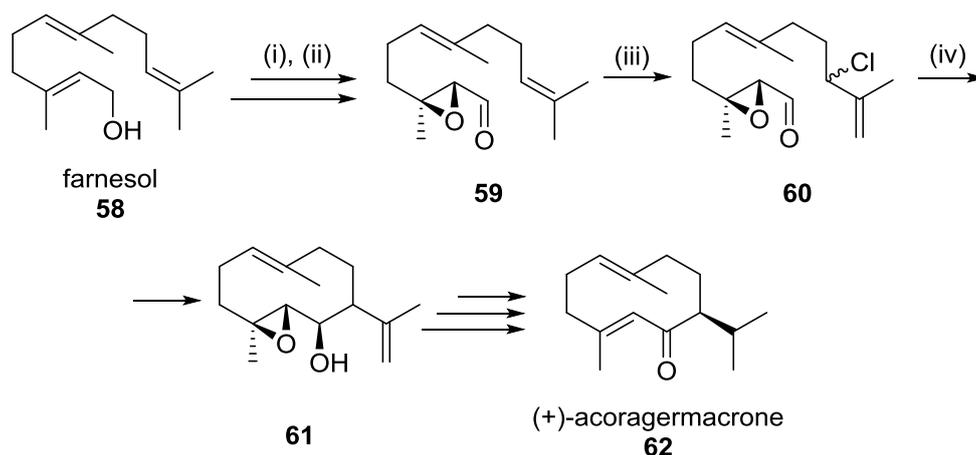
Reagents and conditions: (i) Ti(OiPr)$_4$ (0.1 equiv), (+)-DET (0.12 equiv), *t*-BuOOH (2 equiv), 4Å MS, CH$_2$Cl$_2$, -50 ºC, 2 h (ii) SO$_3$·py (4 equiv), iPr$_2$NEt (5 equiv), DMSO (10 equiv), CH$_2$Cl$_2$, 0 ºC, 0.5 h (iii) PhSeCl (0.12 equiv), NCS (1.1 equiv), CH$_2$Cl$_2$, RT, 1 h (iv) [PdCl$_2$(PPh$_3$)$_2$] (10 mol%), K$_2$CO$_3$ (1.5 equiv), Et$_2$Zn (1.5 equiv), DMA, 50ºC.

Scheme 12. Synthesis of (+)-acoragermacrone (**62**).

1.4.3 Aristolochene synthase (AS) from *Penicillium roqueforti*

Two aristolochene synthases genes have been isolated from *P. roquefortii*[103] and *A. terreus*[104] and, in both cases, have been cloned and overexpressed in *E coli*.[105] Both enzymes possess the characteristic class I terpene fold, but share only around 60% of the amino acid sequence identity.[106]

The proposed catalytic mechanism of AS starts with attack of the distal double bond of FDP (**17**) at C1 and then loss of diphosphate to afford the germacrydienyl cation (**63**). (*S*)-germacrene A (**64**) is then formed after deprotonation at C12 (Scheme 13).[73] This intermediate undergoes a protonation of the double bond on C6 and cyclisation by the attack of the double bond on C2 to afford the bicyclic eudesmane cation (**65**). Subsequent hydride shift from C2 to C3 affords the cation **66**, followed by methyl group migration from C7 to C2 that gives the aristolochyl cation (**67**). Final deprotonation of H$_{si}$ at C8 yields the final (+)-aristolochene (**56**, Scheme 13). The mechanisms and the stereochemistry have been confirmed with various experiments involving isotopic labeled FDP.[107] Cane *et al.* determined the absolute configuration by its comparison with an authentic sample of synthetically prepared (-)-aristolochene.[108]
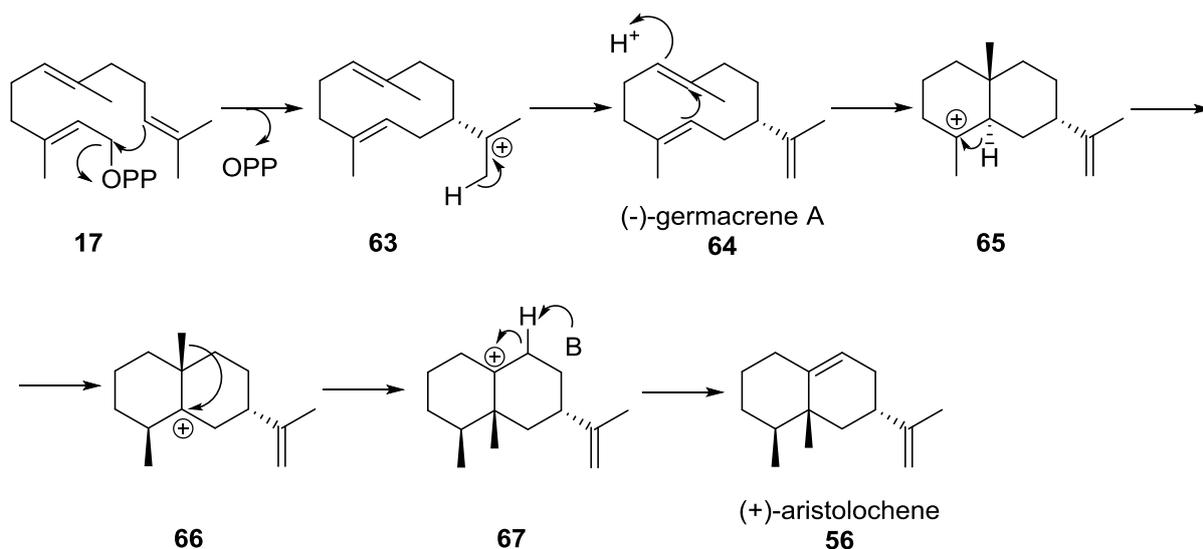
Scheme 13. Proposed catalytic mechanism for aristolochene formation (**56**).

(+)-Aristolochene (**56**) has been found in the defensive secretions of some termites.[109] Feeding experiments[110] demonstrated that aristolochene (**56**) is the precursor of PR-toxin (**68**), which is the major toxin found in *P. roqueforti.*[111] PR-toxin inhibits the synthesis of RNA and the activity of different protein synthases in eukaryotes.[112] Tests in rats reveal toxic properties making it lethal by intraperitoneal or oral administration.[113] The structure of the toxin was solved by X-ray crystallography [114] and spectroscopic methods (**68**, Scheme 14).[115]
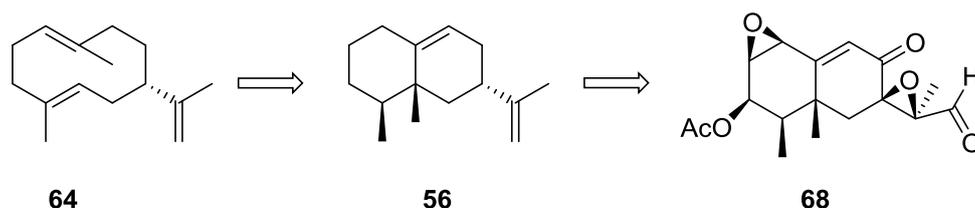
Scheme 14. PR-toxin (**68**) from aristolochene (**56**).

The total synthesis of (-)-aristolochene (**75**) accomplished by Cane *et al.*[116] using (+)-valencene as starting material was lately improved by Pedro *et al.* who used (*R*)-carvone (**69**) as starting material.[117] The synthesis relies on a rearrangement of a heterospecific cationic decalinic intermediate guided by trimethyl silyl group.[118] *R*-carvone (**69**) was treated with Me$_3$SiLi to introduce trimethyl silyl group using the conditions described by Still[119] to afford

70. The decalinic core was prepared through Robinson annulation by reaction of **70** with methyl vinyl ketone to afford **71**. Subsequent reduction and epoxidation of **71** gave epoxide **73**. The key step is the rearrangement of **73**, which is initiated by the Lewis acid catalysed opening of epoxide **73** that generates a tertiary carbocation, subsequent methyl migration and elimination of trimethyl silyl group afford **74** after acetilation of the resulting alcohol. Reduction of ester **74** gave (-)-aristolochene (**75**, Scheme 15).
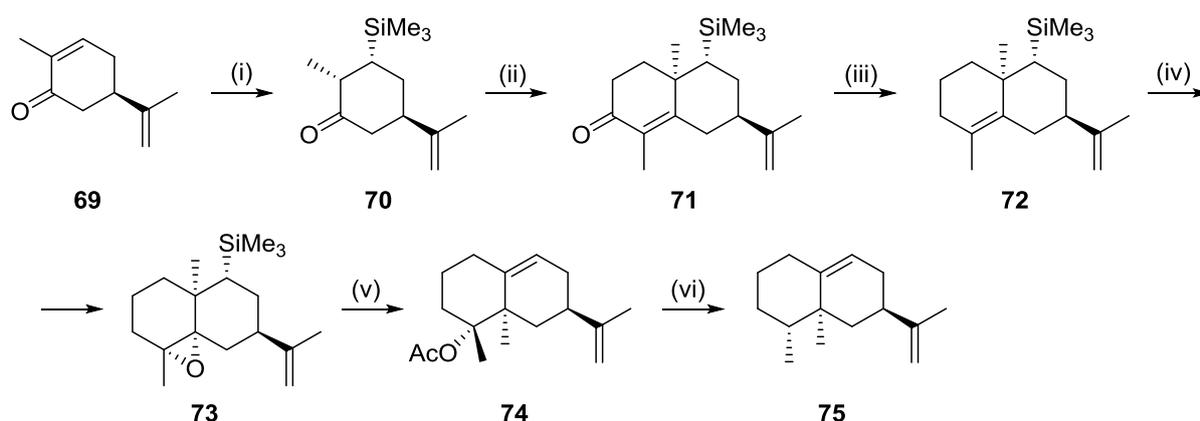
Reagents and conditions: (i) (Me$_3$Si)$_2$ (2.5 equiv), MeLi (2 equiv), THF-HMPA (ii) Methyl vinyl ketone (2 equiv), Ph$_3$CSbCl$_3$ (0.05 equiv), 0 °C and then 1 M KOH/MeOH (iii) LiAlH$_4$ (4.3 equiv), AlCl$_3$ (12.8 equiv), Et$_2$O, 0 °C, 5 min, then **71** , 0 °C (iv) *m*-CPBA (1.5 equiv), NaOAc (2.5 equiv), CHCl$_3$, 0 °C (v) TiF$_4$ (1.5 equiv), CH$_3$CN, -20 °C (vi) 4-DMAP (0.46 equiv), Ac$_2$O-Pyr (1:1) and then K (2.5 equiv), 18-crown-6 (cat), *t*-BuNH$_2$.

Scheme 15. Synthesis of (-)-aristolochene (**75**).

1.4.4   Germacrene D synthase (GDS) from *Solidago canadensis*

Germacrene D is a repellent for some species of aphids.[120] This repellent pheromone is produced as a natural defence mechanism by some plants.[121] Two different sesquiterpene synthases have been cloned from *Solidago canadensis*. Whereas one enzyme produces (+)-germacrene D (**81**), the other produces (-)-germacrene D (**78**).[122] The catalytic mechanisms of the two-germacrene D synthases have been studied with deuterium labelled FDP analogues.

The (-)-enantiomer (**78**) is generated from FDP (**17**) by an initial attack of the distal double bond to C1 to afford germacradienyl cation (**76**). Then, a hydride shift from the proton on C1

to C11 gives the allylic carbocation (**77**) which undergoes a deprotonation at C15 to quench the allylic carbocation affording the final (-)-germacrene D (**78**, Scheme 16).
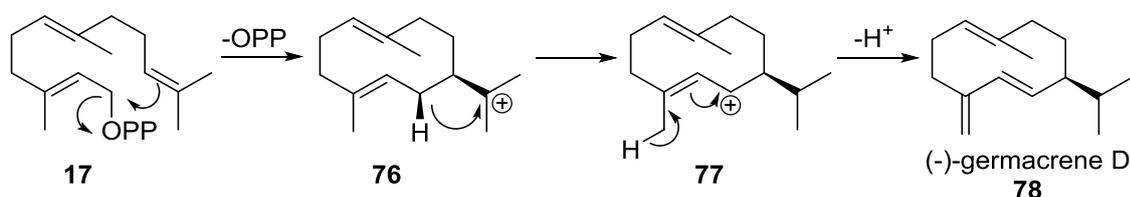
Scheme 16. Proposed mechanism for the formation of (-)-germacrene D (**78**).

The formation of the (+)-isomer starts in the same way with the formation of the same germacradienyl cation (**76**). In this case, the subsequent hydride shift takes place from proton on C10 to C11 to afford the new tertiary carbocation on C10 **79**. The migration of the hydrogen in C1 to C10 affords now allylic carbocation with the opposite stereochemistry **80**. Deprotonation at C15 gives the (+)-enantiomer (**81**, Scheme 17).[123]
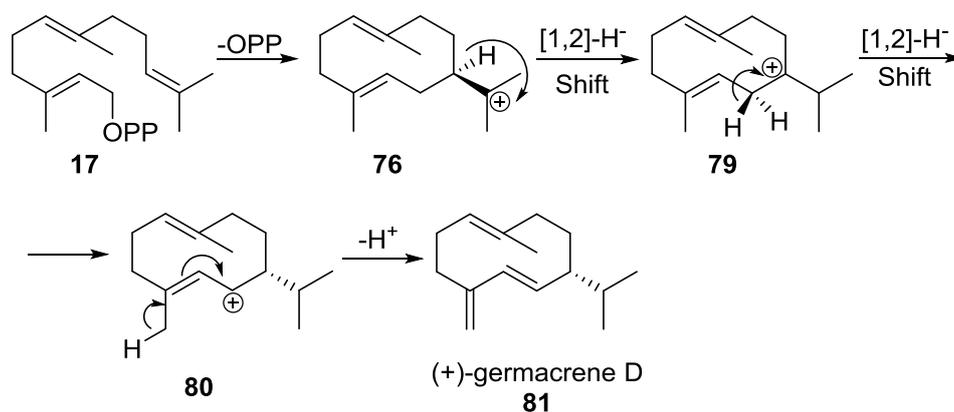
Scheme 17. Proposed catalytic mechanism for the formation of (+)-germacrene D (**81**).

(-)-Germacrene D (**78**) was isolated from ylang-ylang oil with 98% ee[124] in sufficient amount to be used as starting material for the total synthesis of different kind of periplanones.[125] (-)-Germacrene D (**78**) is a natural intermediate in the biosynthesis of periplanone A (**82**) and B (**83**),[126] which acts as sexual pheromone of male *Periplaneta americana.*[127]
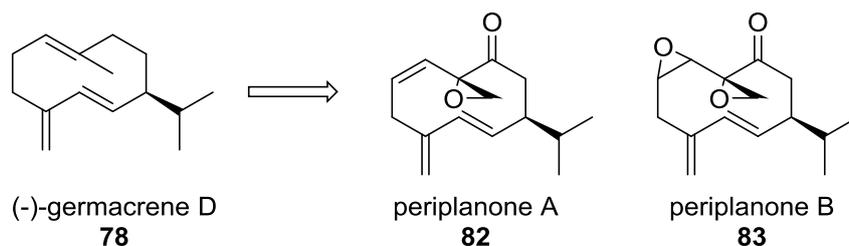
Scheme 18. Periplanones A and B (**82** and **83**) from (-)-germacrene D (**78**).

Due to its potential use as an agrochemical for pest control, a total synthesis of (-)-germacrene D (**78**) was developed by Schreiber *et al.*[128] The synthesis commenced with photocycloaddition of allene (**84**) and α,β-unsaturated ketone **85** followed by stereospecific addition of vinylmagnesium bromide provided allylic carbinol **86** as a mixture of isomers. Oxy-Cope rearrangement of **86** gave cyclobutene **87**, which underwent an electrocyclic ring opening to afford a mixture 2:1 (trans / cis) of ketone **88**. The mixture was equilibrated by photochemical reaction obtaining a mixture 15:1 and trans isomer was separated by HPLC.[129] Selective enolisation of ketone **88** and subsequent sulfenilative trapping gave vinyl triflate **89**. Stereospecific displacement of vinyl triflates with organocopper reagent was performed to afford (-)-germacrene D (**78**, Scheme 19).
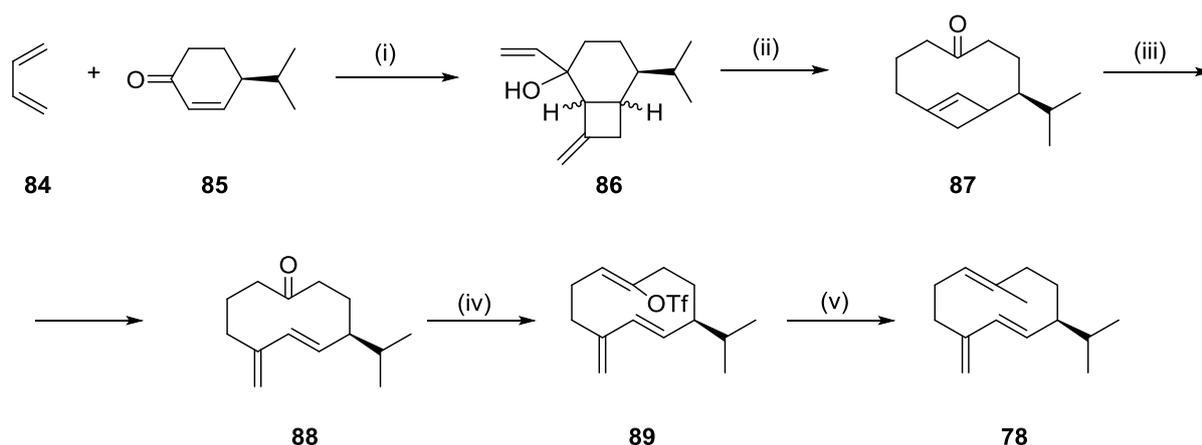
Reagents and conditions: (i) hυ, (450-W Hanovia lamp equipped with a Pyrex filter), Et$_2$O 30 ºC and then CH$_2$=CHMgBr, Et$_2$O, -78 ºC (ii) KH, 5 equiv of 18-Cr-6, THF, 60 ºC, 25 min (iii) Toluene, 175 ºC, 20 h and then hυ (450-W Hanovia lamp equipped with a Vycor filter) benzene. (iv) LiHMDS, HMPA, Tf$_2$NPh, THF -78 ºC (v) Me$_2$CuLi, Et$_2$O, -78 ºC.

Scheme 19. Synthesis of (-)-germacrene D (**78**)

In presence of acid, (-)-germacrene D (**78**) rearranges over time into a complex mixture of cadinenes, muurolenes, amorphanes, ylangane, eudesmanes, isodaucanes, oppositanes and axanes.[130] (-)-Germacrene D (**78**) is stable up to 180 ℃ but at 240 ℃ rearranges to β-ylangene (**90**), β-copaene (**91**), ε-muurolene (**92**) and ε-amorphene (**93**).[131] Isogermacrene D (**94**) (Figure 5) was obtained when a sample of (-)-germacrene D (**78**, Scheme 16) was analysed by GC-MS at injection port temperature of 400 ℃.[132]
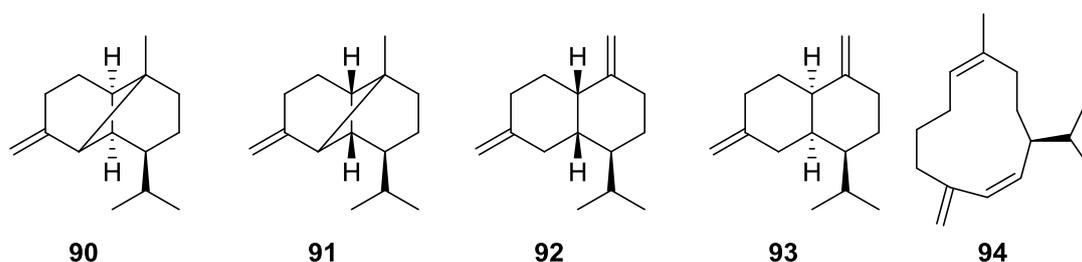
Figure 5. Products obtained from the thermal decomposition of (-)-germacrene D (**78**).

Due to their relevant biological properties, part of this work is aimed at the chemoenzymatic preparation of several analogues of aristoloche (**56**), as well as (+)-germacrene A (**50**) and (-)-germancrene D (**78**) for testing their repellent properties against aphids. These analogues will be tested for improved environmental stability or enhance repellent properties in comparison with their parent compounds.

1.4.5   (+)-δ-Cadinene synthase (DCS) from *Gossypium arboreum*

The *de novo* synthesis of the phytoalexin gossypol from (+)-δ-cadinene was observed in a cell culture of the cotton tree *Gossipium arborerum*, when the fungus *Verticium dahliae* was inoculated.[133] The study of this defence mechanism against fungal or bacterial infections, led to the identification of a 64 kDa sesquiterpene cyclase, (+)-δ-cadinene synthase.[134] The DNA encoding for this enzyme has been isolated, cloned and over expressed in *E. coli*.[135]

The catalytic mechanism of DCS has been discussed in the literature and, although some proposals have been formulated, two mechanisms are still possible for the conversion of FDP

(**17**) to (+)-δ-cadinene (**103**).[136] The first isomerisation step is common to both mechanisms; it begins with the isomerisation of the primary diphosphate on FDP (**17**) to the tertiary diphosphate, (3*R*)-nerolidyl diphosphate (NDP, **95**, Scheme 20) and subsequent ionisation to **96**.[137] Next steps of the two different proposals involve, as the key step, either a 1,10-cyclisation or a 1,6-cyclisation, to achieve the final (+)-δ-cadinene (**103**).
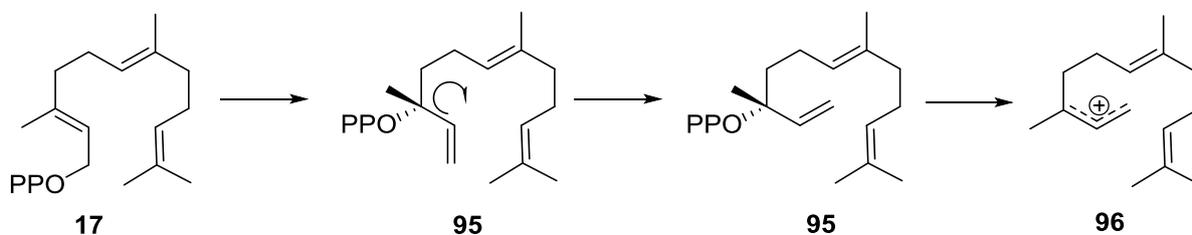
Scheme 20. Isomerisation of FDP (**17**) into (*3R*)-NDP (**95**).

One of the proposed catalytic mechanisms involves a 1,10-macrocyclisation of **96** with the elimination of diphosphate to afford the *cis*-germacradienyl cation (**97**). Subsequent hydride shift leads to the allylic carbocation (**98**). A second cyclisation involving reaction of the double bond on C6 with the carbocation on C1 affords cadinenyl cation (**102**), which is a common intermediate for both mechanisms.[138]

The proposed catalytic mechanism through a 1,6-cyclisation starts with the attack of the double bond in C6-C7 to carbocation on C1 of **96** affording the α-bisabolyl cation (**99**). α-Bisabolyl cation (**99**) undergoes a hydride shift of the hydrogen on C1 to afford the allylic carbocation **100**, followed by a second cyclisation by attack of the distal double bond to carbocation on C1 to give **101**. The next step of this mechanism is a hydride shift from C7 to C11 to achieve the cadinenyl cation **102**.[136]

The last step of the mechanism, common to both pathways, consists of a deprotonation of the cadinenyl cation (**102**) to afford δ-cadinene (**103**, Scheme 21).
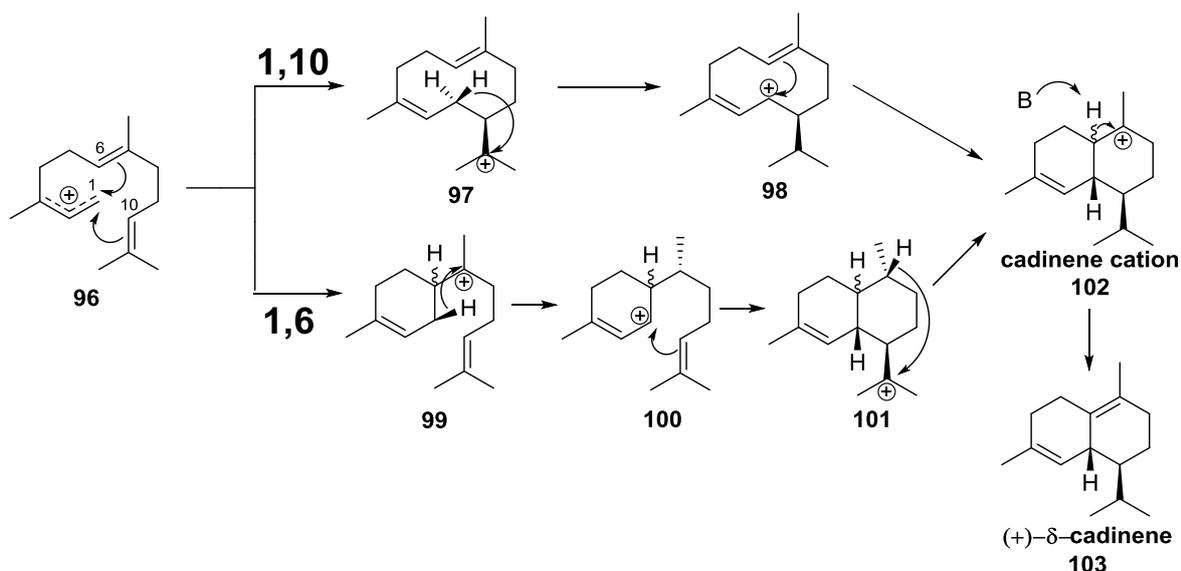
Scheme 21. Two possible pathways for the formation of (+)-δ-cadinene (**103**) from NDP (**95**).

The production of (+)-δ-cadinene (**103**) is the first biosynthetic step in route to *Gossypol* (**104**).[139] This secondary metabolite **104** is the major terpenoid produced by cotton tree and is its main chemical defence.[140] Interestingly, this phytoalexin has been also studied as a male contraceptive drug[141] and as a potential cancer therapeutic drug.[142]

104
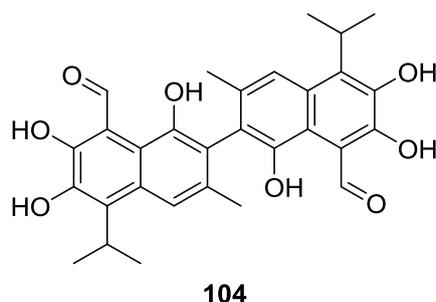
Figure 6. Gossypol (**104**) can be prepared from (+)-δ-cadinene (**103**).

Mizutani developed a simple total synthesis of δ-cadinene (**103**) through stereo selective Robinson annulation between **106** and **107** to form cadinenone (**108**). The oxygen of **108** was removed by thioketal / Ni Raney method to produce δ-cadinene (**103**, Scheme 22).
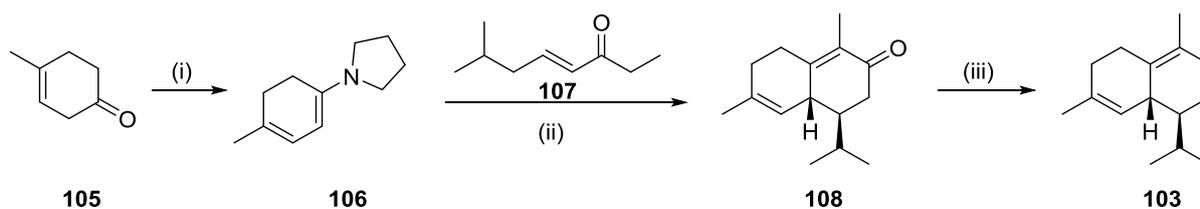
Reagents and conditions (i) Pyrrolidine, benzene, reflux (ii) AcONa / AcOH, benzene, 80 °C (iii) Ethanedithiol, BF_3OEt_2, MeOH, 0 °C and then W_2-Raney Ni, EtOH / H_2O.

Scheme 22. Synthesis of δ-cadinene (**103**).

The biosynthesis of δ-cadinene (**103**) is still being debated, and part of this study is focused on providing an unambiguous solution to this problem.

1.5 Study of terpene synthase mechanism

The reactions promoted by terpene synthases have been the subject of study for a long time and there seems to be general agreement in the literature that these reactions occur through a carbocationic intermediate.[143] Some approaches to the study of enzymatic mechanism of terpene synthases include X-ray crystallography,[144] mutagenesis,[145] aza-analogues of carbocations intermediates[146] and substrate analogues of FDP (**17**), particularly fluorinated FDP analogues.[147]

1.5.1 Crystal structures in combination with mutagenesis

Aristolochene synthase (AS) from *Penicillium roqueforti* provides a suitable example of how the crystal structure of a terpene synthase can be used to design a mutant enzyme that helps to uncover the role of specific amino acid in the catalytic reaction, as well as gaining more insight into the enzymatic mechanism. AS was the first sesquiterpene cyclase from a fungus to be crystallised. The 2.5 Å resolution crystal structure (Figure 7) allows identification of the active site and the study of the key amino acids involved in the reaction catalysed by AS.[148]

For instance, the identification of leucine 108 as part of the active site in the crystal structure of AS, led to the study of the role of this amino acid by preparing mutants of AS, in which leucine 108 was substituted with less bulky amino acids, such as alanine or serine. These mutants of AS did not possess the ability to produce cyclic products, generating instead a mixture of linear hydrocarbons. This result outlines the importance of the leucine 108 for the templating effect that AS has over the flexible FDP (**17**), placing the double bonds in the right orientation for cyclisation.[149]

The presence of aromatic residues in the active site of terpene synthases has been proposed as a key feature for the stabilisation of carbocationic intermediates and thus reducing the energy required for the formation of the desired product and helping to discriminate among other possible pathways.[150] This carbocationic stabilisation promoted by aromatic residues was investigated by preparing AS mutant F178V, which produces germacrene A (**64**) instead of aristolochene (**56**). Therefore, germacrene A (**64**) is an intermediate in the conversion of FDP (**17**) into aristolochene by AS which undergoes a second cyclisation to generate eudemane cation (**65**, Scheme 13, *vide supra*). F178 is hence proposed to stabilise the eudesmane cation (**65**) through π-interaction of its phenyl side chain with the carbocationic centre. The substitution of phenylalanine for valine thereby increases the energetic barrier for the formation of eudesmane cation (**65**), thus germacrene A (**64**) is produced instead.[151]
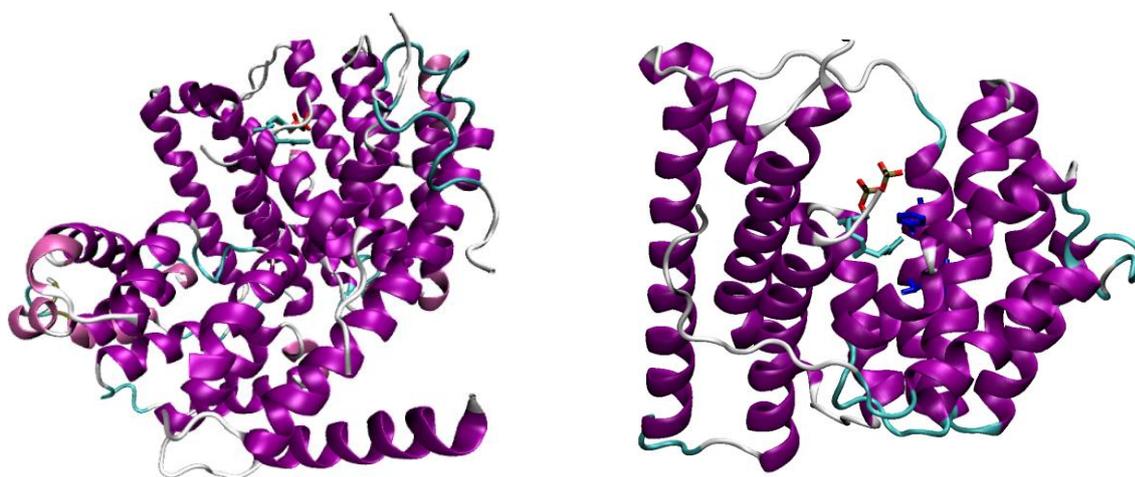
Figure 7. A cartoon representation of the crystal structure of aristolochene synthase from *Penicillium roqueforti* (1DGP.pdb, left) and a model of the active side with FDP (right).

1.5.2   Aza-analogues of carbocationic intermediates

Eudesmane cation (**65**) is predicted to be an intermediate in the catalytic mechanism of AS and this possibility was investigated with aza-analogues that mimic the transition state leading to eudesmane cation (**65**). Iminium salts **109** and **110** were synthesised and evaluated in inhibition studies under steady state conditions, showing that the two compounds were only moderate inhibitors of PR-AS. However, when the studies were repeated in the presence of diphosphate, these two salts became potent inhibitors of AS. This result highlighted pointed out the role of diphosphate in stabilising the eudesmane cation (**65**) and provides convincing evidence that it is an intermediate in the catalysis of AS.[152]
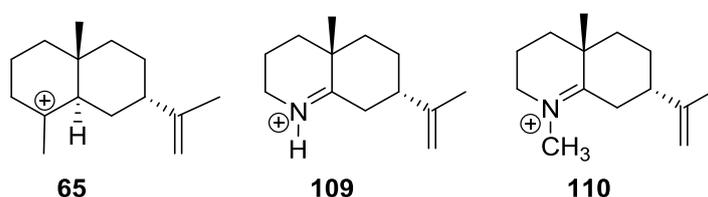
Figure 8. Aza-analogues (**109** and **110**) of eudesmane cation (**65**).

1.5.3   FDP analogues

FDP analogues are useful tools to uncover the cryptic enzymatic mechanism of sesquiterpene synthases. FDP containing fluorine atoms are especially useful. The small size of the fluorine atom does not affect the binding of the analogue to the active site,[153] but can prevent the formation of carbocations β to the fluorine atom and inactivated double bonds. These properties can be useful to provide evidence for the formation of a specific carbocation or outline the role of a double bond.[154] Many examples of fluorinated FDP analogues used as mechanistic probes can be found in the literature.[155] For instance, strong evidence supporting the intermediacy of germacrene A (**113a**) in catalysis by 5-*epi*-aristoloche synthase (TEAS) was gain by characterisation of 6F-germacrene A (**114**) as the product from incubation with 6F-FDP (**111**). The mechanism of this enzyme starts with the displacement of the diphosphate by attack of distal double bond to afford carbocation **112a**, which is quenched by deprotonation to give germacrene A intermediate **113a**. Protonation of double bond on C6 triggers a cyclisation and a reaction cascade that affords 5-*epi*-aristolochene (**53**). However, with 6F-FDP (**111**), the double bond on C6 is deactivated towards protonation by the

presence of the fluorine atom, preventing the subsequent cyclisation thus obtaining exclusively 6F-germacrene A (**114**) as product of the incubation (Scheme 23).[154] 6F-germacrene A (**114**) was also obtained when **111** was incubated with AS.[156]
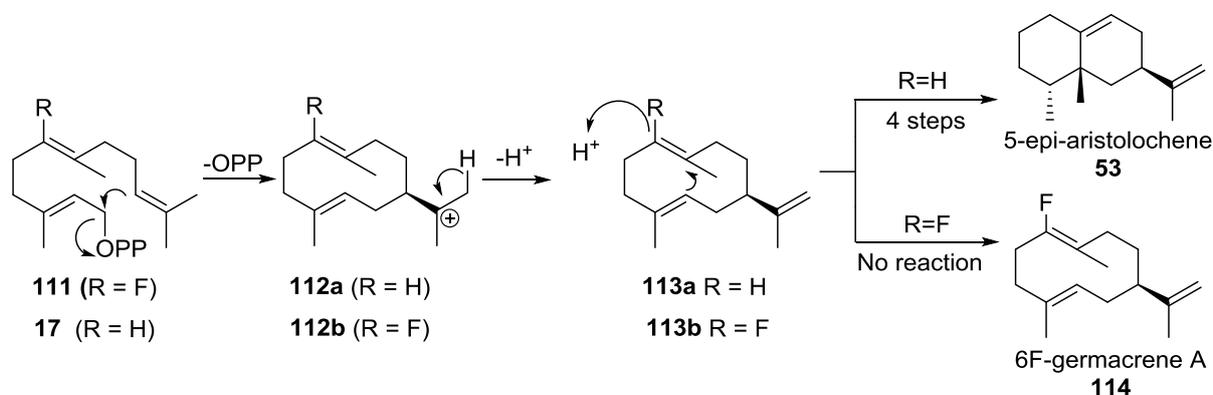
Scheme 23. Formation of 6F-germcrene A (**114**) from 6F-FDP (**111**) by TEAS.

Dihydro FDP analogues have also been used to uncover the enzymatic mechanism of sesquiterpene synthases. For example, the 6,7-dihydro FDP (**120**, Scheme 25) was useful to provide evidence for the intermediacy of nerolidyl diphosphate (NDP, **95**, Scheme 20) in the catalysis of trichodiene synthase. The reaction catalysed by this enzyme is proposed to start with an isomerisation of FDP (**17**) to produce nerolidyl diphosphate (NDP, **95**). NDP undergoes then a 1, 6-cyclisation to afford the bisabolyl cation (**115**). Second cyclisation by attack of the distal double bond to carbocation on C7 to afford **116** is followed by cascade of methyl, hydrogen shifts and a deprotonation (**116** to **119**) to afford the final product trichodiene (**119**, Scheme 24).[157]
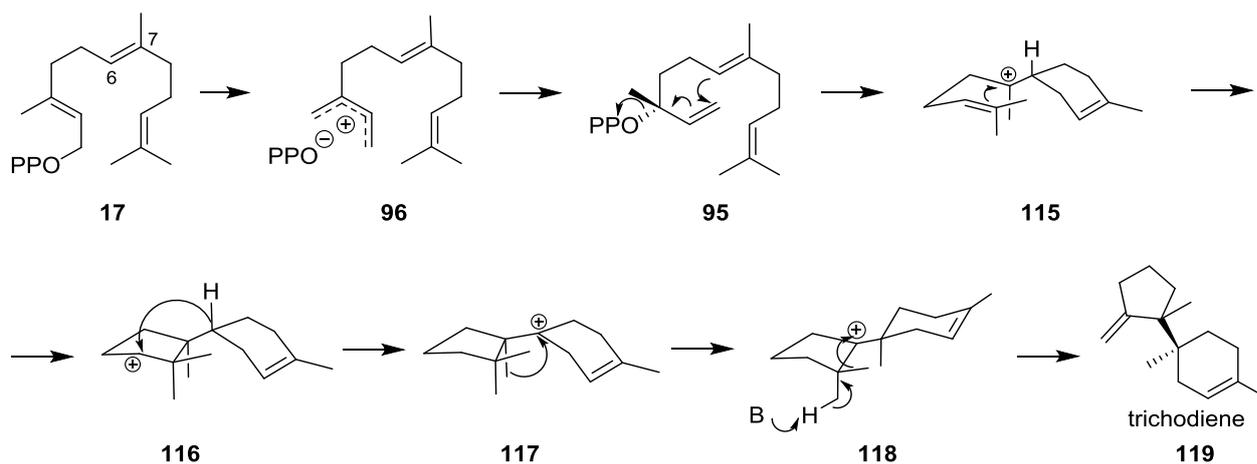
Scheme 24. Catalytic mechanism of trichodiene synthase.

When 6,7-dihydro FDP (**120**) was incubated with trichodiene synthase, the compound is isomerised into 6,7-dihydro NDP (**121**), but the subsequent intramolecular 1,6-cyclisation is in this case impossible due to the absence of the double bond on C6. 6,7-Dihydronerolidyl diphosphate **121** is attacked by a molecule of water and 6,7-dihydronerolidol **122** is recovered from the incubation. Recovery of **122** supports isomerisation of FDP (**17**) into NDP (**95**) as first step in the mechanism of trichodiene (Scheme 25).[158]
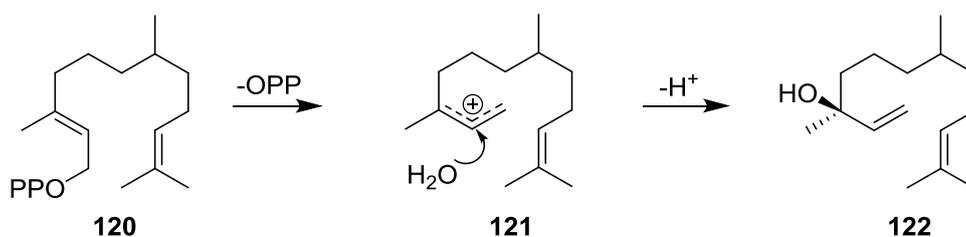
Scheme 25. Outcome of the incubation of trichodiene synthase and 1,6-dihydro FDP (**120**).

1.6   Synthesis of FDP and analogues

Due to the relevance of FDP analogues to the study of sesquiterpene synthases, several different methodologies for isoprenoid chain preparation of FDP have been developed. These different methodologies rely on key reactions for the formation of the hydrocarbon chain, such as Horner-Wadsworth-Emmons olefinations; a sequence of sulfonylation, alkylation,

and desulfonylation; or addition of dienolate to an electrophile. In addition, two different methods have been developed to convert primary alcohols into diphosphates.

1.6.1 Wittig and Horner-Wadsworth-Emmons olefination

Wittig and Horner-Wadsworth-Emmons olefinations have been used to convert a ketone to an isoprene unit. This method has the disadvantage of creating two isomers of the product. However, the two isomers can be separated by chromatography, the overall yield of this process is lower than other methodologies.[159] Despite this issue, this is the preffered method for the synthesis of analogues containing vinylic fluorine atoms. For instance, this approach was used for the synthesis of 2F-FDP (**126**) from geranyl acetone (**123**) and fluorophosphonate **124** (Scheme 26).
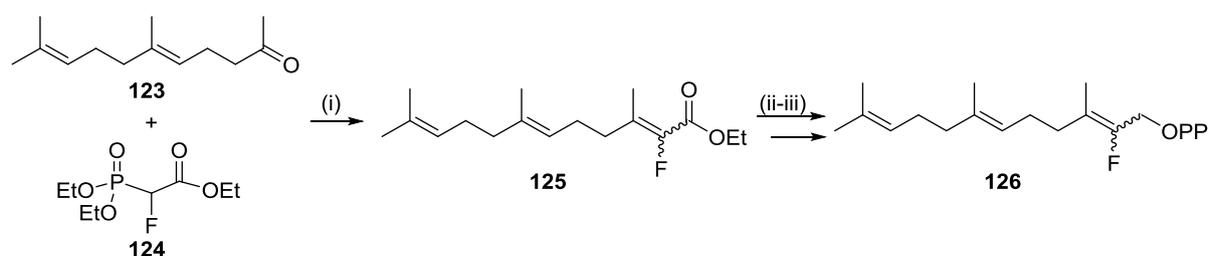
Reagents and conditions: (i) NaH, benzene, RT; (ii) DIBAL-H, CH_2Cl_2, -78 °C to -50 °C; (iii) CBr_4, PPh_3, CH_2Cl_2 and then $(Bu_4N)HP_2O_7$, CH_3CN, RT.

Scheme 26. Synthesis of 2F-FDP (**126**) by means of Horner-Wadsworth-Emmons olefination.

1.6.2 Sulfonylation, alkylation and desulfonylation methodology

Biellmann and Ducep developed and used a sequence of sulfonylation, alkylation, and reductive desulfonylation for the first time in 1969.[160] This method begins with the synthesis of a bromide and a sulfonate. Coupling of these two compounds and subsequent desulfonylation achieves the synthesis of the FDP hydrocarbon chain. For instance, the chain elongation of geraniol (**127**) has been achieved through a synthetic sequence involving sulfonylation of farnesol (**127**), alkylation to **129** and reductive desulfonylation to give **130** (Scheme 27).[161]
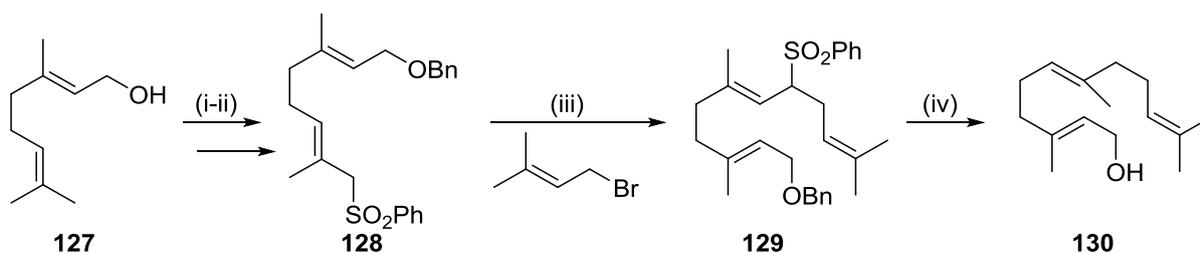
Reagents and conditions: (i) NaH, benzyl bromide, THF, 0 ℃ and then SeO$_2$, EtOH, RT; (ii) PBr$_3$, DCM, 0 ℃ and then PhSO$_2$Na, DMF, RT (iii) n-BuLi, HMPA, THF, -78 ℃ (iv) Li, EtNH$_2$, RT.

Scheme 27. Elongation of geraniol with the sulfonylation, alkylation and reductive desulfonylation methodology.

1.6.3 Weiler-Sum chain elongation

The chain elongation sequence developed by Weiler and Sum[162] in 1981, involves the addition of dienolate **132**, which is generated *in situ*, to an electrophile, followed by the conversion of the resulting β-keto ester into an alkene.[163] This synthetic sequence results in the addition of an isoprenyl moiety to the electrophile. For example, Gibbs applied this methodology to the synthesis of **134**. The sequence starts with the addition of dienolate **132** to electrophile **131** to obtain **133** that is then transformed into **134** (Scheme 28).
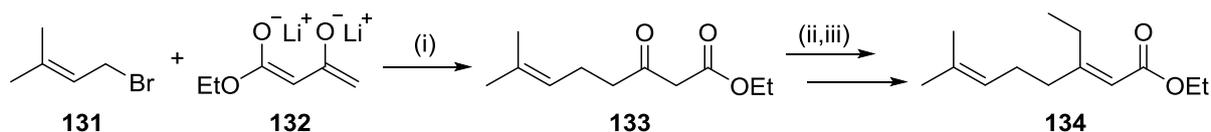
Reagents and conditions: (i) THF, 0 °C (ii) (Me$_3$Si)$_2$NK, THF, -78 °C; 2-(5-chloropyridyl)N(SO$_2$CF$_3$)$_2$, from -78 °C to RT; (iii) EtMgBr, CuCN, Et$_2$O, -78 °C.

Scheme 28. Weiler-Sum chain elongation of **131** to **134**.

1.6.4 Preparation of diphosphates

The first procedure developed for the formation diphosphates by Böhm[164] involves the reaction of the corresponding alcohol with monophosphate salts and trichloroacetonitrile.

This procedure affords a complex mixture of mono-, di- and triphosphates. The crude reaction is then partially purified by cation ion exchange chromatography. Keller developed an improved purification protocol[165] by finding appropriate conditions to purify this complex mixture of compounds by flash chromatography or preparative TLC on silica. However, in both cases the yields obtained were modest (24-31%).

Significantly improved methodology was reported by Davisson *et al.*,[166] who performed the diphosphorylation reaction with different electrophiles, such as isoprenyl chloride, bromide or tosylate, instead of the alcohol by reaction with tris(tetra-n-butylammonium) hydrogendiphosphate. The reaction exclusively afforded the desired diphosphate. The yields observed varied from 35 to 80% depending on the hydrocarbon chain and the leaving group used.

1.7   Optimisation of enzymatic reactions yields

Many natural products that are useful for artificial purposes are produced in very small amount in nature. Consequently, the extraction of these valuable compounds from natural sources is not a viable process to satisfy the market. Total synthesis of natural compounds is mostly an inefficient procedure that is not suitable for industrial production due to its high cost. A significant effort is currently focused on engineered yeast, such as *Sacharomyces cerevisiae,* or bacteria, such as *Escherichia coli,* that can be harnessed to produce high quantities of valuable natural compounds using metabolic pathways.[167]

In spite of the extensive literature about the optimisation of the metabolic pathways of these microorganism for *in vivo* production of natural compounds,[168] few reports has been published exploring the potential of isolated natural enzymes for *in vitro* production of highly valuable chemicals. Strategies to improve reaction yield rely on the modification of the catalyst itself. For example, directed evolution produces a large number of mutant enzymes that contains random mutations and then, all mutant enzymes are screened for improved activity.[169] Alternatively, the *de novo* design of enzymes with computation can produce enzymes for specific tasks. However, these enzymes can suffer from lower reaction yields than natural occurring enzymes.[170] Recently, Baker *et al.*[171] optimised a computational designed Diels-Alderase[172] obtaining an enzyme with enhanced activity (18 times higher than

native enzyme) by remodelling the backbone structure to enable more interactions with substrates using the online game foldit developed by Cooper *et al.*[173]

There are some other reports about optimisation of enzymatic reactions that focus on strategies, such as immobilised enzymes or use of additives. For instance, Klibanov *et al.* reported an overall yield of 0.01% in the production of N-acetyl-L-tryptophan ethyl ester by reaction of N-acetyl-L-tryptophan and ethanol in aqueous media with chymotrypsin as catalyst whereas a 100% yield was obtained with immobilised chymotrypsin.[174] In addition, Khmelnitsky *et al.* reported that some additives such as KCl could dramatically increase the activity of chymotrypsin and subtilisin in organic solvents achieving 100 times the initial activity.[175]

1.7.1 Incubations of sesquiterpene synthases with their substrates

In spite of all these strategies to improve enzyme efficiency, there are only few examples where optimisation of the enzymatic reactions yields has been performed. Especially, there has been very little work published related to the optimisation of terpene synthase efficiency but this little work obtained promising results. For instance, Hohn *et al.* reported an optimisation of the concentration of magnesium for the incubation of aristolochene synthase that enhanced the enzymatic activity 30-fold.[176]

Extensive literature researches reveal that little work has been done to optimise the reaction conditions and there is no a standardised procedure for the incubation of terpene synthases with their substrates. Most common set ups for incubations of terpene synthases with their substrates includes a biphasic mixture of aqueous incubation buffer and organic solvent (usually hexane or pentane). Aqueous incubation buffer often contains glycerol, $MgCl_2$, buffer, β-mercaptoethanol, enzyme and substrate. However, the concentrations used for each component in the aqueous phase changes across the literature even for the same enzyme. Moreover, incubation procedures for extraction of volatile terpene products from the aqueous phase change across the literature and scale up of small incubation set up often result in low reaction efficiencies. All these variations led to inconsistent and often low yields in the enzymatic reactions of terpene synthases.

Part of this work was aimed at the development of a standard procedure for the incubation of terpene synthases with their substrates to maximise conversion and yield of the product(s) (Chapter 3).

1.7.2 Flow chemistry

Traditionally organic reactions have been performed in batch, which mean in a round bottom flask where reactants are mixed together with a magnetic stirrer. In contrast, in flow chemistry reactants are pumped into tubes that are joined one to another. The bulk chemical industry has used this approach extensively for a long time. Only in the last few years, continuous flow systems become popular for the preparation of chemicals on the small laboratory scale. Miniaturised flow devices can perform multi step synthesis continuously. Flow systems allow the integration of several chemical reactions in only one system, as well as other operations such as quench, work up, isolation and purification.[177]

Chemistry in flow systems has several advantages that make it attractive over traditional batch reactions. For instance, flow systems are increasing its popularity due to the safety profile over traditional batch process. Hazards derived from stockpiling explosive, toxic, or sensitive intermediates of synthesis can be overcome by the continuous processing achieved in flow systems. Besides, relatively small volumes and its high surface-area-to-volume ratio that enable fast heat dissipation can prevent a runaway reaction.[178] The precise control over reaction conditions performed in automated flow systems, where mixing and heat transfer is well characterised along with the small volume required for a reaction, allows rapid development of reactions by testing different conditions in a short period of time.[179] In contrast with batch reactions where the mechanism of mixing is complex due to the different variables involve (*e.g.* shape of the vessel);[180] in flow systems, the good characterisation of the mixing and the conditions makes reaction scaling a less complex process for bulk production. Indeed, scaling reactions is easier in flow systems than in batch; three general approaches to scale reactions have been employed: run the process for longer (scaling out), use a larger continuous reactor (scaling up), or use multiple reactors in parallel (numbering up).[181]

One of the most important advantages of flow systems over batch process is the efficient heat and mass transfer, which increase the productivity of the process by increasing the reaction

rate. The latter is especially useful in heterogeneous systems where reaction rate is controlled by mass transfer. In addition, due to its nature, flow systems can be easily combined with other technologies such as microwave irradiation, photochemistry, inductive heating or sonication, which can potentially increase efficiency of the process.[182]

All these properties make flow systems an attractive novel methodology that can help to increase reaction rates and simplify scalability of terpene synthases reactions. Reactions of terpene synthases in a flow reactor were compared with their analogous reactions in batch in the work described in Chapter 3.

1.8   Aims of the project

Some plants have developed a natural chemical defence mechanism against herbivorous insects; the plant releases a sesquiterpene that affects the behaviour of the aphids in order to repel them.[183] (+)-Germacrene A and (-)-germacrene D have been reported as semiochemicals acting as repellents/toxins for different species of aphids, this property makes these two chemical compounds potential candidates for pest control.[184]

Modification of (+)-germacrene A and (-)-germacrene D can result in modulation of their properties, for example, fluorinated germacrene analogues have been reported to have improved stability whereas alkyl substitution can reduce the volatility of these compounds helping them to stay on the leaf for longer period of time. However, the unstable nature of these two volatile compounds has set back a satisfactory synthesis,[86] therefore development of a synthetic approach for the preparation of (+)-germacrene A and (-)-germacrene D analogues has not been closely studied.

For this project, the chemoenzymatic preparation of (+)-germacrene A and (-)-germacrene D analogues by incubation of FDP analogues with the corresponding sesquiterpene syntheses was investigated. Towards this end, a series of FDP analogues were synthesised and tested with GAS and GDS. In addition, these FDP analogues were tested also with AS, due to the ability of this enzyme to sometimes produce the enantiomeric germacrene A from FDP analogues. To obtain the sesquiterpene derivatives in sufficient amount for biological testing and characterisation, an incubation methodology was developed and optimised for improved

conversion and yield. The products obtained with germacrene synthases were assessed for biological activity against aphids, in collaboration with the Rothamsted Research.

Another different aim of this project was the synthesis and design of new mechanistic probes for the study of the reaction catalysed by sesquiterpenene synthases. These novel mechanistic probes contain hydroxyl groups, which were expected to quench the carbocationic intermediates, helping to reveal the mechanisms of these enzymes. These analogues were applied in the study of DCS catalysis, to distinguish between the two possible proposed mechanisms (1, 10 or 1, 6 cyclisation, Section 1.4.5, Scheme 21).

CHAPTER 2

Chemical expansion of the terpenome: synthesis of farnesyl diphosphates analogues

2. Introduction

Alkyl substituted FDP analogues are alternative substrates or inhibitors of protein-farnesyl transferases (FTase). For instance, a group of proteins called Ras proteins are involved in the signal cascades that control cell growth, differentiation and survival, playing crucial role in eukaryotes.[185] Ras proteins increase their hydrophobicity by farnesylation, which is a crucial modification to exert their activity.[186] Since Ras proteins are involved in numerous human carcinomas,[187] the development of inhibitors or alternative substrates that helps to understand the mechanism of FTase has been an area of interest for the pharmaceutical industry.[188] Examples of FDP analogues that behave as an inhibitor with protein FTase are **135d** and **135e**. On the other hand, FDP analogues **135a**, **135b** and **135c** behave as substrates of protein farnesyl-transferase (Scheme 29). There is a relation between the presence of a double bond in the substituent and the ability to act as a substrate.[189] Spielmann and his co-workers have demonstrated that the factors that govern substrate versus inhibitory interactions are quite subtle and complex.[190] The ability of FTase to accept different FDP analogues has been investigated by other groups to incorporate photoaffinity labels,[191] affinity tags[192] and fluorophores.[193]
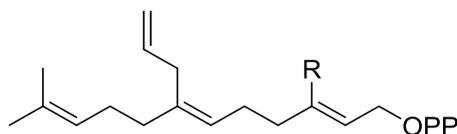
135
R = a) R = allyl, b) R = vinyl,
c) 3-methyl-but-2-enyl,
d) R = isopropyl, e) R = isobutyl

Scheme 29. Example of alkyl substituted FDP analogues.

Although alkyl substituted FDP analogues have been prepared before, prior to this work they had not been tested with germacrene synthases. In this project, alkyl modified FDP were used to chemoenzymatically prepare alkyl modified germacrene analogues. Alkyl groups increase the bulk of the hydrocarbon chain of FDP that must fit in the active site of terpene synthases. Alkyl-modified FDP analogues can fit more readily in the active site of germacrene synthases if the alkyl groups introduced are small. Methyl groups offer minimal increment in the size of hydrocarbon chain than any other alkyl group. Therefore, methyl substituted analogues were

preferred rather than larger alkyl groups. Chemical modifications of natural products by methylation is a rather common strategy to alter or modulate the intrinsic biological properties of secondary metabolites, including methylated sesquiterpenes such as TMTT, a potent aphid-repellent C_{16} homo terpene resembling (*E*)-β-farnesene (**6**, Figure 2).[194] Therefore, it seems reasonable to expect that the chemo enzymatically produced C16 homo germacrenes will most likely display enhanced or altered bioactivities. Evaluation of a set of methyl and dimethyl modified FDP analogues with germacrene synthases and AS to obtain modified germacrenes allowed testing of novel germacrenes for repellent activity against aphids. Two alkyl-FDP analogues previously described in the literature and named 14Me-FDP (**137**), 15Me-FDP (**138**) were prepared for testing with sesquiterpene synthases. In addition, 12Me-FDP (**136**) and 14,15diMe-FDP (**139**) were prepared as novel alkyl-FDP analogues.

Germacrenes are very unstable compounds given the disposition of the double bonds in the hydrocarbon cyclodecadiene core. This disposition can be changed by reducing the number of carbon atoms in the macrocycle, so the new germacrene analogue should have improved stability. Recently, Gibbs *et al.*[195] synthesised a set of FDP analogues that contain one less CH_2 spacer between isoprene units, finding that these FDP analogues can be substrates for protein farnesyl trasferases. This group of analogues were called frame shifted FDP analogues. 8nor-FDP (**140**) was synthesised in order to obtain a nine-membered ring germacrene derivative that would have improved stability.

FDP analogues containing a hydroxyl group were designed to interfere with the carbocationic catalytic mechanism of terpene synthases. The lone electronic pairs of the hydroxyl group can quench a carbocation on the alpha carbon to generate an epoxide, and, in this way, the carbocationic intermediate will be frozen in epoxide form. With this objective in mind, novel 8OH-FDP (**141**) and 14OH-FDP (**142**) FDP analogues were synthesised to explore the mechanism of DCS (Scheme 30).
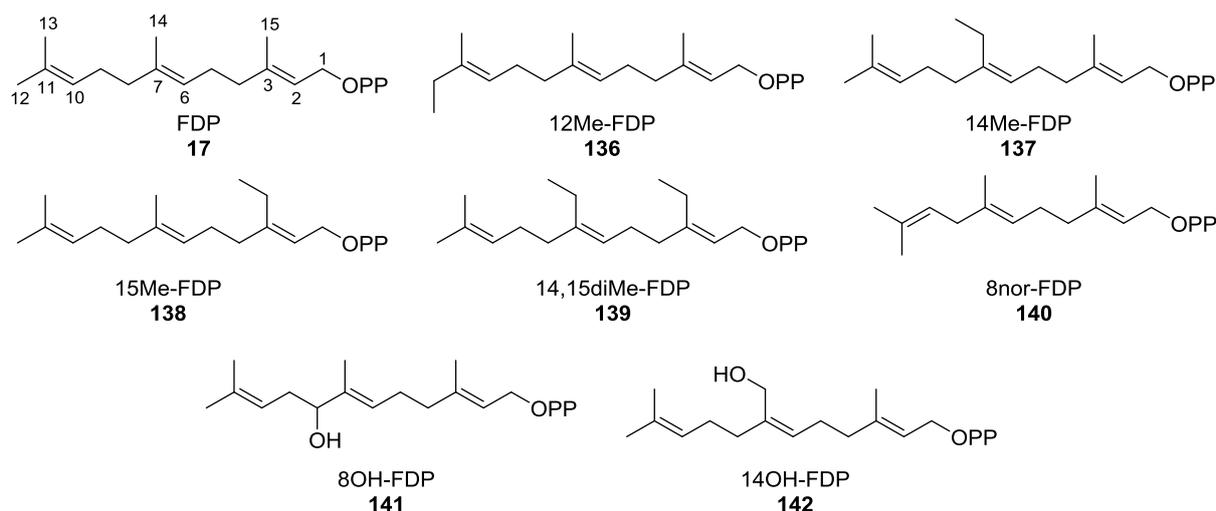
Scheme 30. FDP analogues targeted for synthesis in this study.

2.1 Synthesis of 14Me-FDP (137)

The synthesis of 14Me-farnesol (**165**, Scheme 33 *vide infra*) was first reported by Sen *et al.*, who used it as an authentic standard for aiding characterisation in the study of alternative substrates for prenyl transferases involved in the synthesis of juvenile hormone (JH).[196] Three different types of JH have been isolated from *Lepidoptera* (butterflies and moths), JHI (**143**), JHII (**144**) and JHIII (**145**) (Scheme 31). The most common JH [197] is the JHIII derived from FDP and it has been proved to be an important factor for growth and development in plants and invertebrates.[198]
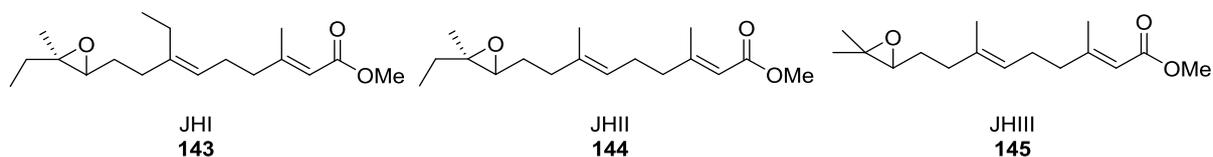
Scheme 31. Juvenile hormones characterised from *Lepidoptera*.

The synthetic methodology used by Sen *et al.* relied on the use of vinyl stannanes and involved eight synthetic steps to afford the geraniol derivative (**160**, Scheme 33).[196] Improved methodology was developed by Rawat *et al*,[199] for the synthesis of 14Me-FDP (**137**). This method adds successive isoprene units with a procedure that allows the introduction of different alkyl groups through displacement of a vinylic triflate intermediate with dialkyl

cuprate. This methodology was also applied in this work for the preparation of 15Me-FDP (**138**) and 14,15Me-FDP (**127**).

Retrosynthetic analysis: A generic farnesyl diphosphate **146** is prepared from the corresponding alcohol **147** that is prepared from ester **148**. The disconnection of the alkyl group (R_2) of **148** led to synthons **149** and **150** that correspond to dialkylcuprate **151** and triflate **152** respectively. Compound **152** may be prepared from β-keto ester **153** and the β-keto ester is disconnected to give synthones **154** and **155** that correspond to dienolate **156** and bromide **157**. Bromide **157** was prepared from alcohol **158** (Scheme 32). The second isoprene unit is disconnected in an analogous way.
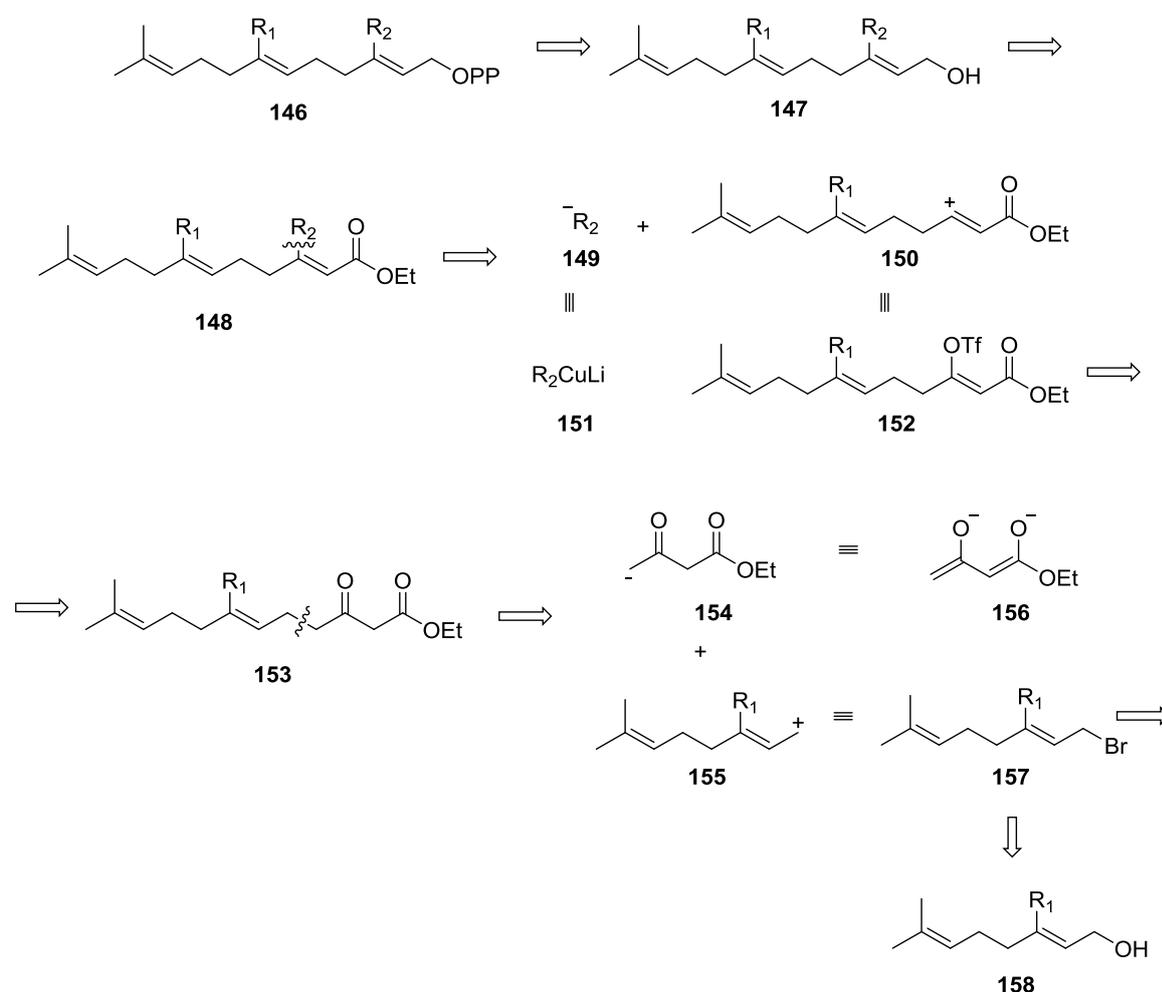
Scheme 32. Retrosynthetic analysis of a generic alkyl substituted FDP analogue, where R_1 and R_2 is any alkyl group.

This synthetic plan was used for the preparation of 14Me-FDP (**137**) with some modifications (Scheme 33). β-keto ester **133** was prepared from dimethylallyl bromide (**131**) following a procedure described by Rawat.[199] However, the vinyl triflate (**159**) was best prepared by a procedure described by Specklin *et al.*[200] Whereas an ethyl group was introduced by Cu (I) assisted displacement of the triflate with ethyl magnesium bromide (Et)$_2$CuLi in 73% yield from β-keto ester **133**. Methyl group in the second isoprene unit was introduced by reaction with the corresponding phosphonate and (Me)$_2$CuLi in 65% yield, as described by Jin *et al.*[201] The reduction of esters **134** and **164** was carried out by the reaction with DIBAL-H at low temperature. Then, the transformation of corresponding alcohols **160** and **165** into suitable leaving group was performed by mesylation and subsequent displacement of mesyl group by bromine or chlorine (Scheme 33).
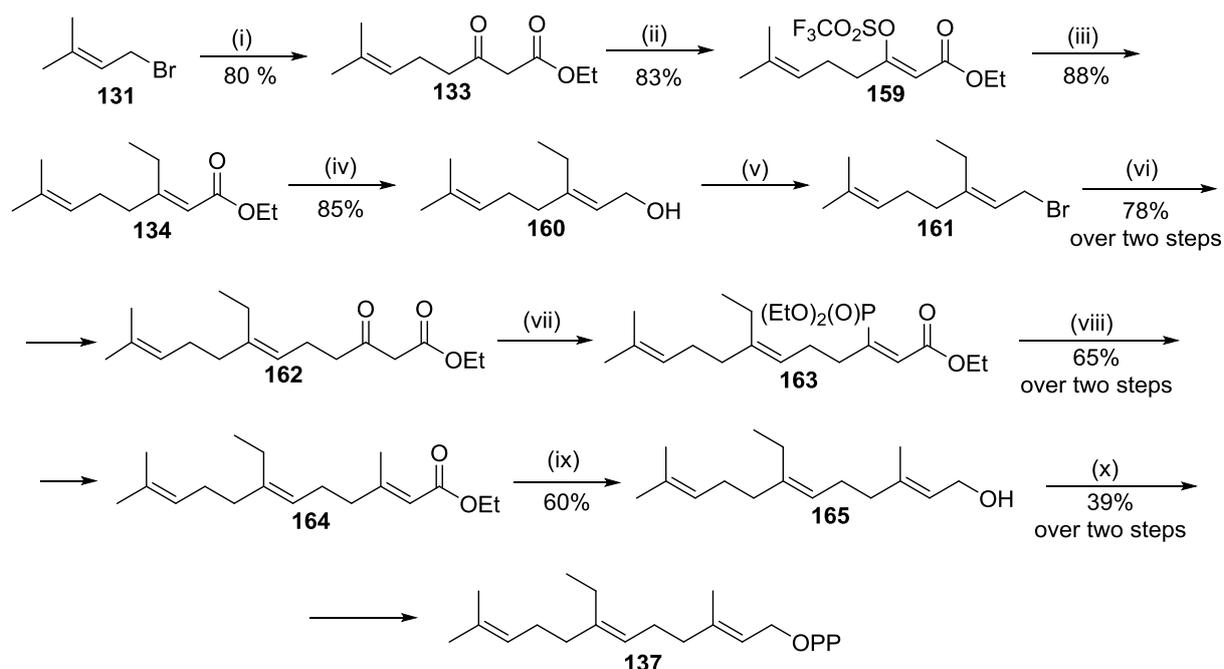
Reagents and conditions: (i) Ethyl acetoacetate, NaH, BuLi, THF, 0 ℃; (ii) Et$_3$N, lithium trifluoromethane sulfonate, trifluoromethanesulfonic anhydride, DCM 0 ℃; (iii) CuI, EtMgBr, THF, -78 ℃ ; (iv) DIBAL-H, toluene, -78 ℃, (v) Et$_3$N, MsCl, LiBr, THF, -45 ℃ to 0 ℃; (vi) ethyl acetoacetate, NaH, BuLi, THF, 0 ℃; (vii) NaH, diethylchlorophosphate, Et$_2$O, 0 ℃; (viii) Me$_2$CuLi, Et$_2$O, -78 ℃; (ix) DIBAL-H, toluene, -78 ℃; x) S-collidine, MsCl, LiBr, DMF 0 ℃; (Bu$_4$N)$_3$HP$_2$O$_7$, CH$_3$CN, then DOWEX 40 W (NH$_4^+$ form) cation exchange.

Scheme 33. Synthesis of 14Me-FDP (**137**)

2.1.1 Preparation of β-keto ester 133
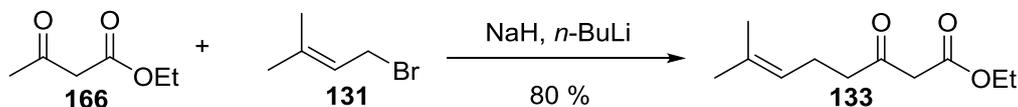
Scheme 34. Preparation of β-keto ester (**133**) from dimethyl allyl bromide (**131**).

The formation of the lithium dienolate **132** from ethyl acetoacetate (**166**) and the reaction of this intermediate with different alkylating agents has been previously reported by Weiler.[202] The dienolate **132** was formed by deprotonation with sodium hydride, followed by further deprotonation with a stronger base, such as butyllithium (Scheme 35). The alkylation was tested by Weiler with different alkylating agents and, in all cases, takes place exclusively at the γ carbon atom of **166**.[202]
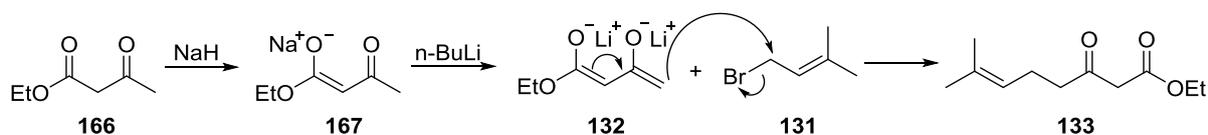
Scheme 35. Mechanism of formation of **133**.

An incomplete reaction was observed when the bromide **131** reacted with 1.2 equivalents of dienolate **132** with yields varying from 37 to 61% (Table 1, entry 1). Increasing the number of equivalents of **132** led to a moderate improvement in the yield of the reaction 60-68% (Table 1, entry 2). Reaction times over 1 hour led to an increase in the by-products observed by TLC; therefore, reductions of reaction time to 1 hour improve the yield to 72-80% (Table 1, entry 3). Jin *et al.*[203] made similar observations.

Entry	Temperature °C	Molar equivalents of **132**[*]	Molar equivalents of nBuLi and NaH*	Reaction time (h)	Yield (%)
1	0	1.2	2	4	37-61
2	0	3	3.3	4	60-68
3	0	3	3.3	1	72-80

*Molar equivalents in relation to **131**

Table 1. Different conditions used for the preparation of **133**.

2.1.2 Preparation of ester 134
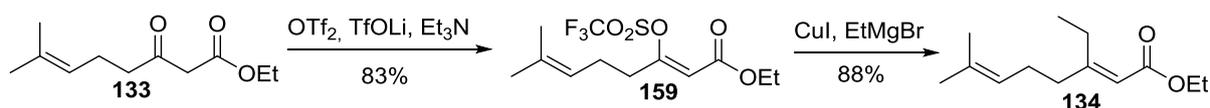
Scheme 36. Preparation of ester **134** *via* triflate **159**.

The ester **134** was prepared from vinylic triflate **159** (Scheme 36). The synthesis of this vinylic triflate has been performed efficiently using a pyridine amine bistriflate (2-(5-chloropyridyl) N(SO$_2$CF$_3$)$_2$) by Rawat.[199] However, this work concluded that the amine salts were difficult to remove, making necessary the purification of the rather unstable vinylic triflate **159** by column chromatography. However, an alternative methodology for selective synthesis of vinylic triflates from β-keto esters has been described by Specklin *et al.*[204] This method uses a mild base, such as EtN(Pr^i)$_2$ in the presence of LiOTf salt, which fixes the

geometry of the enolate double bond *via* metalation, thus achieving exclusively the Z isomer of the product. Premixing the lithium salt and the base with the β-keto ester **133** for at least 10 minutes before adding the triflic anhydride, was especially important for fixing the stereochemistry of the enolate **168** (Scheme 37). Near simultaneous addition of all reagents led to isolation of a mixture of geometrical isomers. The solvent used in the formation of the enolate influences the geometry of the double bond, giving the Z isomer exclusively when dichloromethane was used, while in contrast, mixtures of geometrical isomers are obtained when other solvents, such as diethyl ether, are used.[204]
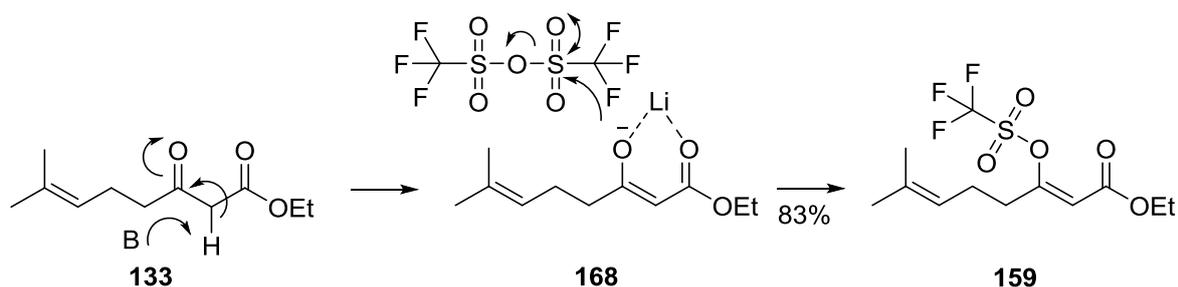
Scheme 37. Stereoselective formation of enol triflate **159**.

Vinyl triflate **159** was synthesised in 83% yield. Although it was possible to purify **159** by flash chromatography column, affording yellow oil, the colour slowly turned to black upon storage for few hours in the fridge, thus highlighting the unstable nature of this compound. The unpurified triflate **159** was considered by ^{1}H NMR spectroscopy to be sufficiently pure for its use without purification in all subsequent reactions and that is the reason why it was used immediately before and after its decomposition.

Although initial attempts to perform the displacement of the vinyl triflate **159** with Et$_2$CuMgBr (prepared *in situ* by reaction of EtMgBr and CuI) resulted in low yield of ~50%, the reaction outcome observed by TLC indicated that the reaction was complete and clean. The procedure of quenching involves the addition of saturated aqueous NH$_4$Cl solution and the subsequent extraction with diethyl ether, which results in the precipitation of the copper salts. The presence of copper salts made the extraction procedure difficult. The procedure developed by Jin *et al.*[203] overcomes this problem by diluting the reaction mixture with diethyl ether and addition of concentrated NH$_4$OH to the quenched solution. The biphasic mixture was stirred for 2 hours. In this latter case, the procedure led to the complete

dissolution of the copper salts and the obtention of a clear biphasic mixture. The workup proceeded without further complication obtaining ester **134** in 88% yield.

2.1.3 Preparation of alcohol 160
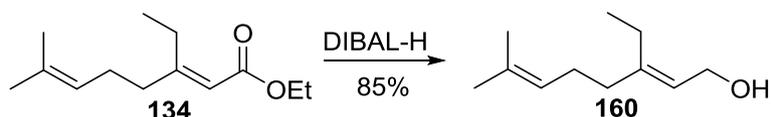
Scheme 38. Preparation of alcohol **160** from ester **134**.

The diisobutylaluminium hydride (DIBAL-H) was chosen to reduce allylic esters to their corresponding alcohols, thus avoiding isomerisation of the vicinal double bond. A preliminary test reaction afforded the allylic alcohol **160** in low yield of 59% (Table 2, entry 1). The original work-up using a saturated aqueous solution of sodium potassium tartrate to chelate aluminium was substituted by a basic quench of the reaction (NaOH$_{aq}$, 2M), but no improvement of the yield was observed (Table 2 entry 2). In subsequent experiments basic (NaOH$_{aq}$, 2M) or acid (HCl, 1M) for extended periods was investigated. The complete dissolution of the aluminium aggregates and consequent improvement in the work-up and yield was achieved by this method (Table 2, entries 3 and 4).

Entry	Equivalents of DIBAL-H	Quenching method	Yield (%)
1	2.5	K^+Na^+ tartrate for 10 min	59 (impure)
2	3	2 M NaOH for 10 min	61
3	3	1 M HCl + DCM stirring for 2 h	85
4	3	2 M NaOH + DCM, stirring for 16 h	93

Table 2. Reaction yields obtained from the various conditions used for quench and subsequent work up of the reaction used for formation of **160**.

2.1.4 Preparation of alcohol 165
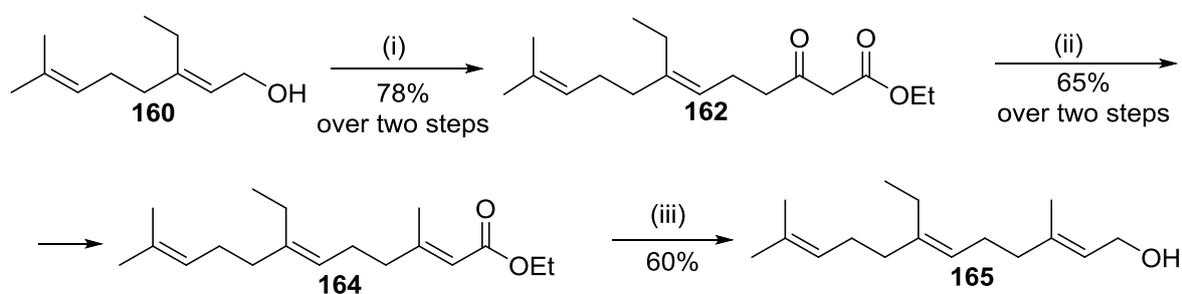
Scheme 39. Preparation of alcohol **165** from alcohol **160**.

The first step for the addition of an isoprene unit to alcohol **160** was the transformation of the alcohol into a suitable electrophile that can undergo alkylation with dienolate **132**. The transformation of alcohol **160** into bromide **161** with the method used by Jin *et al.,*[203] which involves the transformation of the alcohol *via* a mesylate intermediate and subsequent displacement of the mesylate with LiBr in a one-pot reaction, was tested and resulted in a clean reaction. Alkylation of dienolate **132** was performed immediately and without further

purification of bromide **161**. The required β-keto ester **162** was obtained in 78% yield (Scheme 33).

The transformation of β-keto ester **162** with diethylchlorophosphate and NaH afforded the vinylic phosphonate **163** in 86% yield. This procedure afforded exclusively the *Z* isomer through sodium chelation of the intermediate enolate and subsequent *O*-phosphorylation with diethyl chlorophosphate.[203] Alternatively, the *E* isomer can be obtained using tertiary amines, such as Et$_3$N in HMPA as bases.[205]

Phosphate **163** reacted with dimethyl lithium cuprate to afford ester **164**. Ester **164** was analysed by TLC after purification by chromatography and judged pure as only one spot was observed in the TLC plate. Subsequent reduction of ester **164** with DIBAL-H afforded the corresponding alcohol **165**. The purification of alcohol **165** by flash chromatography afforded one pure product as determined by TLC. However, ^{1}H NMR spectroscopic analysis displayed additional signals that were not expected giving the apparent purity of the product (Figure 9B). GC-MS analysis revealed the presence of a second product that display a molecular ion with *m/z* = 204 (Figure 9A). The by-product was identified as (2*E*, 6*E*)-7-ethyl-11-methyldodeca-2,6,10-trien-1-ol, (Figure 9B, **169**) originating from direct reduction of the phosphonate **163**. The by-product **169** accounts for the 29% of the total product judging from relative integrals on signals centred at 4.07 and 4.03 ppm that correspond to protons marked as H$_b$ in the ^{1}H NMR spectrum (Figure 9B, **169**). The formation of the by-product **169** was previously observed by Jin[201] in the dimethyl cuprate displacement reaction of **163**. Its formation was overcome by the addition of methyl iodide followed by its stirring for 2 hours at -45 ºC, before quenching the reaction.
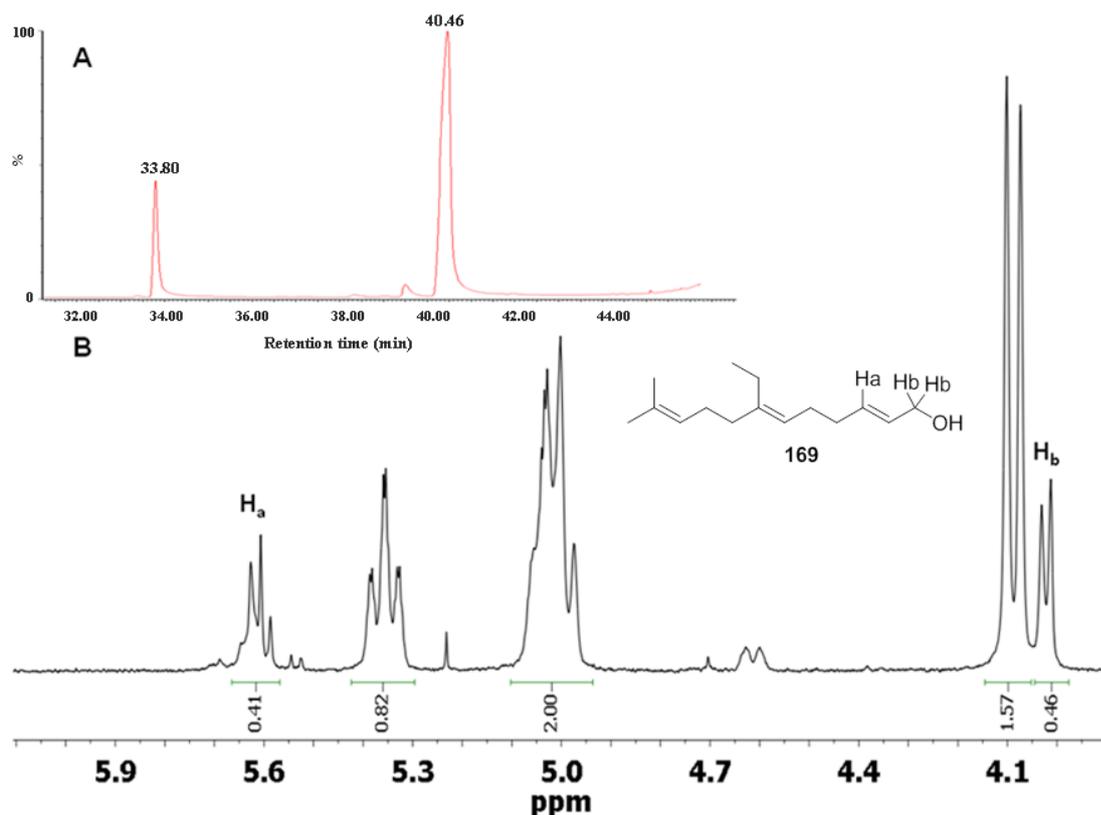
Figure 9. A) Gas chromatogram of the mixture of product **169** and **163** B) ^{1}H NMR spectrum of the mixture of products (in the range δ_H 4 - 6 ppm).

Following the procedure of Jin,[201] the displacement of phosphate **163** resulted in the complete disappearance of **163**. Ester **164** was obtained in 65% yield and the subsequent reduction afforded the alcohol **165** in 60% yield.

2.1.5   Preparation of diphosphate 137

The first step in the preparation of the diphosphate **137** is the preparation of tris(tetra-n-butylammonium) hydrogendiphosphate salt (**171**). Diphosphoric acid is first generated from disodium dihydrogen diphosphate (**170**) by exchange of the sodium ions for protons on a cation exchange column, followed by immediate titration to pH 7.3 with 40% (w/w) aqueous tetra-n-butylammonium hydroxide (Scheme 40).
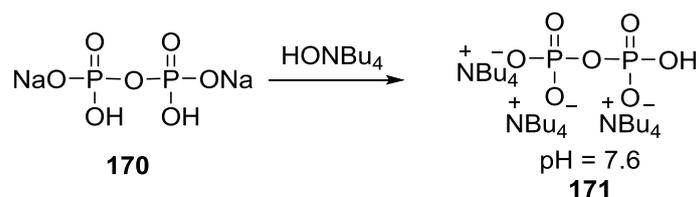
Scheme 40. Formation of tris(tetra-n-butylammonium) hydrogen diphosphate (**171**).

The alcohol **165** was converted into the chloride **172** using the procedure described by Collington and Meyers.[206] This procedure starts with the conversion of the alcohol into a mesylate group by reaction with methanesulphonyl chloride and Et_3N and subsequent displacement of mesylate by chlorine. The mentioned chloride **172** was immediately used in the next reaction. Direct displacement of the allylic chloride **172** by the diphosphate salt **171** afforded the diphosphate salt **173**. This salt was passed through a cation exchange column (Dowex50W-X8, ammonium form) to obtain the tris ammonium salt of diphosphate **173**. Ammonium salts are easier to purify by HPLC and are solid, which make them easy to handle (Scheme 41).
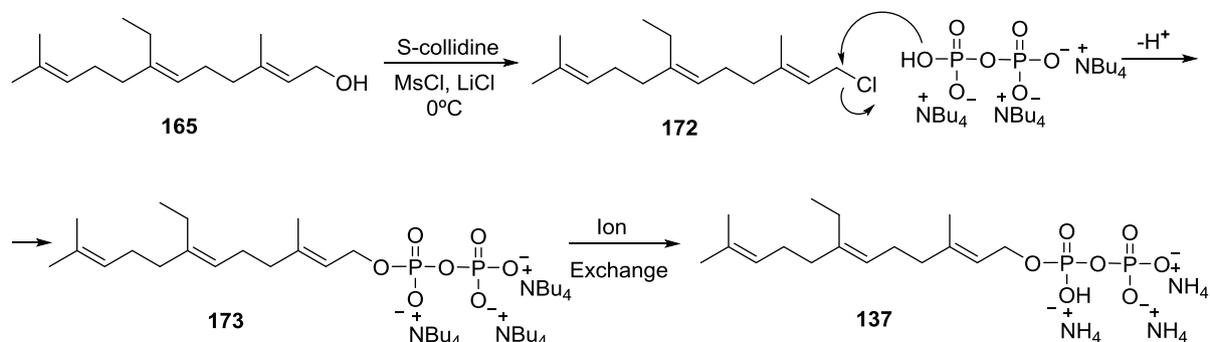
Scheme 41. Preparation of the FDP analogue **137**.

Crude salt was purified by two different methods. Methanol trituration of the solid obtained from the lyophilised fractions from the ion exchange column has been shown to be a good method to quickly obtain an impure FDP analogue (5-10% impurity of PPi by as judged ^{31}P-NMR).[207] Purification of the remaining residue after methanol trituration by reverse-phase HPLC afforded pure diphosphate salt **137** in 39% yield.

2.1.6 Conclusions

The methodology developed by Rawat *et al.*[199] was optimised and modified using the procedures reported by Specklin[204] regarding selective enolate formation and by Jin[203] regarding the displacement of vinyl triflates with lithium dialkyl cuprates. 14Me-FDP (**137**) was obtained in 6% yield over twelve steps by combining these two methods.

2.2 Synthesis of 15Me-FDP (138)

The first report related to the preparation of 15Me-FDP (**138**) was published by Ogura *et al.*,[208] who prepared the isopentenyl diphosphate derivative **174** as an inhibitor of farnesyl diphosphate synthase. However, incubation of analogue **174** and diphosphate **175** with farnesyl diphosphate synthase gave as a product the chemoenzymatic prepared 15Me-FDP (**138**, Scheme 42).
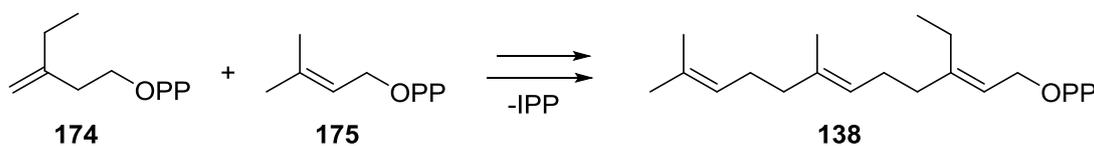
Scheme 42. Chemoenzymatic preparation of 15Me-FDP (**138**).

Later on, a synthetic method was published by Montellano *et al.* according to Horner-Wadsworth-Emmons' olefination of geranyl acetone with diethyl carboethoxyethyl phophonate, affording a mixture of isomers.[209] A synthetic methodology used to modify the hydrocarbon chain involving a coupling with vinylic triflate was first reported by Dawe.[210]

15Me-FDP (**138**) was prepared following the isoprene chain elongation developed by Weiler and Sum.[162] Thus, alkylation of geranyl bromide (**176**) with dienolate **132** yielded the β-keto ester **177**. A second reaction *via* triflate **178** according to Rawat[199] allowed coupling with EtMgBr, generating ester **179**. Subsequent reduction of the ester **179** and diphosphorylation of alcohol **180** afforded 15Me-FDP (**138**, Scheme 43).
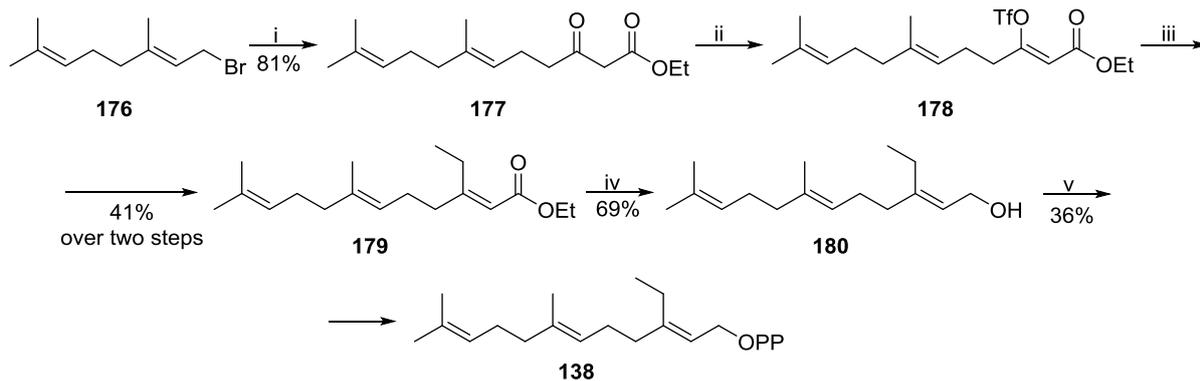
Regents and conditions: i) ethyl acetoacetonate, NaH, BuLi, 0 ºC; ii) Et$_3$N, lithium triflate, triflic anhydride, 0 ºC; iii) CuI, EtMgBr -78 ºC; iv) DIBAL-H -78 ºC v) S-collidine, MsCl, LiBr, DMF, 0 ºC and then (Bu$_4$N)$_3$HP$_2$O$_7$, CH$_3$CN, then DOWEX 40 W (NH$_4^+$ form) cation exchange.

Scheme 43. Synthesis of 15Me-FDP (**138**).

2.2.1 Preparation of diphosphate 138

The addition of bromide **176** to a solution of dienolate **132** gave the β-keto ester **177** in 81% yield.

Although the preparation of enol triflate **178** proceeded as usual, judging by TLC, the analysis of the crude mixture by ^{1}H NMR spectroscopy did not show the expected signal for the alkene proton of **178** at 5.75 ppm. The signals corresponding to the OCCH$_2$CO protons of the ester **177** observed at 5.1 ppm disappeared and only one absorption is observed in the olefinic region at 5.8 ppm integrating for one proton. The absorptions corresponding to the ethyl ester could still be observed. Hence, it is proposed a cyclisation triggered by acid, possibly coming from hydrolysis of triflic anhydride to triflic acid (pKa = -12).[211] The proposed mechanism for the formation of cyclic product **183** starts with the protonation of the expected product **178** to generate tertiary carbocation **181**. This intermediate undergoes a cyclisation by attacking the middle double bond to generate cyclic carbocation **182**. Deprotonation of this intermediate affords the proposed product **183** (scheme 44).
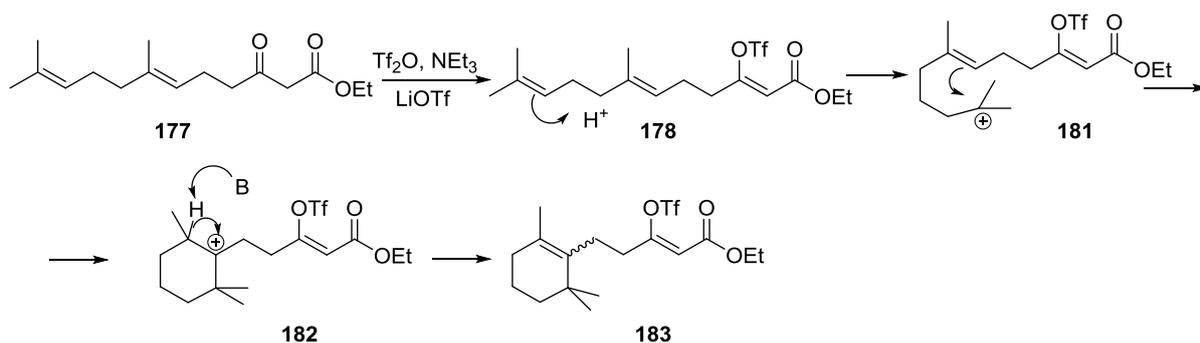
Scheme 44. Possible mechanism for the formation of the proposed cyclic product **183**.

Vinylic triflate **178** was finally obtained by adding an excess of Et₃N to the reaction. The base quenched the acid coming from the triflic anhydride and the reaction proceeded without further complication. Triflate **178** was used in the next step without further purification.

The ethyl group was introduced by reaction with Et₂CuLi of the vinylic triflate **178**. Further analysis of the reaction by GC-MS did not show any by-product from the direct reduction of the vinylic triflate, therefore, the addition of ethyl iodide was not necessary in this case. Ester **179** was synthesised in 41% yield over two steps. Alcohol **180** was obtained in 69% yield by the reduction of ester **179** with DIBAL-H. Methanol trituration of the crude diphosphorylation reaction afforded a first fraction of the 15Me-FDP (**138**) in 18% yield (5% impure). The purification of the remaining crude solid after trituration with methanol by HPLC afforded a second fraction of pure colourless solid in 36% yield.

2.2.2 Conclusions

Methodology developed for the synthesis of 14Me-FDP (**137**) was successfully applied to the synthesis of 15Me-FDP (**138**). Cyclisation of vinyl triflate **178** triggered by acid was overcome by adding an excess of base and the final diphosphate **138** was obtained in 8% yield over six steps.

2.3 Synthesis of 14, 15 diMe-FDP (139)

The synthesis of alcohol **187** *via* vinyl cuprate coupling has been previously reported.[212] An alternative synthesis was performed by allyl barium coupling of chloride **184** and bromide **185**. In both cases, the synthesis suffers from poor step economy with 17 steps to afford alcohol **187** and poor yield. Alcohol **187** was used in the study of the insect juvenile hormone.[196]
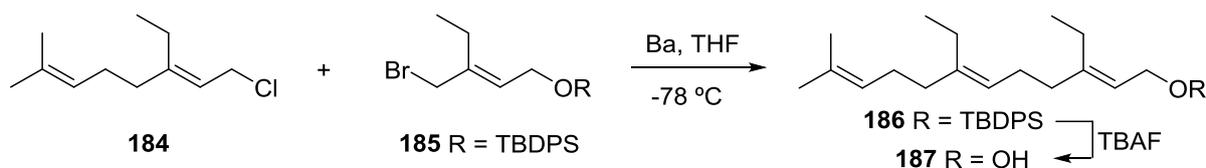
Scheme 45. Synthesis of alcohol **187**.

2.3.1 Preparation of diphosphate 139

The synthesis of 14,15-diMeFDP (**139**) was performed from β-keto ester **162** (Scheme 33). Ester **189** was obtained in 52% yield from β-keto ester **162**. The reduction of the ester gave alcohol **187** in 73% yield. The diphosphorylation of **187** afforded a colourless solid after lyophilisation of fractions obtained from ion exchange column. The methanol trituration of the solid afforded a first fraction of diphosphate **139** as colourless solid in 36% yield. The purification of the remaining solid by HPLC afforded a second fraction of **139** in 19% yield (Scheme 46).
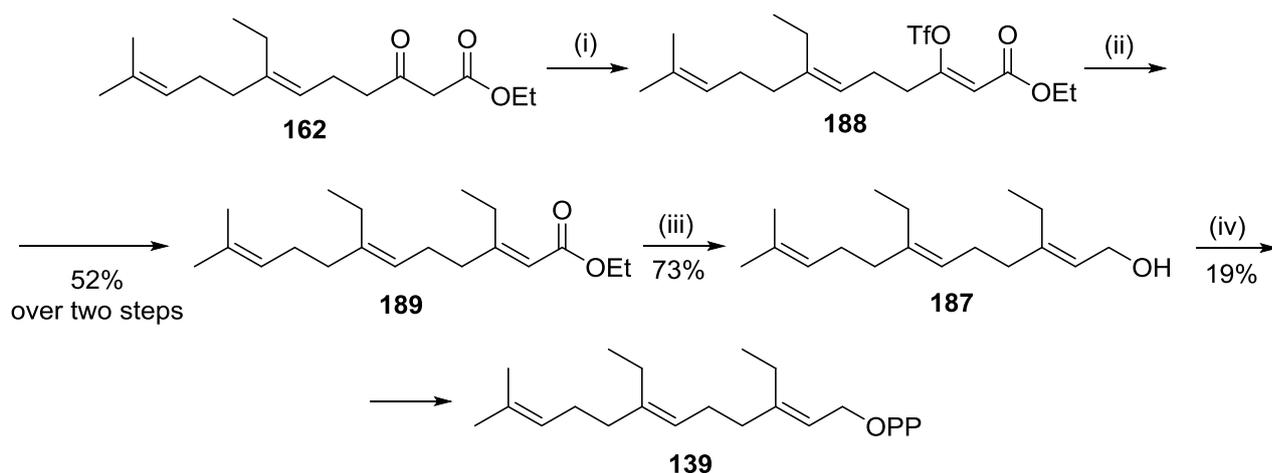
Reagents and conditions i) Et₃N, lithium triflate, triflic anhydride, 0 ºC; ii) CuI/EtMgBr (1:2) -78 ºC; iii) DIBAL-H -78 ºC iv) S-collidine, MsCl, LiBr, DMF 0 ºC and then (Bu₄N)₃HP₂O₇, CH₃CN, then DOWEX 40 W (NH₄⁺ form) cation exchange.

Scheme 46. Synthesis of 14,15diMe-FDP (**139**).

2.3.2 Conclusions

14,15Me-FDP (**139**) was prepared for the first time using a methodology developed for the synthesis of 14Me-FDP (**137**). Whereas this methodology achieved diphosphate **139** in 5% yield over twelve steps, a previous methodology reported by Ewing *et al.*[196] involved seventeen steps to afford alcohol **187** in 3% yield.

2.4 Synthesis 12Me-FDP (136)

Literature review revealed that 12Me-FDP (**136**) had never been synthesised prior to this study. However, in 1970, Corey performed a synthesis of ether **193** with analogous hydrocarbon chain as 12Me-FDP (**136**). This compound was prepared as an intermediate in the synthesis of an analogue of JHII (**132**, Scheme 31). The synthesis involved a modified Wittig olefination between triphenyl phosphonium salt **190** and aldehyde **191**, deprotonation of resulting betaine with *sec*-butyl lithium and addition of paraformaldehyde to afford alcohol **192** which is reduced to **193** (Scheme 47).[213]
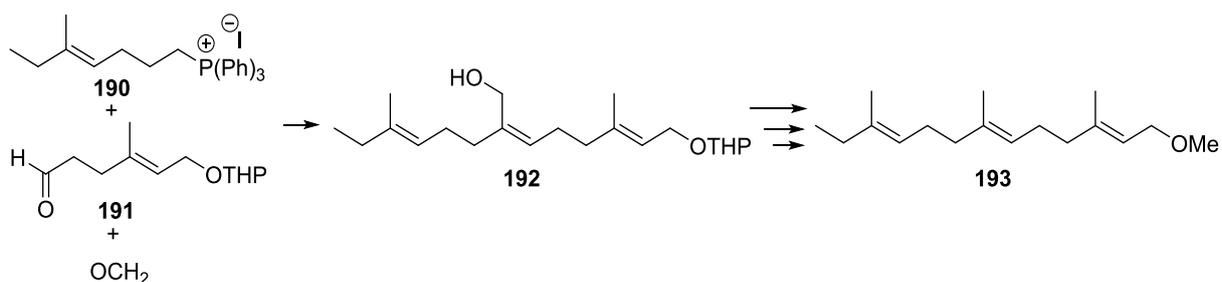
Scheme 47. Previous published synthesis of modified FDP hydrocarbon chain in C12.

Later on, Mori[214] described an improved synthesis of the hydrocarbon chain of 12Me-farnesol (**209**, Scheme 50) from farnesyl acetate (**194**). The synthesis starts with the oxidation of farnesyl acetate (**194**) with selenium dioxide to afford the allylic alcohol **195**. The alcohol **195** was further oxidised to α,β-unsaturated aldehyde **196** by reaction with MnO_2. This aldehyde is converted into diene **197** by Wittig olefination. Finally, a selective diimide reduction of the terminal double bond afforded the final product **198** (scheme 448).[214] However, this synthesis presented several problems, including the isomerisation of the double bond on C2 when the terminal double bond was reduced.[215]
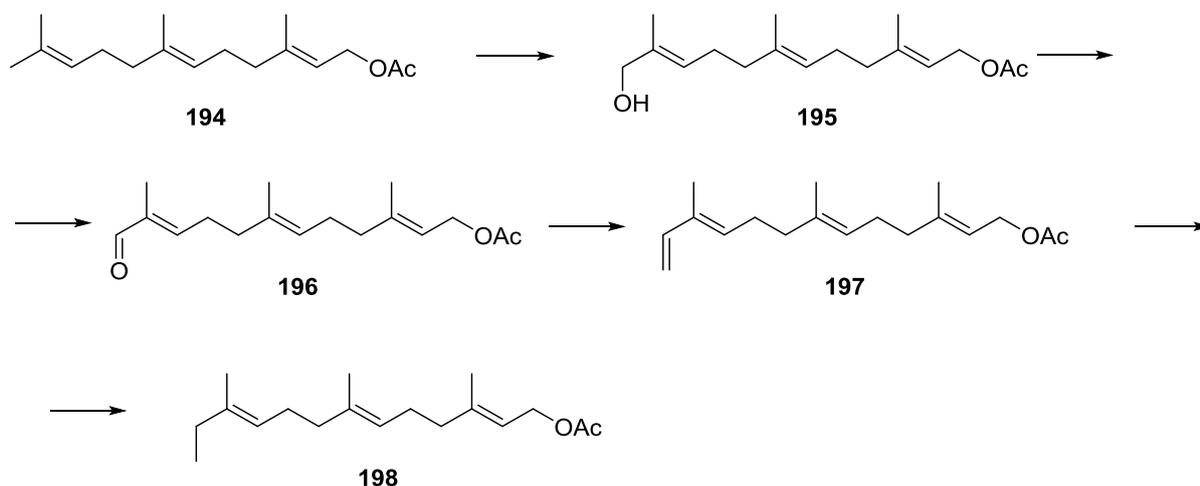
Scheme 48. Synthesis of the hydrocarbon chain of 12Me-FDP (**136**) by Mori.

Retrosynthetic analysis: Diphosphate **136** was prepared from the corresponding alcohol that was obtained from hydrolysis of THP ether **199**. The disconnection of methyl group on C12 of **199** gave synthons **200** and **201**, which correspond to dimethyl cuprate lithium **202** and

bromide **203** respectively. Bromide **203** is obtained from alcohol **204** that derivates from **205** (Scheme 49).
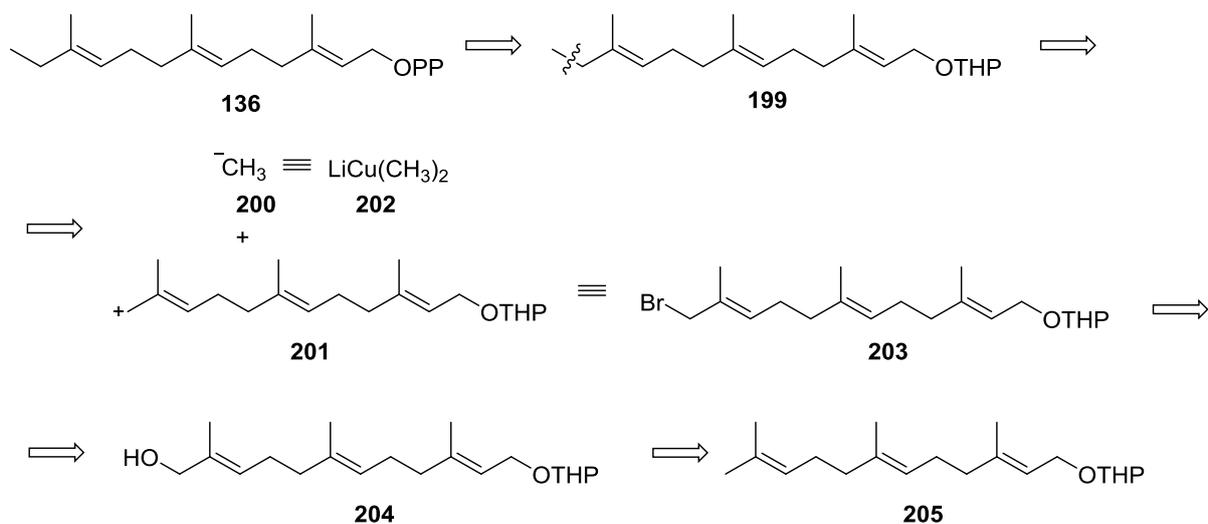
Scheme 49. Retrosynthetic analysis of 12Me-FDP (**136**).

The synthesis starts with the THP protection of *trans, trans*-farnesol (**206**). The THP protected farnesol **207** was then oxidised with selenium dioxide in CH_2Cl_2 and *t*-BuOOH as a co-oxidant to afford the allylic alcohol **204** in 21% yield.[216] The exclusive *E* stereochemistry of the allylic oxidation with SeO_2 is well established.[217] The transformation of the resulting hydroxyl group to a leaving group allowed displacement with lithium dimethyl cuprate to afford THP ether **205** in 81% yield. Subsequent deprotection of the THP ether **205** and diphosphorylation of alcohol **209** afforded 12Me-FDP in 29% yield over two steps (**136**) (Scheme 50).
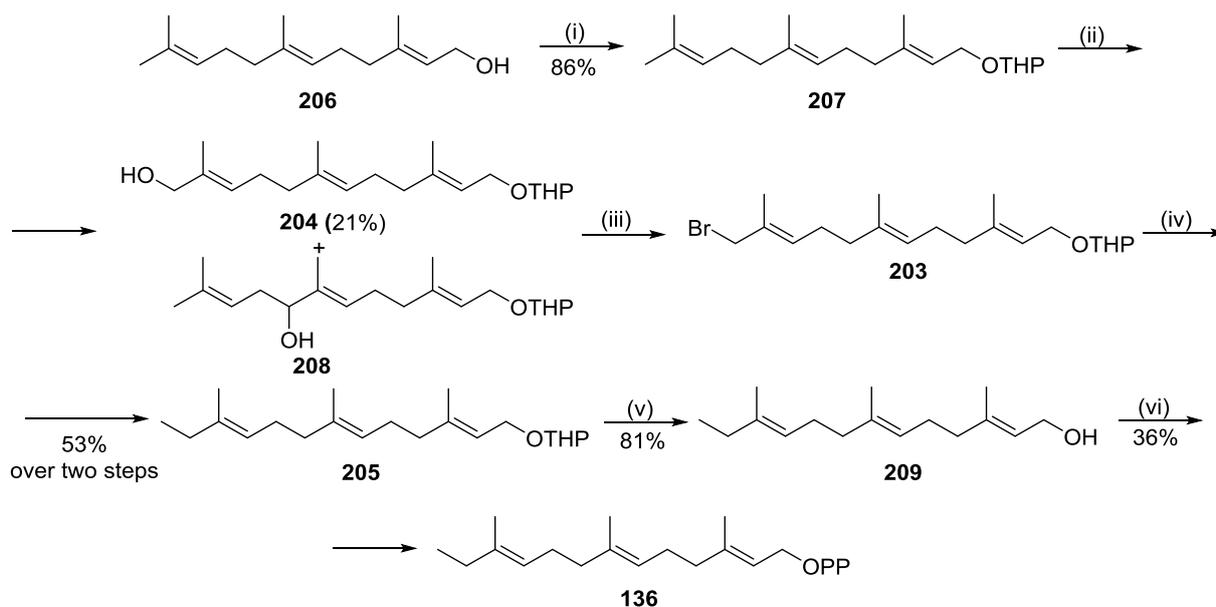
Reagents and conditions (i) PPTS, DHP, RT; (ii) SeO$_2$, *t*-BuOOH (70% in H$_2$O) (iii) Et$_3$N, MsCl, LBr THF, -45°C to RT ; (iv) Me$_2$CuLi, Et$_2$O, -78 °C; (v) PPTs in MeOH (vi) S-collidine, MsCl, LiBr, DMF 0 °C; (vii) (Bu$_4$N)$_3$HP$_2$O$_7$, CH$_3$CN, then DOWEX 40 W (NH$_4^+$ form) cation exchange.

Scheme 50. Synthesis of 12Me-FDP (**136**).

2.4.1   Preparation of alcohol 204

The THP protection of farnesol (**206**) was performed using pyridinium *para*-toluenesulfonate (PPTS) and dihydropiran (DHP). This clean reaction afforded **207**, after purification, as colourless oil in 86% yield. The selenium dioxide oxidation of the THP ether **207** gave the expected primary alcohol **204** along with some contamination of the secondary alcohol **208**, as previously reported by Labadie *et al.*[216] These two alcohols were separated by flash chromatography on silica using a mixture of hexane and ethyl acetate as eluent. The primary alcohol **204** was obtained in 21% yield.

2.4.2   Preparation of 12Me-FDP (136)

As it has been previously described for alcohol **160** (Scheme 33), the conversion of the allylic alcohol **204** into bromide **203** was performed. Alcohol **204** was then converted into a

mesylate by reaction with methanesulfonyl chloride and Et$_3$N, the mesylate group was then displaced with LiBr in a one-pot reaction. However, the displacement of the mesylate at 0 ºC led in this case to a slow reaction that was incomplete after 3 hours. The reaction was repeated, warming from -45 ºC to room temperature after LiBr addition. A colour changed from white milky to a yellow solution was observed. The reaction was completed in 1.5 hour as judged by TLC. The slow reaction was attributed to the hindered position of the allylic alcohol. The proximity to the methyl group to the alcohol can hinder the attack of the bromide, increasing the difficulty of the substitution. The allylic bromide was used in the next step without further purification.

The preliminary reaction of bromide **203** with MeLi at 0 ºC afforded a complex mixture of products as indicated by TLC, suggesting that this reactive bromide (**203**) was clearly not stable under the conditions used. The reaction was repeated at -78 ºC using dimethyl lithium cuprate as a nucleophile, and the purification of the crude obtained after 30 minutes of reaction afforded a yellow oil in 53% yield over two steps. Although ^{1}H NMR spectroscopy indicates the presence of some impurities, the sample was judged sufficiently pure for carrying on to the next step.

Stirring the THP ether **205** in MeOH with PPTS overnight afforded the alcohol **209** in 81% yield. However, a close examination of the ^{1}H NMR spectrum of alcohol **209** revealed that the impurity persisted. However, repeated attempts to purify the sample were unsuccessful (Figure 10, impurities were marked as *, +).
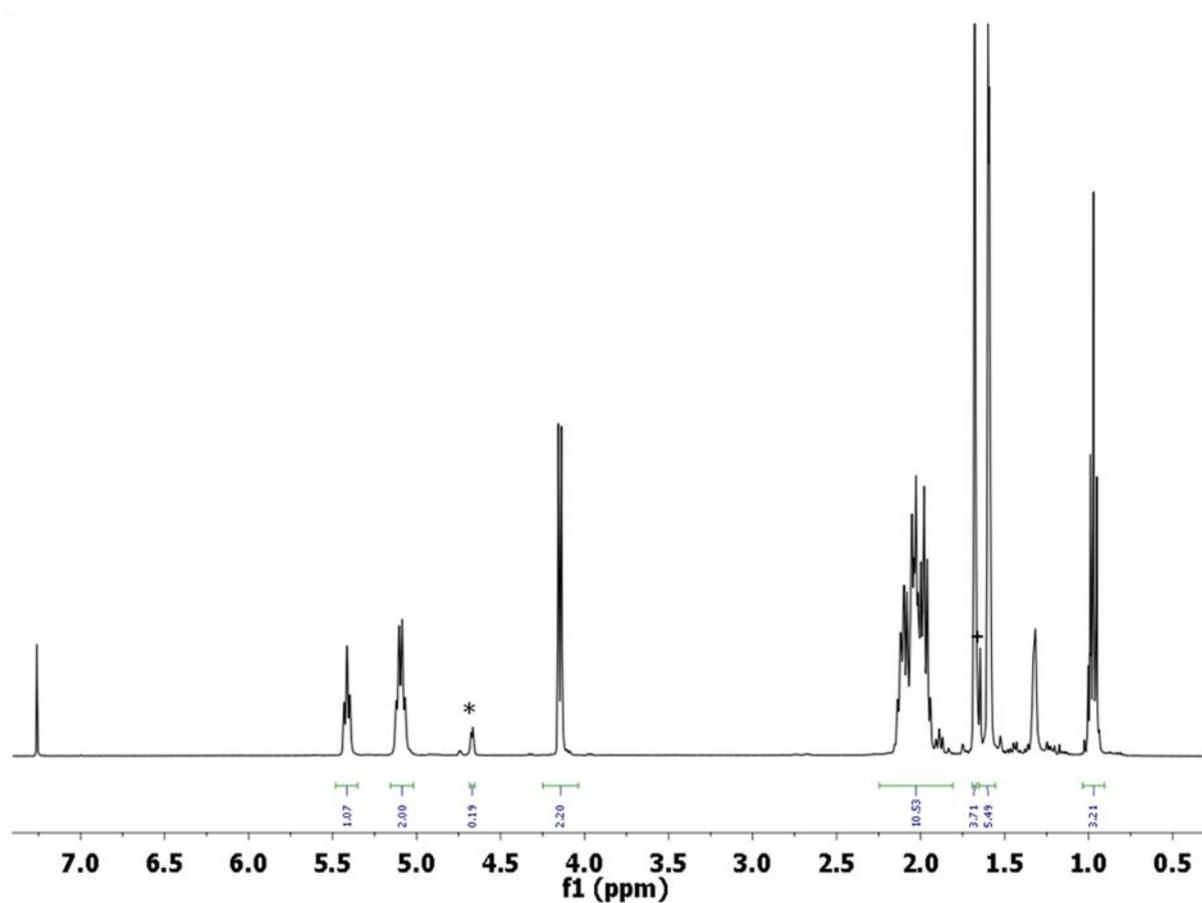
Figure 10. ^{1}H NMR spectrum (250 MHz, CDCl$_3$) of alcohol **209** with the signals corresponding to the impurity marked with *, +.

The proposed mechanism for the formation of the inseparable impurity starts with the displacement of bromine **203** by Me$_2$CuLi. The organocuprate intermediate **210** undergoes addition of methyl group and elimination of CuMe to afford THP ether **211**. The similarities between the structures of the two products obtained explain the inability to purify the compound. In addition, the broad doublet observed at 4.67 ppm matches with the signals expected for the two protons of the terminal double bond of **211** and a small signal overlapping with the singlets at 1.6 ppm could be a doublet corresponding to the allylic methyl group of **211**. The integration of the signals allowed calculation of the grade of the impurity was less than 10% (Scheme 51).
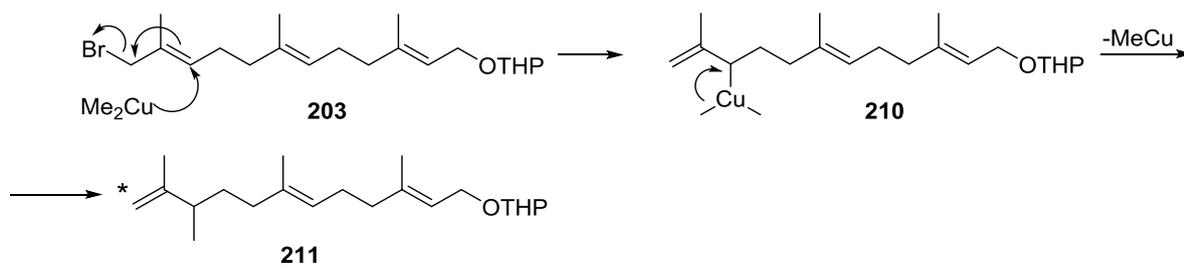
Scheme 51. Proposed mechanism for the formation of the inseparable by-product **211**.

Despite the presence of the impurity, the product was judged sufficiently pure to continue the synthesis. Therefore, alcohol **209** was converted in the corresponding diphosphate salt. Methanol trituration of the crude fraction collected from the ion exchange column gave 12Me-FDP (**136**) as a colourless solid in 36% yield.

2.4.3   Conclusions

New methodology for synthesis was developed for preparation of the novel FDP analogue 12Me-FDP (**136**). This new approach avoided the isomerisation on C2 reported in procedure developed by Mori. Consequently, Mori achieved synthesis of alcohol 209 in 3% yield,[214] whereas methodology reported here achieved alcohol **209** in 8% yield. The preparation of the novel FDP analogue named 12Me-FDP (**136**) was achieved in 3% yield over five steps.

2.5   Synthesis of 8nor-FDP (140)

FDP analogues with increasing or decreasing number of carbon atoms between double bonds were denominated frame shifted by Gibbs.[218] Frame shifted FDP analogues were synthesised for the first time by Gibbs during inhibition studies of mammalian FTase. Gibbs successfully applied Wenkert's methodology[219] for the transformation of carbon-oxygen sigma-bonds into carbon-carbon sigma-bonds to the synthesis of 8nor-FDP (**140**). The key synthetic step of this route is the addition of lithium dihydrofuran (**212**) to the allylic bromide **131**, and subsequent stereospecific nickel-catalysed ring opening reaction by methyl magnesium bromide affording the trisubstituted alkene **213**. Alcohol **213** was then converted into iodide **214** and

the isoprene chain elongation was achieved by means of a Negishi's cross-coupling[220] reaction between the iodide **214** and the vinylic iodide **215** to afford the alcohol **216** (Scheme 52).[221] A different synthetic route was developed in this study for the preparation of 8nor-FDP (**140**).
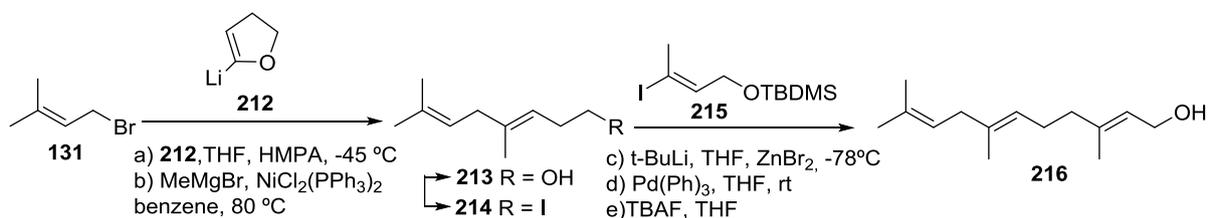
Scheme 52. Synthesis of 8-nor-farnesol (**216**) proposed by Gibbs.

Retrosynthetic analysis: Diphosphate is prepared from the corresponding alcohol **216** that is obtained from the deprotection of THP ether **217**. The first disconnection is the dimethylvinyl tail of **217** to give synthones **218** and **219**. Synthones **218** and **219** correspond with bis-dimethylvinyl cuprate **220** and bromide **221** respectively. **221** Is prepared from the corresponding alcohol **222** (Scheme 53).
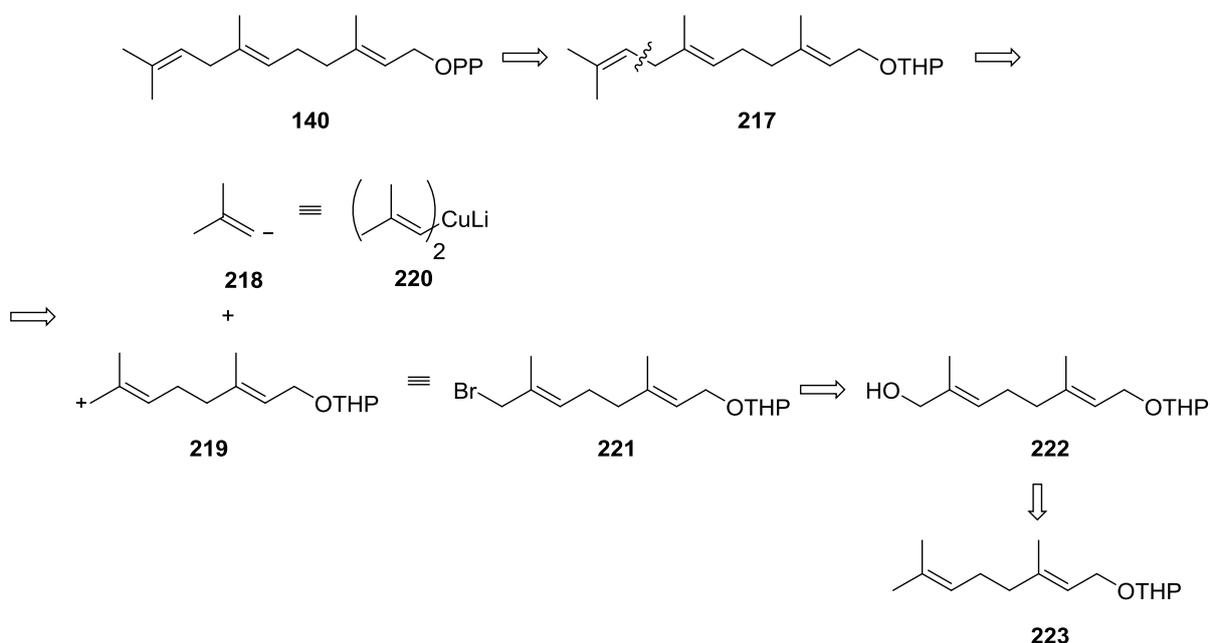
Scheme 53. Retrosynthesis of 8nor-FDP (**140**).

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The synthesis starts with the THP protection of commercially available *trans*-geraniol (**224**), followed by allylic oxidation with selenium dioxide to afford allylic alcohol **222**. The hydroxyl group is converted to bromide to afford **221**. Then, the bromide **221** is treated with dialkyl copper **220** to afford THP ether **217**. The deprotection of the THP ether will afford alcohol **216** and the final diphosphorylation will afford the target compound **140** (Scheme 54).
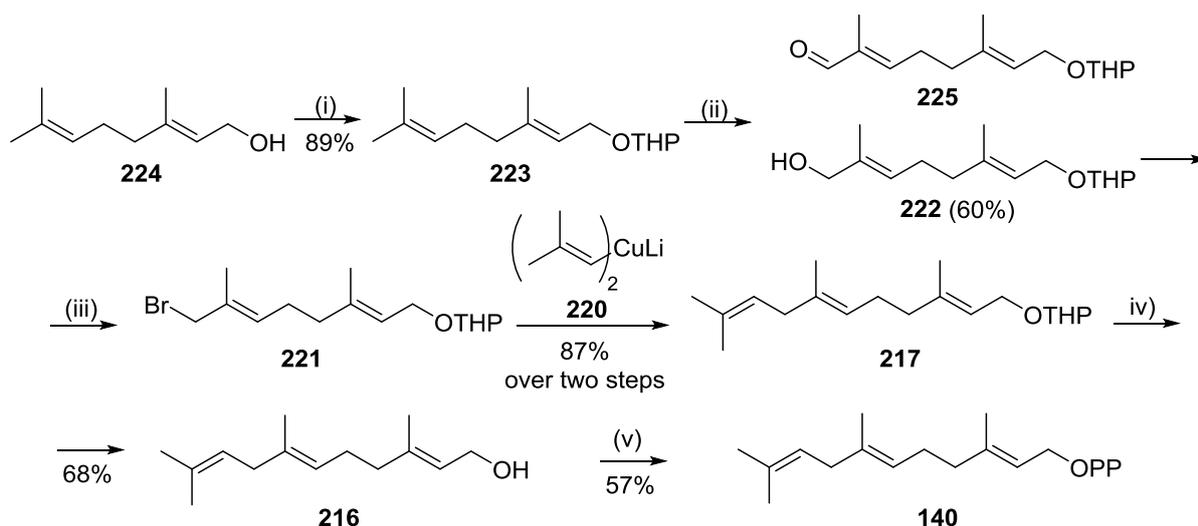
Reagents and conditions: i) DHP, PPTS, DCM, RT. ii) SeO$_2$, t-BuOOH, salicylic acid, DCM 0 ℃ iii) MsCl, Et$_3$N, LiBr, THF, -45 ℃ to RT iv) PPTS, MeOH, RT v) S-collidine, MsCl, LiBr, DMF 0 ℃ and then (Bu$_4$N)$_3$HP$_2$O$_7$, CH$_3$CN, then DOWEX 40 W (NH$_4^+$ form) cation exchange.

Scheme 54. Synthesis of 8nor-FDP (**140**).

2.5.1 Preparation of alcohol 222

Protection of geraniol was achieved in 89% yield by acid catalysed reaction with dihydropyran. The oxidation of THP ether **223** afforded the allylic alcohol **222** in 60% yield. In this case, salicylic acid was added to the reaction mixture to minimise over oxidation of alcohol **222** to the corresponding aldehyde **225** (Scheme 54).[222]

2.5.2   Preparation of THP ether 217

Alcohol **222** was converted into allyl bromide **221** by conversion to mesylate and displacement with LiBr, as described for alcohol **160** (Scheme 33). After 1 hour, the reaction was complete as judged by TLC. The crude product was used in the next step without further purification. Displacement of the allylic bromide **221** with the cuprate **220** gave the THP ether **217** as major product in 87% yield. However, the ^{1}H NMR spectrum of **217** displays small signals that suggest that the sample is contaminated with an inseparable compound (Figure 11, *****, **#**).
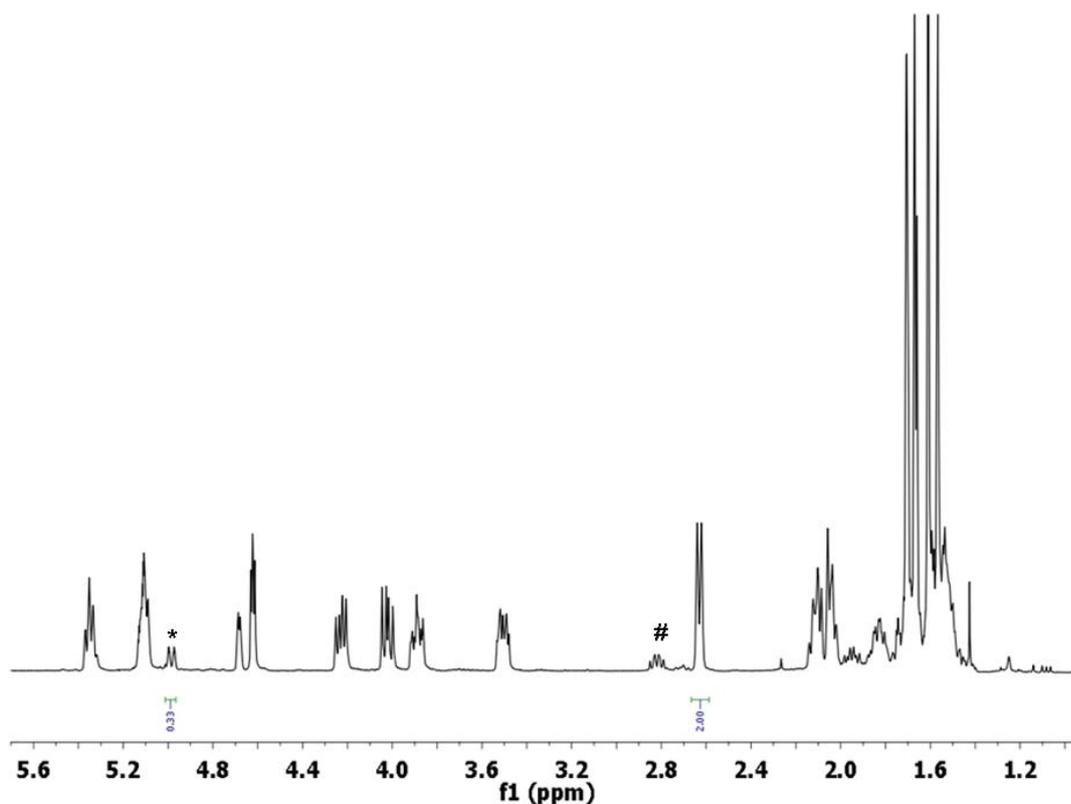
Figure 11. ^{1}H NMR spectrum (400 MHz, CDCl$_3$) of the THP ether **217** impurity is marked with * and #.

The product is presumably formed through an SN$_2$' mechanism through intermediate organocuprate **226** that undergoes a [1, 3] sigmatropic rearrangement to afford **227** (Scheme 52). According to this structure, the broad doublet observed at 4.98 ppm corresponds to the

two protons on the terminal double bond of **227** marked with a * and the quadruplet at 2.28 ppm marked with **#**, corresponds to the double allylic proton of **227**. The integrals for these protons show that ether **217** is contaminated with 14% of **227** (Figure 11).
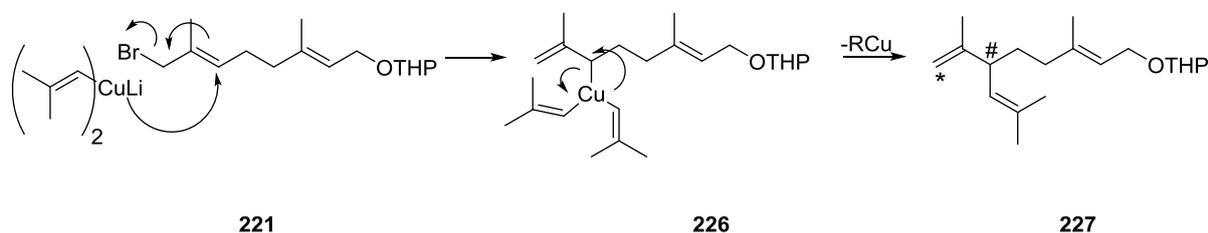
Scheme 55. Proposed mechanism for the formation of the inseparable by product **227**.

The impurity persisted after several attempts when purifying **217** by column chromatography on silica. The synthesis was continued with this unidentified impurity.

2.5.3 Preparation of 8nor-FDP (140)

PPTS mediated deprotection of THP ether **217** afforded alcohol **216** in 68% yield. The diphosphorylation of alcohol **216** and purification by ion exchange column and reverse phase HPLC afforded diphosphate **140** as a white solid in 57% yield (Scheme 54).

2.5.4 Preparation of a mixture of 8-nor-farnesenes: (*E*) E-8-nor-β-farnesene (**228**), (*E*)-8-nor-α-farnesene (**229**), (*Z*)-8-nor-α-farnesene (**230**).

The mixture of (*E*)-7,10-dimethyl-3-methyleneundeca-1,6,9-triene (**228**), (*3E,6E*)-3,7,10-trimethylundeca-1,3,6,9-tetraene (**229**) and (*3Z,6E*)-3,7,10-trimethylundeca-1,3,6,9-tetraene (**230**) was prepared by dehydration with PPTS in dichloroethane (Scheme 56). The farnesene mixture was purified using a pipette charged with small amount of silica, and analysed by GC-MS (section 4.2.6, Figure 40).
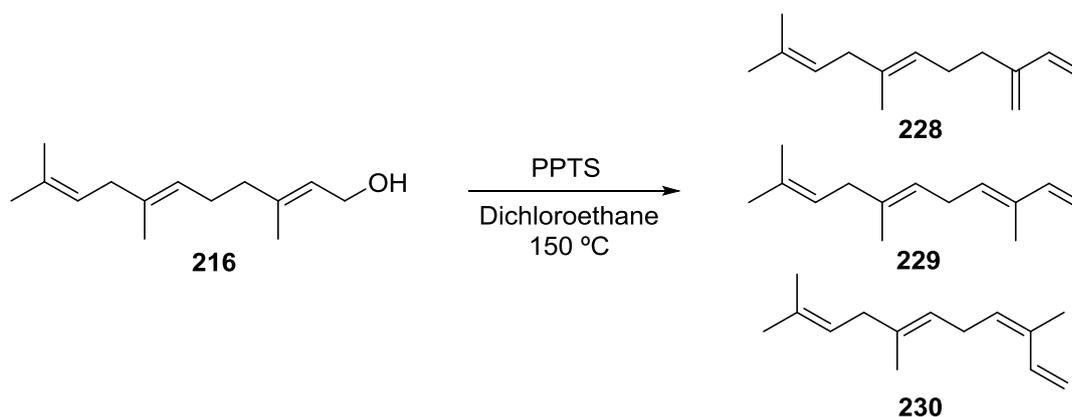
Scheme 56. Preparation of 8nor-farnesene mixture.

2.5.5 Conclusions

8Nor-FDP was prepared through a new synthetic route that relies on the addition of dialkyl cuprate **220** to allylic bromide **221** as a key step. By using this methodology, the synthesis of this novel FDP analogue was achieved in 18% yield over 6 steps.

2.6 Synthesis of 8OH-FDP (141)

No report of the synthesis of FDP analogues containing a hydroxyl group has ever been published. A retrosynthesis designed to plan the synthesis of 8OH-FDP (**141**) with an addition of Grignard reagent as a key step is described here.

Retrosynthetic analysis: Diphosphate **141** is prepared from the acetyl alcohol **231** that is disconnected on C8 to give synthones **232** and **233** that correspond to Grignard reagent **234** and aldehyde **235** that is prepared through oxidation of **236** (Scheme 57).
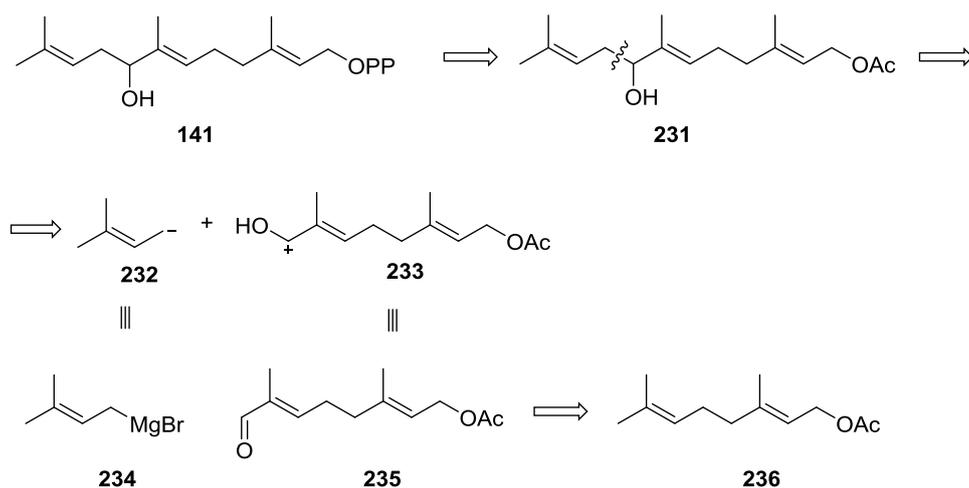
Scheme 57. Retrosynthetic analysis of 8OH-FDP (**141**).

Aldehyde **235** was prepared by selenium dioxide oxidation of commercially available geranyl acetate **236** and subsequent oxidation of the resulting alcohol **237** with PDC to afford aldehyde **235**. The Grignard reagent **234** should be made from the commercially available bromide **131** by reaction with Mg. The diphosphorylation will be performed by selective tosylation of the primary alcohol *via* the usual diphosphorylation reaction to afford the 8OH-FDP **141** (Scheme 58).
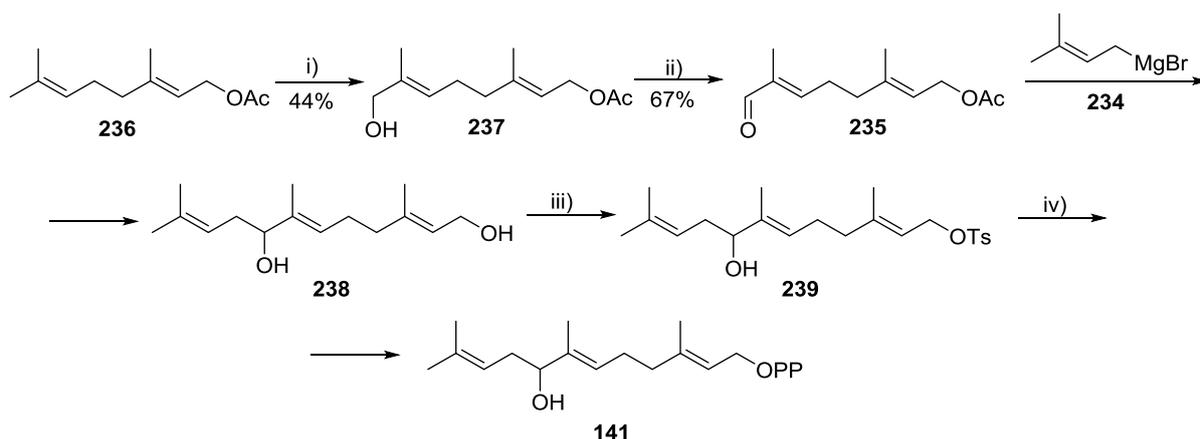
Reagents and conditions: i) SeO$_2$, t-BuOOH, salicylic acid, 0 °C ii) PDC, molecular sieves 3Å, 0 °C iii) TsCl, Py, RT iv) (Bu$_4$N)$_3$HP$_2$O$_7$, CH$_3$CN, then DOWEX 40 W (NH$_4^+$ form) cation exchange.

Scheme 58. Synthesis of 8OH-FDP (**141**).

2.6.1 Preparation of aldehyde 235

As it has been explained above, the oxidation of geranyl acetate **236** with selenium dioxide was performed for ether **223** (Scheme 54) achieving **237** in 44% yield. Oxidation with PDC led initially to the required aldehyde in poor yield. This reaction yield was improved by the addition of molecular sieves (3 Å) to the reaction mixture, and further filtration of the reaction mixture through celite®, which notably facilitated the workup of this transformation. The aldehyde **235** was ultimately obtained in 67% yield.

2.6.2 Preparation of the diol 238

The addition of the Grignard reagent **234** to the aldehyde **235** was performed with a large excess of **234** to trigger simultaneous deprotection of the acetal group. The Grignard reagent was initially formed by reaction of magnesium tunings (previously activated in an oven at 200 ºC) with the bromide **131** in freshly distilled diethyl ether, and the reaction was started by addition of a small crystal of I_2. However, no reaction was observed, and only starting material was recovered from the reaction mixture.

In a 2nd attempt, the reaction was carried out according to the modified procedure of Barbier. This protocol is similar to Grignard's procedure, but the major difference is that Barbier reaction is performed in one pot.[223, 224] Under Barbier's conditions, consumption of all starting material led to a new product with the expected TLC polarity. However, the ^{1}H NMR spectrum of the product did not show the expected absorptions of the required product **238**. Integration of the characteristic triplets at 5.32 and 5.24 ppm reveals that the new product contains only two double bonds. Two additional double doublets centred at 5.84 ppm and 4.97 ppm were suggestive of the presence of a terminal double bond. The Barbier product was finally characterised as diol **240**, which could be formed following the SN$_2$' like mechanism depicted in Scheme 59.
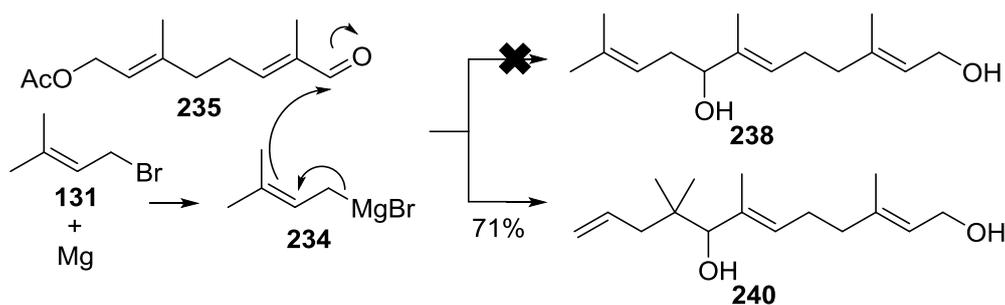
Scheme 59. Proposed reaction mechanism explaining formation of diol **240**.

A literature search revealed that the SN_2' like reaction described in Scheme 59 had been previously reported using similar Mg, Co or Zn based reagents with bromide **131**.[225]

Alternatively, alcohol **238** was obtained in low yield through the oxidation of farnesyl acetate **194**.[216] Thus, oxidation of farnesyl acetate afforded a mixture of primary alcohol and secondary alcohol that could be separated by chromatography column. The reaction gave the secondary alcohol **241** in 13% yield. Hydrolysis of the acetate afforded the diol **238** in 87% yield (Scheme 60).
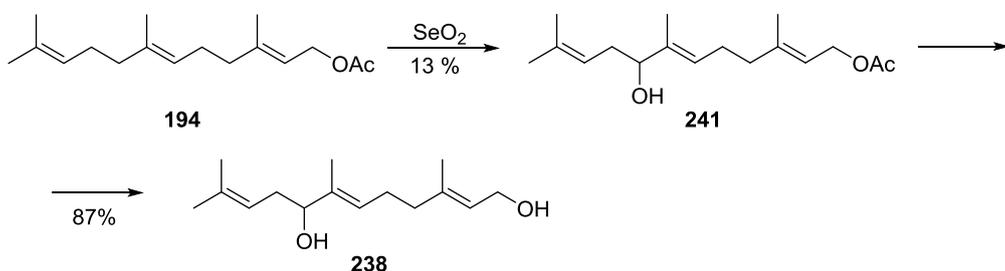
Scheme 60. New synthetic route for the synthesis of **238**.

2.6.3 Preparation of 8OH-FDP (141)

A p-toluenesulfonyl (tosyl) group was selected as the leaving group to carry out the diphosphorylation reaction given that it is expected that the tosyl chloride would selectively react with the primary alcohol group of diol **238** due to its bulk. After 24 hours, TLC analysis revealed a product that was not UV active. The compound was isolated by flash chromatography on silica gel and analysed by ^{1}H NMR spectroscopy. The signal at $\delta_H = 4.14$

ppm corresponding to the CH$_2$OH protons had slightly shifted to 4.15 ppm. Mass spectrometry identified the product as the allylic chloride **242**. This chloride resulted from displacement of the initially formed tosylate (Scheme 61). The final diphosphate 8OH-FDP (**141**) was formed from **242** using the standard phosphorylation conditions, resulting in decomposition of the compound. Finally, diphosphate was prepared by Dr Juan A. Faraldos using a procedure described by Giner *et al.*, which involves protection of the secondary alcohol with an acetate group and deprotection with NH$_3$ (aq) after diphosphorilation.[226]
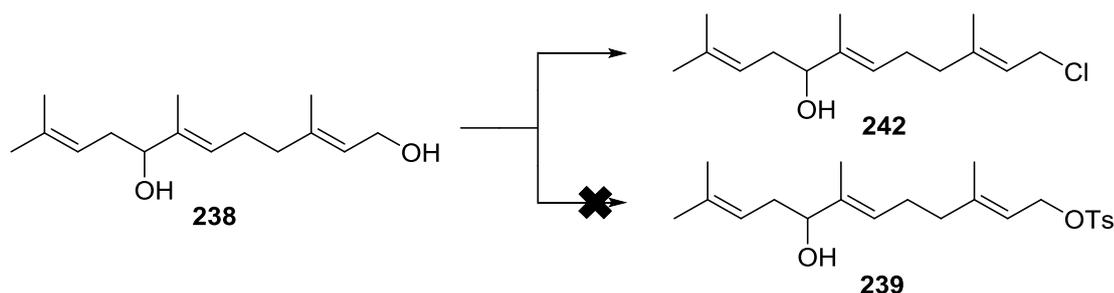
Scheme 61. Preparation of **242**.

2.6.4 Conclusions

The synthetic route initially proposed proved to be unsuitable for the preparation of 8OH-FDP (**141**) due to the unexpected reactivity of the Grignard reagent **234**. Alternative synthesis by means of an allylic oxidation of farnesyl acetate (**194**) is a low yield, but direct route to prepare diol **238**. Selective phosphorylation of diol **238** was difficult and a tedious sequence of protection/deprotection was required to prepare 8OH-FDP (**141**, Scheme 62).[226]
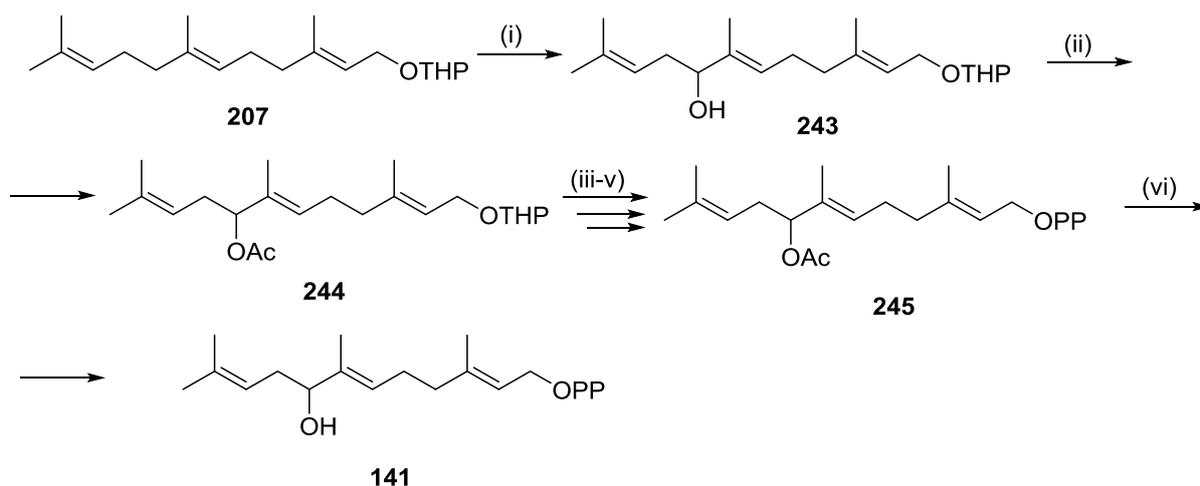
Reagents and conditions: i) SeO_2, t-BuOOH, salicylic acid, 0 °C ii) Ac_2O, py, RT iii) MeOH, PPTS, RT iv) S-collidine, MsCl, LiBr, DMF 0 °C v) $(Bu_4N)_3HP_2O_7$, CH_3CN, then DOWEX 40 W (NH_4^+ form) cation exchange vi) NH_3 (aq)

Scheme 62. Preparation of 8OH-FDP (**141**).

2.7 Synthesis of 14OH-FDP (142)

Literature review revealed that the 14OH-FDP has never been synthesised before. However, Tago has reported the synthesis of plaunotol (**248**),[227] which is the homologous C20 hydroxy derivative of geranylgeranyl diphosphate. The proposed synthesis was performed by means of Wittig olefination between the phosphonium **247** and ketone **246** (Scheme 63). Plaunotol has been reported to have an antibiotic activity against *Helicobacter pylori*, which is the bacterium responsible for gastritis,[228] gastric ulcers[229] and duodenal ulcers.[230] In addition, *H. pylori* has been classified by the World Health Organisation as a potential cancer-causing agent.[231]
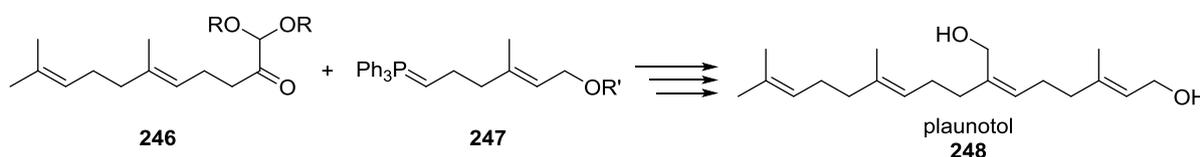
Scheme 63. Key step of the synthesis developed by Tago. R = Et, Cy or Pr^i, R' = Me.

It was therefore decided to prepare compound **142** *via* an analogous Wittig olefination reaction with ketone **250**, which was to be prepared by Rubottom oxidation of **267** (Scheme 64). Phosphonium salt **251** was to be prepared as described by Tago, *via* degradation of THP-protected geraniol.[227]
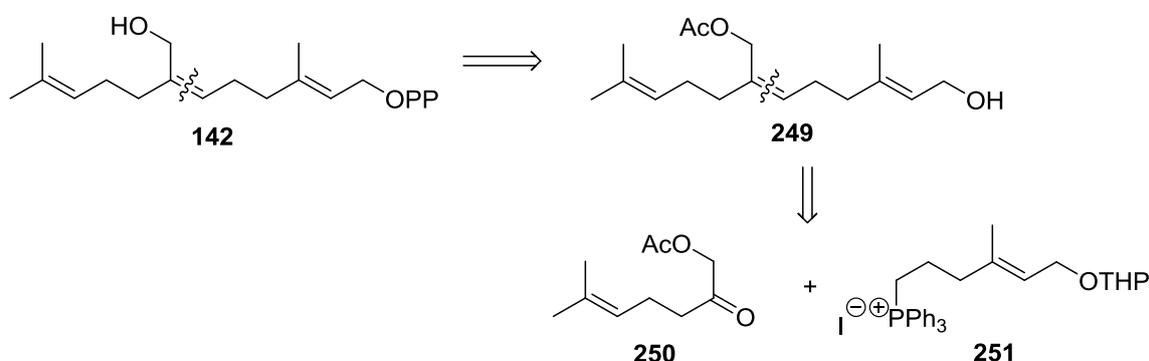
Scheme 64. Retrosynthetic analysis of 14OH-FDP (**142**).

2.7.1   Preparation of phosphonium salt 251

The synthesis of the phosphonium salt **251** was made by degradation of THP protected geraniol **223** (Scheme 66). Correct selection of the protecting group in this case was crucial given that the conditions for deprotection should not cleave acetate group to allow the final diphosphorylation reaction. Whereas THP is removed under acidic conditions, acetate needs basic conditions, Therefore, THP should be a compatible group for the conditions used in this synthesis. Chemoselective oxidation of the distal double of THP ether **223** afforded the epoxide **252** in 69% yield. Although the ^{13}C NMR spectrum of **252** could not be interpreted due to the high level of complexity of the spectrum, the product was characterised by ^{1}H NMR spectroscopy and mass spectrometry. This complexity can be explained by the formation of diastereoisomers due to the following circumstances: 1) the presence of the chiral and racemic THP protecting group, and 2) the resulting mixture of isomers upon epoxidation that produce a mixture of two pairs of enantiomers (**252a** / **252b** and **2.55c** / **252d**) and two pairs of diastereoisomers (**252b** / **252d** and **252a** / **252c**) (Scheme 65).

252a

252b

252c

252d
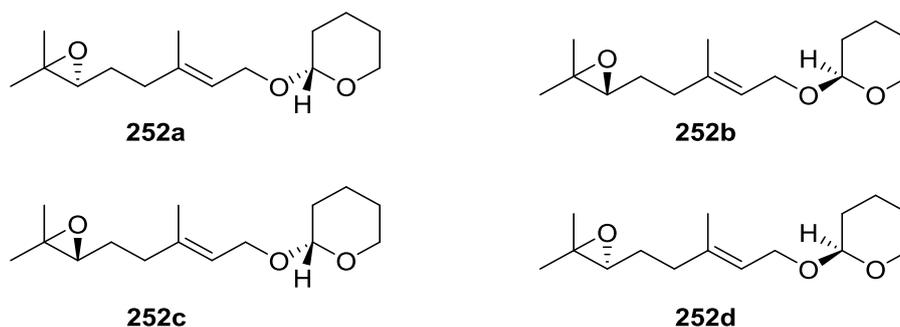
Scheme 65. Different stereoisomers obtained from epoxidation of **223**.

Cleavage of the epoxide **252** with a mixture of H_5IO_6 / $NaIO_4$ afforded aldehyde **253** in moderate yield (28%). Subsequent sodium borohydride reduction of **253** afforded the corresponding alcohol **254** in 83% yield. Iodide **255** was prepared from alcohol **254** by tosylation of the alcohol and displacement of this leaving group with NaI. Phosphonium salt **251** was obtained as a white solid in 67% yield by refluxing iodide **255** and PPh$_3$ in freshly distilled diethyl ether for 48 h (Scheme 66).
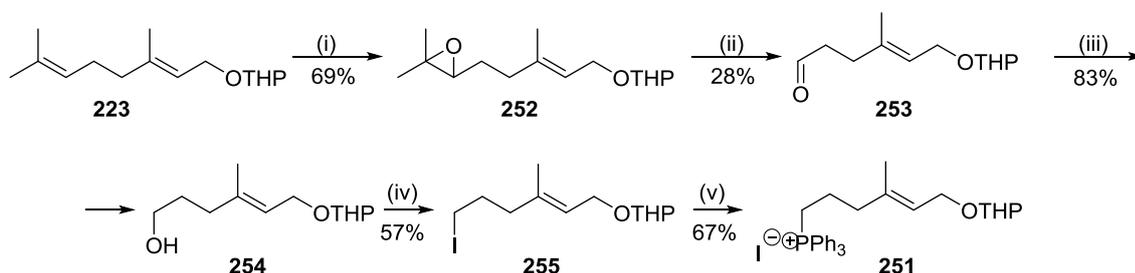
Reagents and conditions: i) mCPBA, DCM 0 °C ii) H_5IO_6 / $NaIO_4$, H_2O/THF 0 °C iii) NaBH$_4$ EtOH 0 °C iv) TsCl, Py, DCM and then NaI in anhydrous acetone v) PPh$_3$, Et$_2$O.

Scheme 66. Synthetic route for the preparation of the phosphonium salt **251**.

2.7.2   Preparation of ketone 250

The key step in the formation of acetyl α-hydroxy ketone **250** *via* silyl enol ether **257** relies on its successful Rubottom oxidation (Scheme 67).[232] Ketone **256** was treated with LDA in THF at -78 °C and subsequently with TMSCl to afford **257** in 90% yield. Silyl enol ether **257** proved to be stable upon storage in the fridge for months under anhydrous conditions.
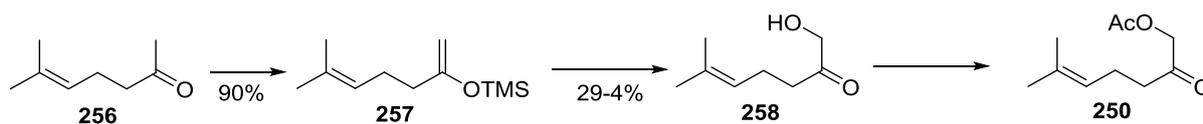
Scheme 67. Synthetic route to the ketone **250**.

The epoxidation of enol ethers with peroxy acids (Rubottom oxidation) results in α-hydroxy ketones after the epoxide ring opening. The reaction starts with the attack of the peroxy acid to the enol **259** to afford a highly unstable epoxide **260**. Epoxide ring opening is then affected by the surrounding acid to afford initially a carbocation **262**, which is quenched by additional acid to afford ester **263**. Attack of the hydroxyl group to the ester releases the silyl ether to generate hydroxyl carbonate **264**, which is subsequently hydrolysed to the final α-hydroxy ketone **265** (Scheme 68). Although a selective Rubottom oxidation at low temperatures has been used successfully in an alternative synthesis of plaunotol as described by Kikumasa,[233] no procedure for the key oxidation was reported.
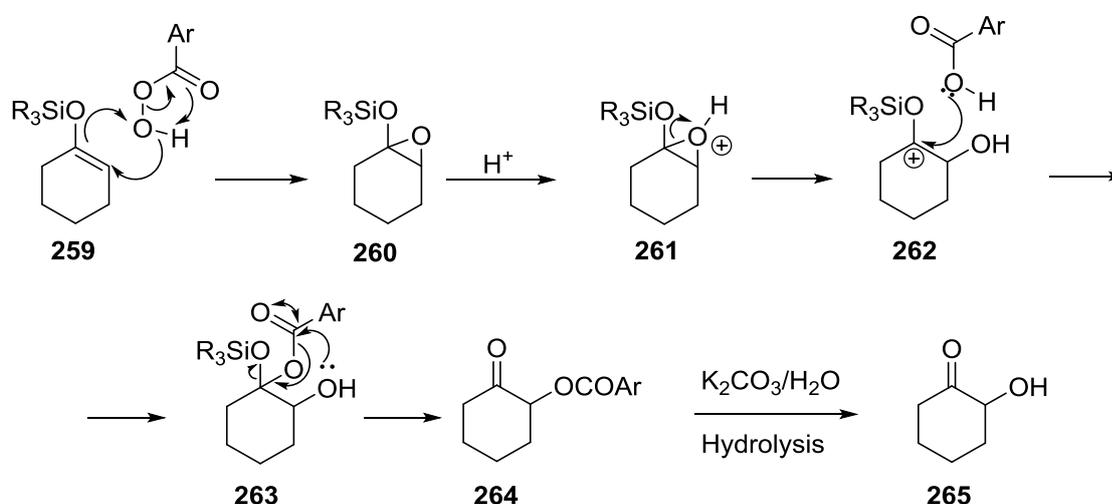
Scheme 68. Mechanism for the Rubottom oxidation.[234]

The Rubottom oxidation of enol ether **257** was carried out by adding dropwise a solution of *m*CPBA to a solution of **257,** both in DCM, and stirring for 3 h at -78 ºC. Acid hydrolysis with aqueous HCl followed by basic hydrolysis with KCO$_3$ While this procedure afforded the

desired product **258** only in small traces, large amounts of ketone **256** were recovered (Scheme 67).

TLC analysis on silica using previously deactivated plates (by dipping the plate in 10% Et_3N solution in ether and allowed to dry) indicates that the reaction mixture after 3 h at -78 ºC contains a large amount of silyl enol ether **257** and ketone **256**. The initial hydrolysis of the starting material can be explained by the presence of benzoic acid in the commercial *m*CPBA used. Thus, the *m*CPBA was purified by washing an ethereal solution of *m*CPBA with an aqueous phosphate buffer at pH = 7.5.[235] Reaction of silyl enol ether **257** with the freshly purified *m*CPBA, followed by acid / basic hydrolysis afforded the expected hydroxy ketone **258** in 4% yield. Intriguingly, TLC monitoring of the reaction did not show any evidences of ketone **256** and large amount of the starting material enol ether **257** was recovered after work up.

In a further attempt at this reaction, both temperature and reaction time were increased (-45 ºC for 4 h). Under these conditions, a hydroxy ketone **258** was obtained in 29% yield. All attempts to scale up this reaction resulted in complex mixtures of products, from which the required product could not be isolated by purification.

During the synthesis of 2*S*-hydroxymutilin, Wang *et al.* developed a modification of the original Rubottom procedure to allow selective oxidation (*m*CPBA) of silyl enol ethers in the presence of additional (terminal) double bonds under buffered (pyridine/acetic acid) conditions at -20 ºC.[236] Following these conditions (-20 or -45 ºC), similar untreatable mixtures of products were again observed upon hydrolysis. The consistently low yields and poor reproducibility of the Rubottom oxidation of **257** prevented a successful synthesis of **250** following this seemingly successful protocol in previous cases.

2.7.3 Preparation of ketone 269

As an alternative, α- keto acetal **269** was then prepared as depicted in Scheme 69 to attempt a novel synthesis of 14OH-FDP (**142**) following the same olefination strategy previously described (see Scheme 64).
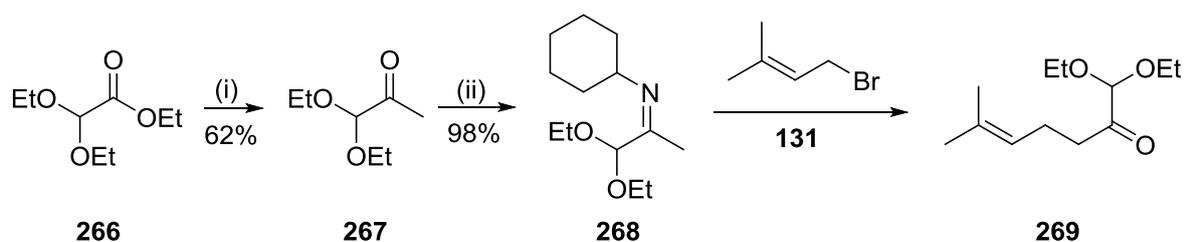
Reagents and conditions: i) CuI, MeMgBr, THF, 0 ℃ and then **266**, -78 ℃ ii) 4Å MS, cyclohexylamine, DCM, RT.

Scheme 69. Synthesis of **269**.

The first attempts to prepare **267** *via* direct addition of one equivalent of MeMgBr were unsuccessful, resulting in the formation of the corresponding alcohol (41% yield) from double addition of methyl magnesium bromide to ester **266**. The literature search revealed that most of the reported Grignard additions to ethyl esters resulting in alkyl ketone formation have been made by reaction of the corresponding chlorinated Grignard reagent.[237] Alternatively, R_2CuLi reagent can also been successfully employed.[238] The reaction of compound **266** at -78 ℃ in THF with Me_2CuLi gave ketone **267** in 62% yield. The subsequent formation of the enamine **268** was performed by direct reaction of cyclohexylamine in the presence of molecular sieves 4 Å affording the target compound in 98% yield.

The treatment of enamine **268** with LDA at 0 ℃ followed by the addition of bromide **131** did not afford the expected ketone **269**. The ^{1}H NMR spectrum of the major product isolated from crude reaction displays two singlets at 4.67 and 4.27 ppm, accounting for one proton each. These two signals correspond to two different tertiary carbons of two different acetals. The spectrum also displays two doublets at 3.04 and 2.58 ppm, which integrate for one proton each. The chemical shift of these signals and multiplicity (AB system) suggest that they belong to two diastereotopic CH_2 protons located in the proximity of two quaternary centres, one being chiral/prochiral. Multiplets centred at 3.77 and 3.62 ppm integrating for 2 and 6 correspond to the ethyl group of two acetals. Additionally, the multiplet centred at 1.21 ppm integrates for 15 protons, which correspond to four methyl groups of the acetals and one additional methyl group (Figure 12).
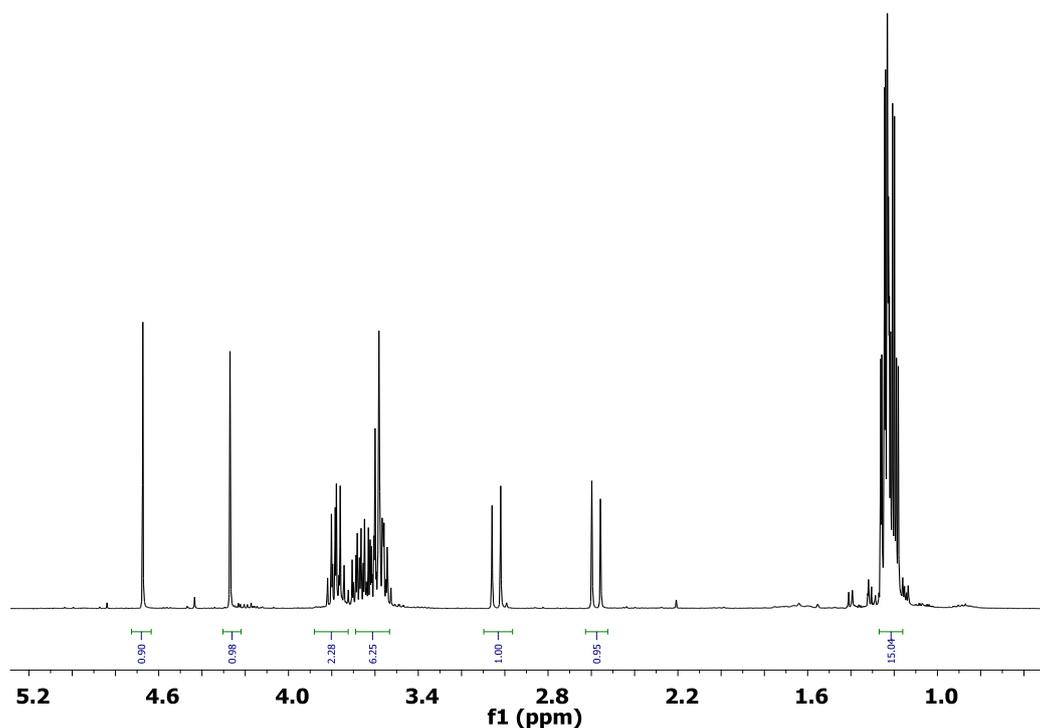
Figure 12. ^{1}H NMR spectrum of product obtained from the treatment of compound **271** with LDA.

Based on this data, it can be concluded that a dimer of **267** is formed by condensation of **270** giving **271** (Scheme 70).
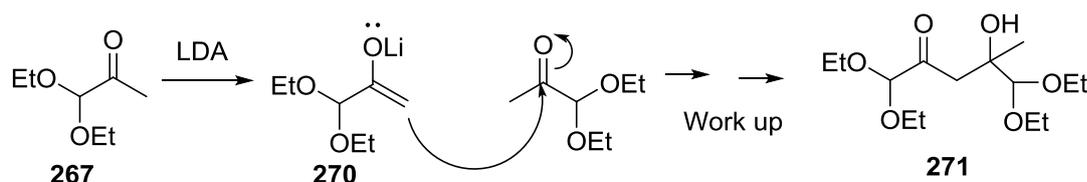
Scheme 70. Mechanism of aldol reaction of **267**.

A new synthetic approach was proposed for the synthesis of ketone **269**. β-Keto ester **272** was prepared by Claisen condensation between ester **266** with ethyl acetate.[239] After solvent removal (4 h) by high vacuum, the sample was relatively clean as judged by ^{1}H NMR spectrum. However, attempts to further purify the crude product by chromatography on silica resulted in the loss of the acetal group. Therefore, the compound was used without further

purification. The crude weight accounts for 81% yield. Compound **273** was produced by deprotonation of β-keto ester **272** and alkylation with bromide **131**. The ^{1}H NMR spectrum of the crude displays the expected signals for compound **273** and hence, it was used in the next step without further purification expecting that the by-products could be removed by simple evaporation. After base catalysed decarboxylation of **273**, careful neutralisation of the basic reaction media, extraction of the product and removal of the solvent and volatile impurities under high vacuum afforded **269** in 30% yield over two steps (Scheme 71).

Reagents and conditions: i) NaH, EtOAc, THF, reflux ii) NaH, **131**, THF 0 °C iii) NaOH, H$_2$O, EtOH, reflux.

Scheme 71. Successful synthetic route to acetal **269**.

2.7.4 Wittig olefination

Scheme 72. Wittig reaction.

The addition of **269** to a solution of the ylide of **251**, which is prepared using *n*-BuLi at -78 °C for 30 minutes, and subsequent stirring (5 h) at -45 °C (Scheme 72), afforded aldehyde **276** (Scheme 74) as a mixture of isomers instead of the expected acetal **274**. This was indicated by signals at 10.09 and 9.35 ppm in ^{1}H NMR spectrum. The spectrum displayed two signals at 10.09 and 9.35 ppm indicating the formation of a 1:3 isomeric mixture of aldehydes, which were reduced to alcohols **277** with NaBH$_4$ (Scheme 74). NOE experiments were performed to identify each isomer. The ^{1}H NMR spectrum of alcohol **277** displays two singles at 4.62 and 4.52 ppm, corresponding to the protons on C14 for each isomer. Analysis of the integrals indicated approximately the same isomeric distribution as in the aldehyde precursors **276**, 1:3 respectively. Whereas the irradiation of signal at 4.62 ppm (minor isomer) results in no appreciable NOE enhancements, the irradiation of signal at 4.52 ppm

(major isomer) led to 1% NOE enhancement of the triplet (5.47 ppm), corresponding to the proton on C6. Unfortunately, this observation identified the major isomer as E isomer **275** (Scheme 73).

Scheme 73. NOE effect was used to identify the major isomer of **275** as E-configured.

The reaction of ketone **269** with phosphonium salt **251** was expected to afford the *cis* isomer given that a similar reaction with an analogue of ketone **246** containing one more isoprene unit tested by Tago *et al.* has proven to give a selectivity (*Z : E*) between 4:1 and 61:1 depending on the conditions.[227]

Wittig reaction was carried out and proportion of Z isomer was increased by stirring the crude product in a mixture of THF/acetic acid (3:2) affording **276** as mixture of isomers *Z/E* (6:4) in 30% yield after chromatography column on silica (Scheme 74).
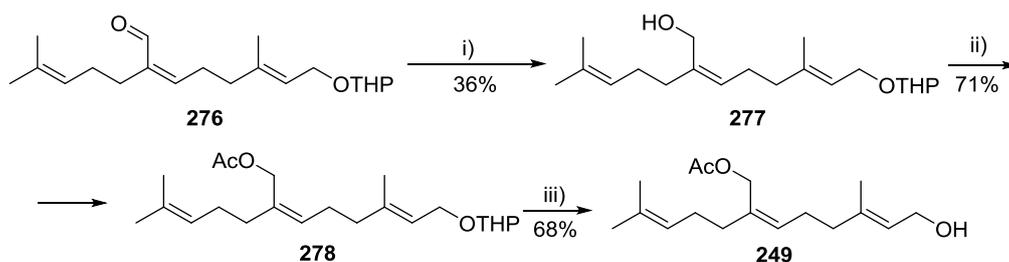
Reagents and conditions: i) NaBH$_4$, EtOH 0 °C ii) Py, acetic anhydride, DCM, RT iii) PPTS, MeOH, RT.

Scheme 74. Preparation of alcohol **249**.

Aldehyde **276** was reduced to alcohol **277** by reaction with NaBH$_4$. The two isomers of the alcohol **277** were separated by vacuum chromatography on silica,[240] and the desired *trans-*

isomer was isolated as pure isomer in 36% yield. The acetate protection of alcohol **277** was achieved in 71% yield. THP deprotection of **278** yielded acetate **249** in 68%.

The quantities generated by this approach were not enough to carry out the planned diphosphorylation/deacetylation sequence needed for enzymatic evaluation. The extremely low yield (<1%), clearly discounted this approach for the preparation of 14OH-FDP (**142**).

2.7.5 Conclusions

As previously described by Tago,[227] phosphonium salt **251** was prepared instead by the synthesis of ketone **250**, through a Rubottom oxidation that seemed to be an inefficient method due to its poor reproducibility. In addition, the reaction suffered from low yield when scaling up was performed. Alternatively, keton **269** was prepared through a sequence of Claisen oxidation and decarboxylation. Wittig reaction between ketone **269** and salt **251** has poor selectivity, thus Z isomer was obtained in low yield. As a result, the overall yield was low. Therefore, this methodology is discounted to accomplish the synthesis of diphosphate **142**.

Perhaps in the current case, the modification of the Wittig olefination used by Corey[213] could significantly reduce the number of steps leading to the desired alcohol (Scheme 75). Unfortunately, this new proposal was not tested due to time restrictions.
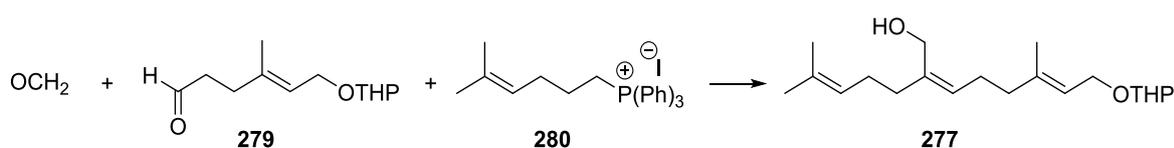
Scheme 75. New proposed synthesis for the preparation of 14OH-FDP (**142**).

2.8 Overall conclusions

The methodology developed by Rawat *et al.*[199] for the synthesis of alkyl modified FDP analogues was optimised and applied to the synthesis of 14Me-FDP (**137**), 15Me-FDP (**138**) and 14,15Me-FDP (**139**). Whereas 14Me-FDP (**137**) was obtained in 6% yield over twelve steps, previously reported synthesis of 14Me-FDP (**137**) was achieved in 5% yield.[199]

Whereas 15Me-FDP (**138**) was prepared in six steps in 8% yield, previously reported synthesis by Montellano *et al.* afforded 15Me-FDP (**138**) in less than 2% yield.[209] Therefore, these two examples show the improvement achieved by the method reported herein. 14,15Me-FDP (**139**) was synthesised for the first time using this method in 5% yield.

A new methodology for the preparation of FDP analogues by the addition of dialkyl cuprate to an electrophile was developed and applied to the synthesis of 12Me-FDP (**136**) and 8Nor-FDP (**140**). 12Me-FDP (**136**) was synthesised for the first time in 3% yield. Whereas 8nor-FDP (**140**) was previously synthesised by Rawat *et al.* in 13%,[218] the methodology presented herein achieved 8Nor-FDP (**140**) in 18% yield. However, this method is limited by the formation of by-products, which difficults the purification of the final product.

The synthesis of a new 8OH-FDP (**141**) was performed through an allylic oxidation of farnesyl acetate. Dihydroxy farnesol **238** was achieved in low yield. The initial plan for selective phosphorylation of **238** was discarded due to the decomposition of chlorine **242** under phosphorilation conditions. Tedious sequence of protection/deprotection was designed to prepare 8OH-FDP (**141**, Scheme 62).

The synthesis of 14OH-FDP (**142**) was performed by means of Wittig olefination. As previously described by Tago,[227] phosphonium salt **251** and keton **269** were prepared through a sequence of Claisen oxidation and decarboxylation. Wittig reaction between ketone **269** and salt **251** afforded aldehyde **276** in poor yield and selectivity. Alcohol **249** was achieved in poor overall yield and therefore this methodology was discounted to accomplish the synthesis of diphosphate 14OH-FDP (**142**).

CHAPTER 3

Innovative methodologies for the enzymatic production of valuable semiochemicals

3. Introduction

The study of the procedures reported in the literature reveals a great variation in experimental conditions and in the reported yields and/or conversions of incubations with terpene synthases. These variations explain the poor reproducibility of enzymatic yields and problems encountered in obtaining terpenes in sufficient quantity to be fully characterised.[241]

The reactions of terpene synthases with their substrates are usually performed in aqueous solutions that contain substrate, enzyme, $MgCl_2$ and buffer. This mixture is overlaid with an organic solvent to extract terpenes from the aqueous media. In a typical incubation procedure, this biphasic mixture is incubated for 24 h and then vortexed to extract organic compounds from the aqueous media to the organic solvent. The organic solvent is analysed after phase separation (Figure 13).
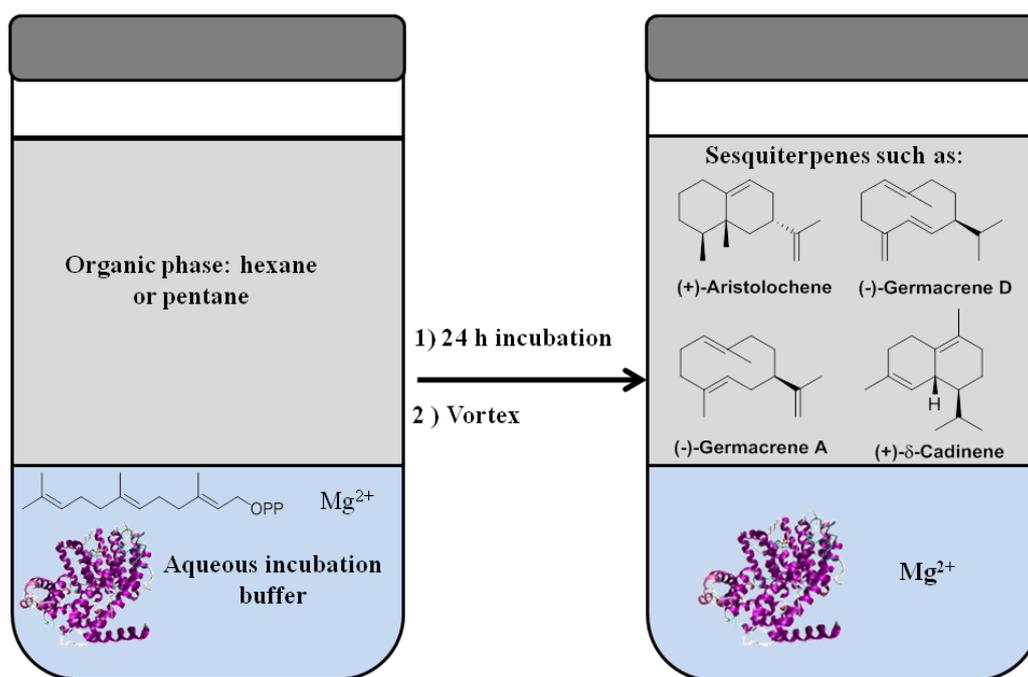
Figure 13. Standard set-up for incubations with sesquiterpene synthases.

In this chapter, efforts aimed at the development of a rationally optimised procedure for the *in vitro* production of valuable sesquiterpenes by optimising reaction conditions and minimising

the product losses of volatile products upon workup are described. Toward this end, a set of experiments to determine the optimal concentration of Mg^{2+}, substrate and enzyme with the four sesquiterpene synthases (DCS, AS, GDS and GAS) used in this project were performed. In addition, different extraction methods, organic solvents and work-up procedures were tested to simplify the current, and often-inefficient work-up steps during the sesquiterpene synthase mediated reactions. Additionally, flow chemistry methodology to improve the performance of sesquiterpene synthases was explored.

3.1   Optimal magnesium concentration

3.1.1   (+)-δ-Cadinene synthase (DCS) from *Gossypium arboreum*

The optimal Mg^{2+} concentration for DCS was determined by measuring the enzymatic conversions using a GC-FID apparatus and α-humulene as internal standard. The GC-FID methodology was later validated by comparison with the well-established radiochemical-assay employed for GAS and GDS.

Incubations of DCS and FDP (Chapter 1, Scheme 21) in buffer (25 mM HEPES, 5 mM DTT, 12 µM DCS, pH 7.5) with concentration of $MgCl_2$ ranging from 3 to 12 mM were performed in a final volume of 500 µL. Preparations were overlaid with 1 mL pentane containing the internal standard α-humulene (35 µM). The mixture was incubated for 30 minutes. Hydrocarbons were extracted by 30 s vortex and organic extracts were analysed by GC-FID. The optimal concentration of $MgCl_2$ was 6 mM as judged by the decreasing conversion observed at higher concentration, as shown in Figure 14.
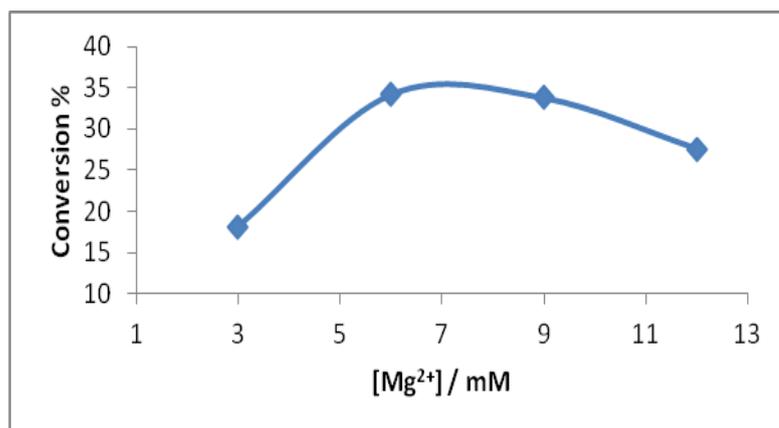
Figure 14. Plot of conversion of FDP against Mg^{2+} for DCS displays decreasing conversion at concentrations higher than 6 mM.

The optimal concentration obtained by GC-FID assay was compared with the value obtained by radio-assay with [1-^{3}H]-FDP, which was performed as described above for aristolochene synthase.[242] Assays (final volume 250 μL) were initiated by addition of an enzyme solution (50 μM, 25 μL) to 100 μM [1-^{3}H]-FDP (240000 dpm/nmol) in buffer (25 mM HEPES, 5 mM DTT, pH 7.5) with a concentration of MgCl$_2$ ranging from 1 to 9 mM. After incubation for 12 min, reactions were stopped by addition of 100 mM EDTA and overlaid with pentane (1 mL). Once the samples had been vortexed for 10 s, the pentane was removed and the sample extracted with pentane in the same way (2 x 1 mL). The pooled pentane extracts were filtered through approximately 50 mg of silica, then emulsified with Ecoscint O (15 mL) and finally analysed by scintillation counting. The optimal concentration of MgCl$_2$, as shown in Figure 15, was 6 mM. This value is in agreement with the value obtained with GC-FID assay, which means that both methods were considered suitable for optimising sesquiterpene synthase incubations for product conversion.
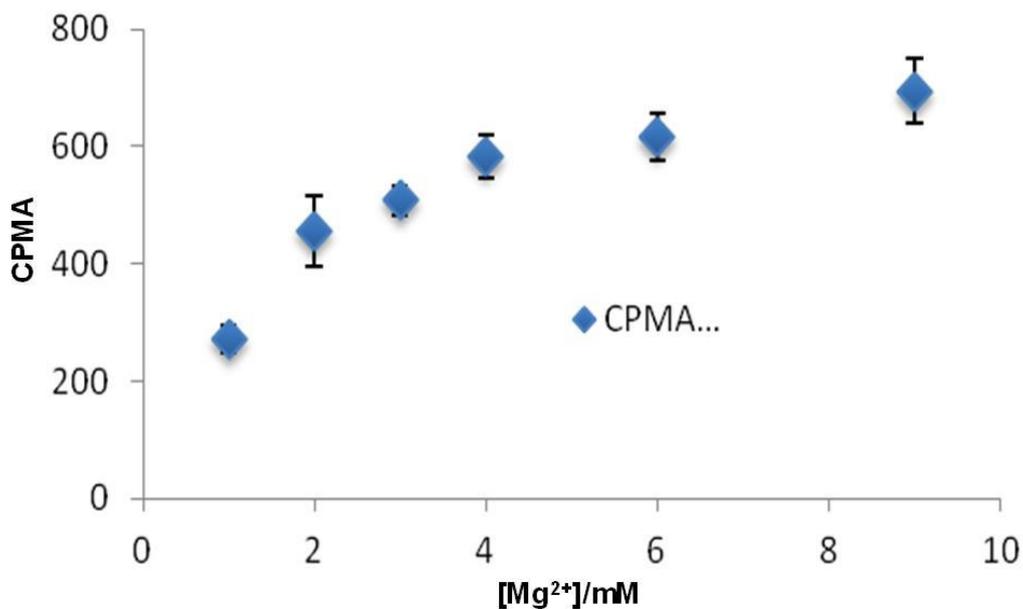
Figure 15. Experiment under kinetic conditions using ^{3}H-FDP. CPMA: counts per minute to detemine optimal Mg^{2+} with DCS.

3.1.2 Aristolochene synthase from *Penicillium roqueforti* (AS)

The incubations of AS and FDP (Chapter 1, Scheme 13) in buffer (20 mM Tris, 5 mM 2-mercaptoethanol, 15% glycerol, 12 μM AS, pH 7.5) with concentration of MgCl$_2$ ranging from 3 to 12 mM were performed as described above for DCS (*vide supra*). The conversion was measured by GC-FID analysis. As shown in Figure 16, optimal concentration was 3 mM. This value is in agreement with the data published by Hohn.[176]
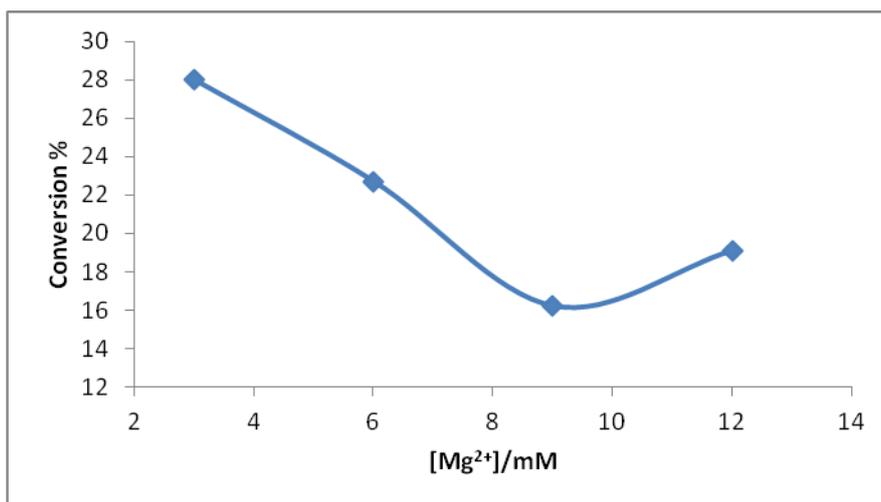
Figure 16. Study performed by GC-FID to determine optimal Mg^{2+} concentration with AS.

3.1.3 (-)-Germacrene D and (+)-germacrene A synthases from *Solidago canadensis* (GDS and GAS, respectively)

By using radio-labelled FDP, the optimal metal requirements of GAS (Chapter 1, Scheme 9) and GDS (Chapter 1, Scheme 16) were estimated previously as 3 mM and 10 mM, respectively.[243]

3.2 Optimal substrate concentration

At concentration above ≈ 0.5 mM of FDP, a white precipitate was observed immediately after dissolving FDP in a buffer containing Mg^{2+} at 10 mM. The precipitate was only poorly soluble in protic (MeOH/CHCl$_3$) or aprotic polar solvent, such as DMSO, thus hampering the full characterisation by NMR spectroscopy. The low solubility, HRMS/LRMS (m/z = 505) and ^{31}P NMR spectroscopy (δ_P 3 ppm in CDCl$_3$) data were, however, in agreement with the values previously reported for difarnesyl phosphate (**282**, Scheme 76). [244]
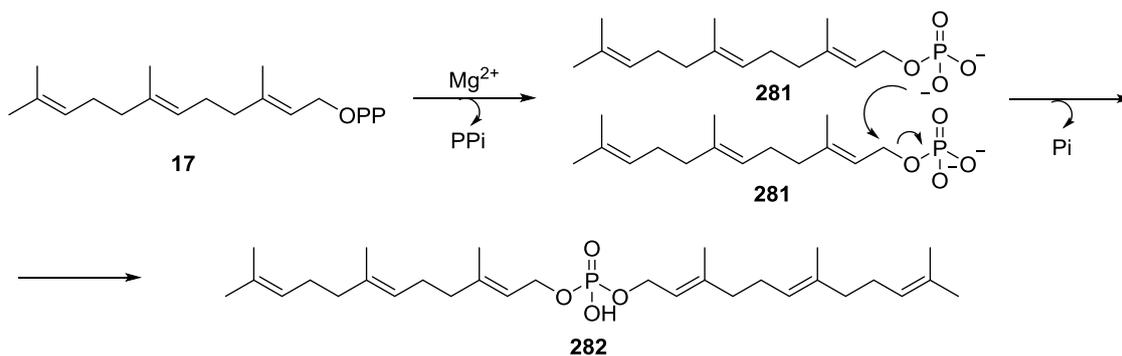
Scheme 76. Proposed mechanism for the Mg^{2+} triggered conversion of FDP (**17**) into **282**.

In order to estimate the concentration of FDP just below the critical concentration for formation of **282**, different buffer solutions ($[Mg^{2+}]$ = 10 mM) containing FDP in the concentration range (0.1 to 0.5 mM) were prepared. Then, the precipitate was removed by filtration. The samples were analysed by reverse phase HPLC (same conditions as specified in section 6.3.4 for purification of **137**) and the area of the peak of remaining and soluble FDP was plotted against the initial concentration of FDP (Figure 17).
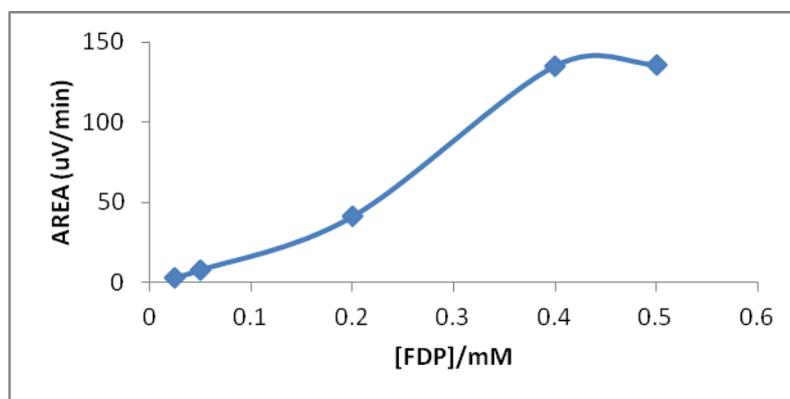
Figure 17. [FDP]^{initial} *vs* Area of the peak in the HPLC corresponding to remaining soluble FDP.

The HPLC peak area corresponding to FDP represents the amount of FDP that did not undergo conversion into **282** (i.e. [FDP]$_{final}$) and remains in solution. As shown in Figure 17, a [FDP]$_{intial}$ plateau is reached at ca. FDP = 0.4 mM, which corresponds to the maximum FDP concentration before the beginning of the conversion into **282** and is the highest

concentration that it is possible to reach in buffer solution and, therefore, the maximum concentration available for enzymatic reaction.

The reaction outlined in Scheme 76 only occurs at a concentration above 0.4 mM. A possible explanation for this observation will be the formation of micelles at this concentration. FDP has a polar head and a hydrophobic tail, which makes this compound a surfactant. Surfactants group themselves in aqueous solution into micelles putting together all the polar head and tails. The formation of micelles occurs above a critical surfactant concentration called critical micelle concentration (CMC).[245] This disposition can trigger the conversion of FDP into **282** by increasing the proximity of polar heads in the micelle.

3.3   Optimal enzyme concentration

3.3.1   (-)-Germacrene D synthase

A series of analytical incubations (24 h) were performed with GDS in concentrations ranging from 4 to 12 μM at fixed optimal concentrations of FDP (0.35 mM) and Mg^{2+} (10 mM) (section 3.2 and 3.1.3, respectively). Conversion was measured by GC-FID by comparison of the peak areas of the pentane extractable products and α-humulene (35 μM) that was used as an internal standard. Figure 18 shows that the conversion of the reaction starts to decline at 6 μM enzyme concentration. Higher concentrations most likely lead to enzyme aggregation, which explains the decrease in enzymatic activity. The highest conversion under these conditions was 43%.
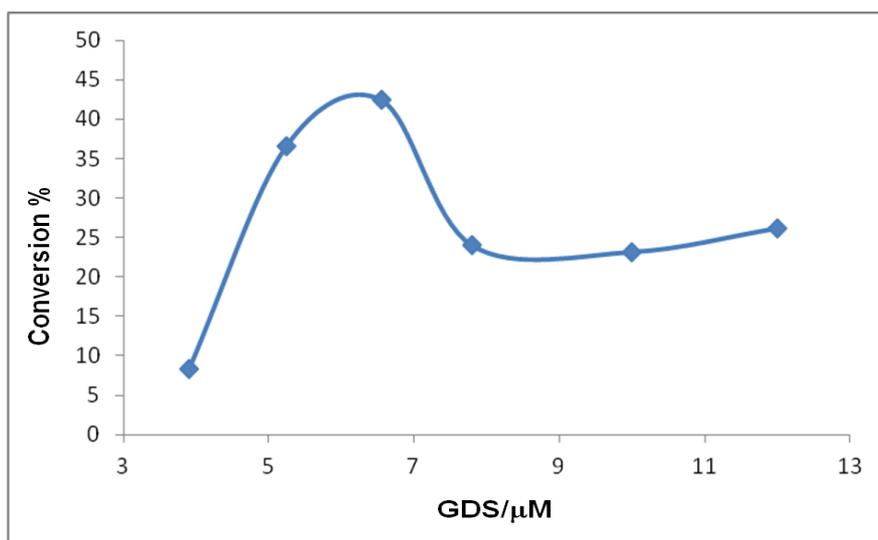
Figure 18. Conversion of FDP with varying GDS concentrations.

The enzymatic aggregation process can be partially prevented by addition of a detergent to the buffer that can avoid interactions between enzyme units. The four following detergents: CHAPS, TRITON X100, TWEEN 20, TWEEN 80, all at 1%, were tested in buffers containing a fixed concentration of enzyme (12 μM) (Table 3, entries 2-5) in 24-hours incubation. In all cases, a substantial improvement in the enzymatic conversion was observed.

Entry	Detergent	Conversion %
1	-	43
2	TRITON X100 (**285**)	94
3	TWEEN 20 (**283**)	92
4	TWEEN 80 (**284**)	85
5	CHAPS (**286**)	76

Table 3. Conversions with GDS when detergent is added to the incubation buffer.

Although for synthetic purposes the best conversion was obtained with Triton X100 (Table 3, entry 2, **285** Scheme 77), due to the insolubility of the amphoteric detergent CHAPS (Scheme 77, **286**) in organic solvents, such as $CDCl_3$ or C_6D_6 (Table 3, entry 5), this detergent was preferred for characterisation purposes over the others because signals of the other detergents can be seen in the ^1H NMR spectrum.

TWEEN 20 (**283**) R

TWEEN 80 (**284**) R

w+x+y+z=20

triton X100
(**285**)
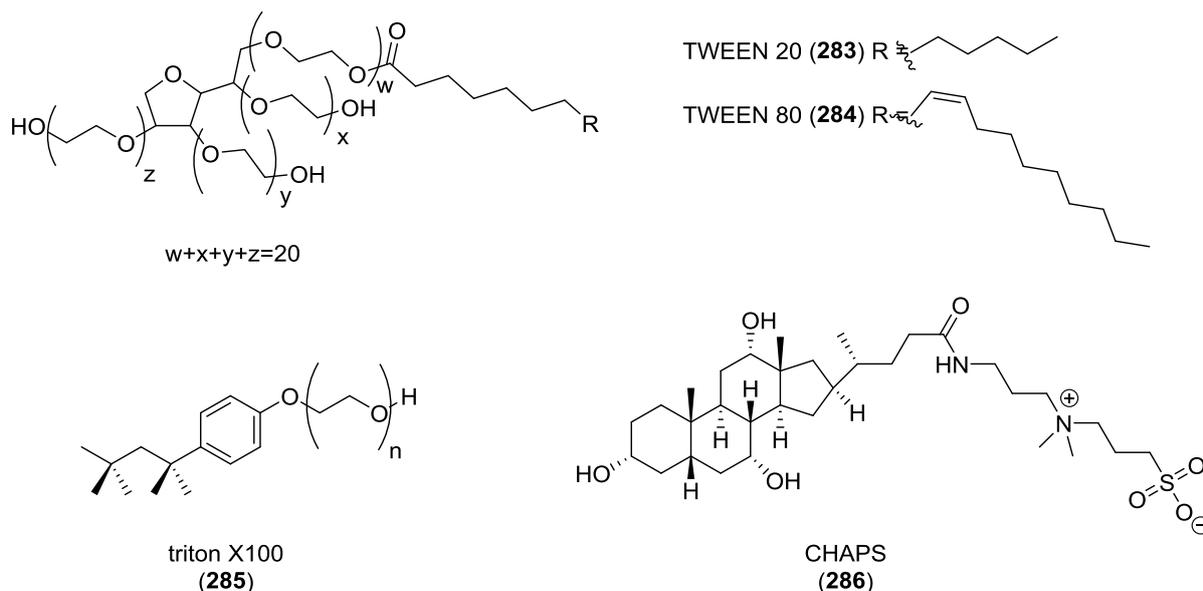
CHAPS
(**286**)

Scheme 77. Structure of TEEWN 20 (**283**), TEEWN 80 (**284**), triton 100X (**285**), CHAPS (**286**).

3.3.2 (+)-δ-Cadinene synthase

The same experiment was performed with increasing concentration of DCS ranging from 6 to 18 μM at fixed optimal concentrations of FDP (0.35 mM) and Mg^{2+} (6 mM) (sections 3.2 and 3.1.1 respectively). Here too, the slope of the curve obtained decreases at high concentration of the enzyme (Figure 19). The result suggests enzymatic aggregation; similarly to previous experiments, the conversion was increased by inclusion of 1% CHAPS (**286**) protein detergent to achieve 45% conversion at 12 μM of DCS, which is a 3-fold improvement over incubation carried out without detergent.
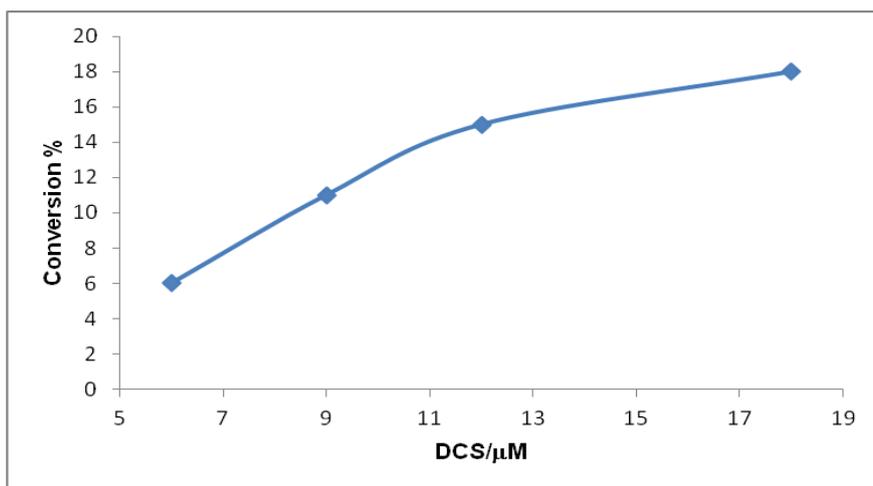
Figure 19. Conversion of FDP to δ-cadinene with varying DCS concentrations.

3.3.3 (+)-Germacrene A and aristolochene synthases

Following similar protocols, the optimal concentration of both GAS and AS was studied. Analytical incubations (24 h) were performed with enzyme concentration ranging from 6 to 18 µM at fixed optimal concentrations of FDP (0.35 mM) and Mg^{2+} (3 mM) (Section 3.2, 3.1.3 and 3.1.2) for both enzymes. In contrast to the previous experiments with GDS and DCS, the experiments with AS and GAS do not suggest that enzymatic aggregation occurs for these enzymes at high concentration judged by the absence of a plateau in the conversion *vs* enzyme concentration curves obtained (Figures 20 and 21).
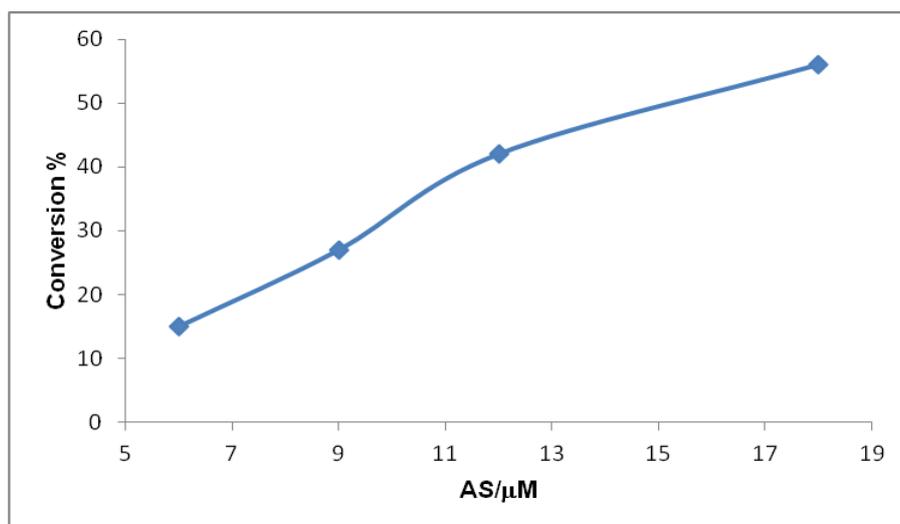
Figure 20. Conversion of FDP to aristolochene with varying AS concentrations.
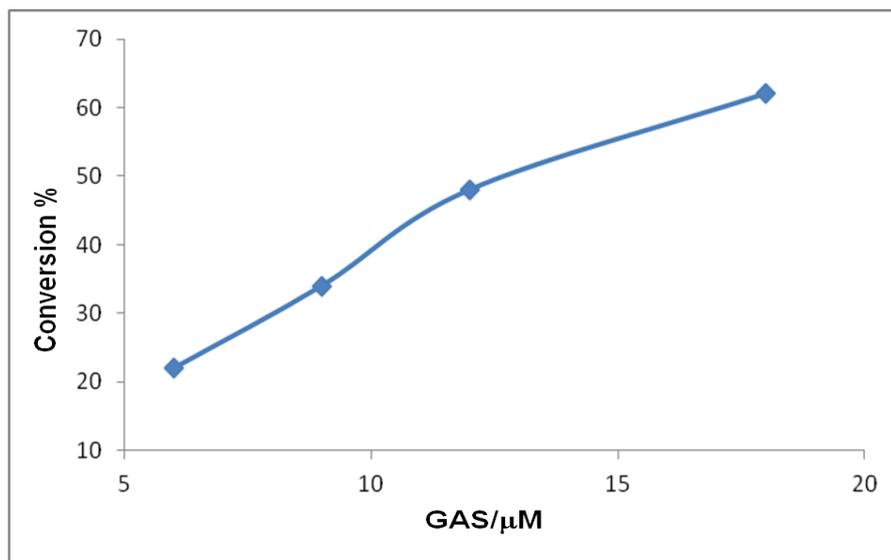
Figure 21. Conversion of FDP to (+)-germacrene A with varying GAS concentration.

Linear correlation between concentration of enzymes (GAS and AS) and conversion suggest that quantitative conversion can be achieved by increasing the enzyme concentration. However, a concentration higher than 18 μM in a large volume of buffer would require a large quantity of enzyme. Therefore, incubations (in mg scale) for enzymatic product characterisation purpose can be performed with 18 μM. This enzyme concentration value is considered a good balance between enzyme economy and relatively high conversion.

Not surprisingly, since GAS and AS do not aggregate at higher enzyme concentration no improvement in conversion was observed in the presence of 1% detergent (e.g. CHAPS).

3.3.4 Conclusions

Interestingly, enzymes that performed best at higher concentration of Mg^{2+} (GDS and DCS 10 mM and 6 mM respectively) aggregate at high concentration of enzyme and, therefore, benefit from inclusion of detergents, whereas enzymes that performed best at low concentration of Mg^{2+} (GAS and AS 3 mM), do not undergo aggregation and, therefore, do not benefit from inclusion of detergents. This observation suggests that Mg^{2+} plays a role in the aggregation process possibly by chelating with amino acids present on the surface of the

different enzyme units acting as a nexus between them and, therefore, triggering aggregation. Detergents can avoid aggregation by surrounding enzyme molecules, thus preventing interaction between them.

The reaction conditions for the enzymatic production of (+)-germacrene A and (-)-germacrene D, δ-cadinene and aristolochene were optimised for Mg^{2+}, enzyme and substrate concentrations. Under the optimised conditions, the sesquiterpenes were obtained in moderate to excellent 45-94% yields. In both cases (GDS and DCS), the enzymatic conversion can be enhanced (3-5-fold) by the addition of detergents (1%). Although for characterisation purposes CHAPS is the preferred detergent, from a synthetic point of view, 1% TRITON X100 allows near quantitative conversions (94% with GDS), and hence appears to be superior.

3.4 Novel, improved methodologies for incubation of sesquiterpene synthases

Methods for the enzymatic production of terpenes in sufficient amount to be characterised have been described previously.[246] However, classic incubation procedures are often inefficient, and, in some cases, not even described in sufficient detail to allow reproduction. Furthermore, the overall yields are, in general low, and hence, of no apparent synthetic utility. Against this backdrop, and as part of this project, substantial efforts were dedicated to the design and development of a new protocol capable of maximising the conversion and isolated yields of the reactions catalysed by sesquiterpene synthases. At the design stage, one of the most important questions, which needed to be addressed, was whether conversions could be enhanced by minimising the amount of organic solvent used for product extraction. In this manner, the isolation of volatiles could be efficiently optimised by reducing the product losses encountered during the evaporation of the solvent. The new methodology was initially developed for semi-preparative scale (mg scale) preparations of (-)-germacrene D using optimised conditions [30 °C, GDS (12 μM), FDP (0.4 mM), Mg^{2+} (10 mM)] in biphasic systems with deuterated organic solvents, which allows the quick and easy identification of products by NMR spectroscopy. This methodology was further extended to other natural compounds, such as (+)-germacrene A and δ-cadinene, and their analogues using alternative FDP substrate surrogates. The optimisation of this new route to valuable terpenes included a

study of different reaction chambers and deuterated solvents. After some experimentation, it was found that the best vessel to carry out semi-prep incubations with GDS, was an ACE pressure tube. The longitudinal geometry of this reaction device minimised the contact between the organic and aqueous phases, thus avoiding denaturation of the biocatalyst at the interface. As a plus, incubations following this novel set-up also benefit from the pressure proof seal of the reaction vessel itself, which effectively minimised losses of volatile products. Furthermore, after some analytical trials, it became evident that d_6-benzene (or d_8-toluene) was prohibited due to the formation of an enzyme precipitate on the interface. The best extracting solvent turned out to be $CDCl_3$; extraction being performed using an automated rotor operating at 4 ºC for the continuous extraction of volatiles over a 12 h period. After extraction, the organic phase was separated using a Biotage phase separator, whereby the organic lower layer passed through the filter and the aqueous layer remained in the tube. The $CDCl_3$ solution was directly collected and transferred into a standard NMR tube for the subsequent spectroscopic analysis of products.

3.4.1 Semi-preparative enzymatic production of sesquiterpenes.

The following general procedure highlights the suitability of the novel approach for the direct examination of products by NMR spectroscopy. Reactions were performed in sealed ACE tubes containing incubation buffer (15 mL), FDP (or analogue, 0.35 mM), CHAPS (150 mg, 1% for GDS and DCS), enzyme (e.g. GDS 12 μM) and $CDCl_3$ (2 mL, deactivated over basic alumina). Each incubation solution was gently agitated for 24 h at 30 ºC. Extraction of enzymatic products into the organic layer was best effected by rotation at 4 ºC for 12 h. After extraction, the aqueous layer (a milky suspension) and the organic layer were separated using a Biotage phase separator. The resulting $CDCl_3$ solution was dried by filtration through a Pasteur pipette containing anhydrous Na_2SO_4. The pooled $CDCl_3$ extracts were concentrated to ~ 0.4 mL under a gentle stream of nitrogen and analyzed by NMR methods.

Four different modifications and improvements of the general procedure (A) were made to meet specific needs. In order to facilitate and speed up the phase separation step, in some cases, the addition of methanol was necessary to break emulsions formed in the extraction process. This emulsion, that contains a trapped product, remained in the phase separator along with aqueous phase (procedure B). The separation was best achieved by subjecting the

reaction vessel and contents to freeze/thawing cycles in liquid nitrogen. This process allowed a cleaner phase separation without the spectroscopic interference of the methanol (procedure C). The purification and structural identification of the enzymatic products was facilitated by excluding β-mercaptoethanol (spectroscopic interference) from the aqueous reaction buffer and by filtering the $CDCl_3$ extracts through basic alumina and dry Na_2SO_4 (procedure D). Figure 22 illustrates the progressive improvement (A to D) of the extractive procedure and increased purity of the enzymatic product in D.
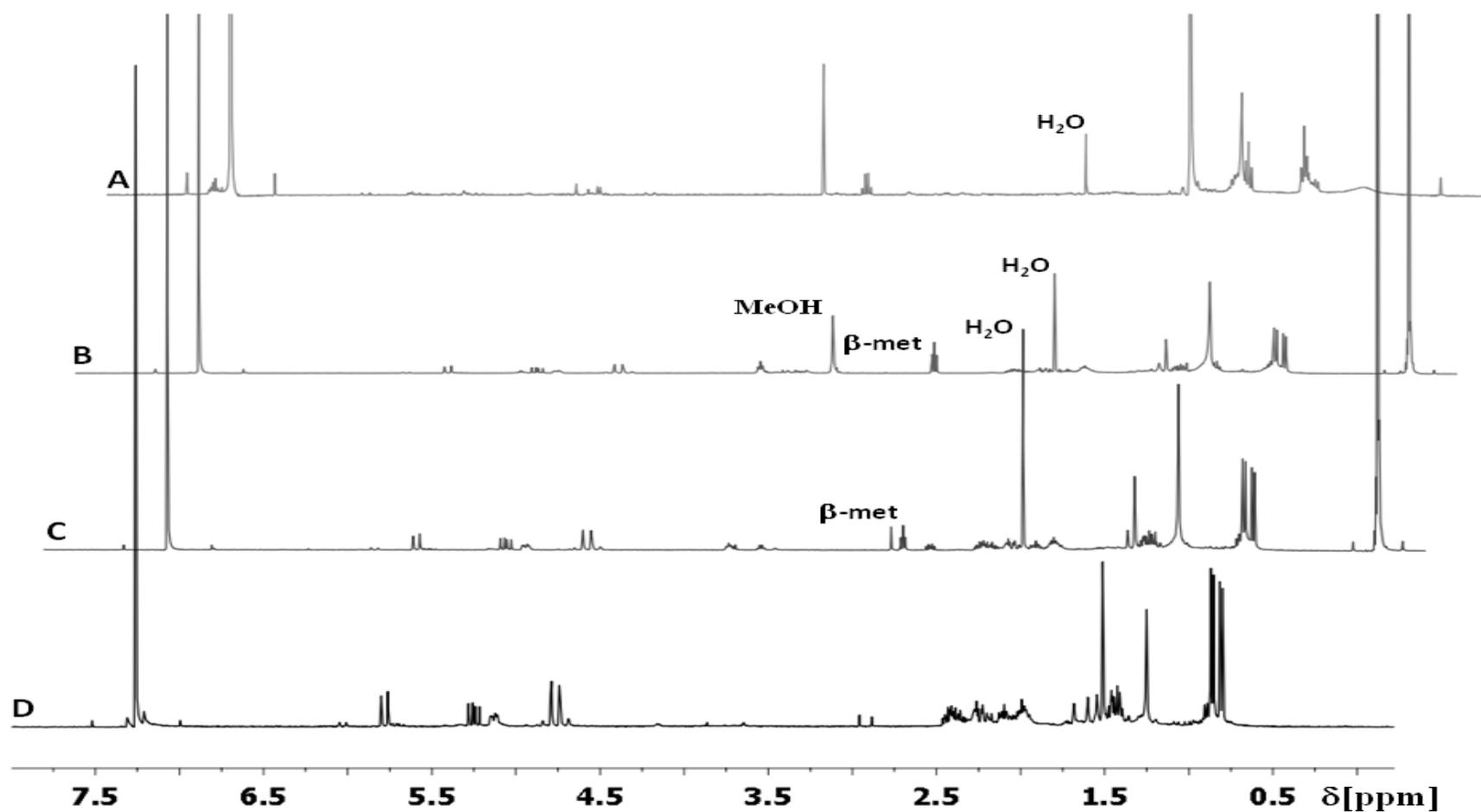
Figure 22. ^{1}H NMR spectra of the products arising from preparative incubation. The concentrations of GDS and FDP were fixed and enzymatic reaction was tested under different conditions. A) General procedure, B) addition of MeOH, C) Freeze/thawing cycles D) Freeze/thawing cycles without β-mercaptoethanol after filtration through basic alumina and Na_2SO_4.

In order to quantify the improvement observed with different protocols, each sample was taken to the same final volume by evaporation, as already described. The integration of the solvent signal and the clear signal at 5.78 ppm (normalised to 1) allowed comparison between procedures. As shown in Table 4, the solvent/product ratio was progressively enhanced by a simple modification of general method A (entry 1), reaching a substantial 10-fold increase in product extracted following D conditions (compare entries 1 and 4).

Entry	Procedure	Integral of $CHCl_3$ at 7.26 ppm	Integral of signal at 5.78 ppm
1	A	53.3	1
2	B	10.9	1
3	C	6.1	1
4	D	5.3	1

Table 4. Relative integrals for solvent peak obtained with each procedure.

3.4.2   Conclusions

Optimal FDP, enzyme and Mg^{2+} concentration values were used in this section to develop a protocol for the incubations of terpene synthases. Optimisation of this protocol was achieved by reducing product loss during evaporation and work-up (elimination of emulsions). Breaking the emulsion formed during work up was found to be of paramount importance for optimising product extraction from aqueous phase, as shown in Figure 22. In addition, reduction of product loss by evaporation was achieved by using ACE sealed tubes as reaction vessel and using deuterated solvent that allowed direct analysis by NMR spectroscopy, avoiding evaporation of large amount of solvents. All these improvements are combined in standard procedure D. Procedure D was successfully used for preparative scale preparation (and characterisation) of sesquiterpenes derivatives (see Chapter 4) obtained from the incubation of unnatural FDP analogues with sesquiterpene synthases, as described in Chapter 2.

3.5 *In vitro* production of natural and unnatural sesquiterpenes by continuous extraction in flow

In previous works, our group has demonstrated the synthetic utility of terpene enzymes in the production of α-pinene/verbenone and (+)-germacrene A and D analogues.[247] However, the slow reactions rates characteristic of terpene synthases, together with the moderate yields obtained simply by scaling up the analytical incubation conditions, led to consideration of flow chemistry as a potentially attractive and alternative route to valuable terpenoids.

The high current interest in continuous-flow processes performed in microfluidic systems[248] is driven by their advantages over classical batch approaches. Miniaturisation of flow devices allows an enhanced mass and heat transfer owing to a very large surface-to-volume ratio. Apart from organic synthetic protocols, biocatalytic reactions have been facilitated by biphasic flow. Esterifications in ionic liquid/heptane mixtures,[249] hydrolysis of esters in water/decane solvents[250] and reductions of carbon–carbon double bonds[251] or aldehydes[252] are accelerated in biphasic flow systems because of the efficient product removal from the aqueous phase. Microwave- and ultrasound-facilitated biphasic reactions have already been reported.[253]

Pre-steady-state kinetic studies of terpene synthases have revealed that the overall rate of terpene catalysed reactions is limited by product release to the aqueous media (i.e. mass transfer ratio between the non-aqueous enzymatic active site and the aqueous media-buffer).[254] The solubility of terpenes in water is remarkably low (10-30 mgL^{-1})[255] and saturation of the aqueous buffer is expected to occur quickly, which further slows down the catalytic process. For fast removal of terpenes from aqueous media, flow reactors offers an increase the contact area between aqueous and organic phases by reducing the diffusion distance. This can lead to higher reaction rates by enhancement of mass transfer.[256]

Based on this extractive advantage of flow chemistry, efforts were made to accelerate the naturally slow catalytic cycle of terpene synthases by quick removal of their water-insoluble terpene products; a continuous extraction protocol was devised. The method was initially developed for native terpenes, but easily adapted to generate non-natural sesquiterpenoids.

3.5.1 Flow reactor design

The flow reactor was designed with a T-shape inlet, as depicted in Figure 23, to ensure segmented flow. This kind of flow is characterised by a series of regular segments of different phases. Each segment is vortexed by its interaction with the wall of the tube causing a continuous refresh of the interface; this convective effect is called the Taylor flow.[257] Segmented flow has proved to increase the efficiency of the mass transfer between two different phases and, consequently, the yield of the process.[258]
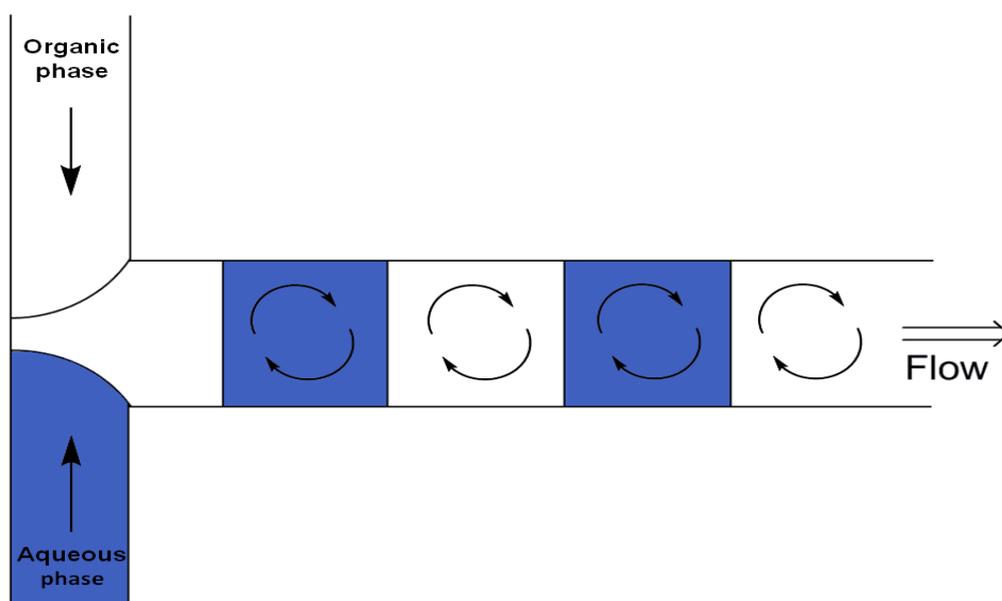
Figure 23. T-shape inlet to create a segmented flow.

In initial trials, solutions of substrate FDP, GDS, and pentane (with internal standard for measuring conversion by CG-FID, see section 3.1) were individually injected into the flow reactor (Figure 24). Alternatively, flow reactor using solutions of premixed enzyme-substrate and pentane were also tested (Figure 25). Under these conditions, both experiments displayed an identical conversion value. These results indicated that in the set-up outlined in Figure 25 the enzyme is reversibly inhibited before contact with organic solvent by saturation of the aqueous solution with the product. Therefore, the reaction rate could be controlled by product extraction in the presence of an organic solvent. In addition, the initial reaction before contact with organic solvent is negligible in comparison with the overall reaction. These inferences were further corroborated by the identical conversion value obtained from a continuous

quenching experiment using EDTA in the collector chamber (Figure 25). This experiment indicates that the batch (slow reaction happening in the collector) contribution to the total conversion is negligible, and hence illustrates the extraction advantages and reaction rate enhancements of the design. The apparatus depicted in Figure 25 was used for all subsequent experiments at fixed substrate concentration of 0.35 mM using pentane as the organic solvent for extraction.
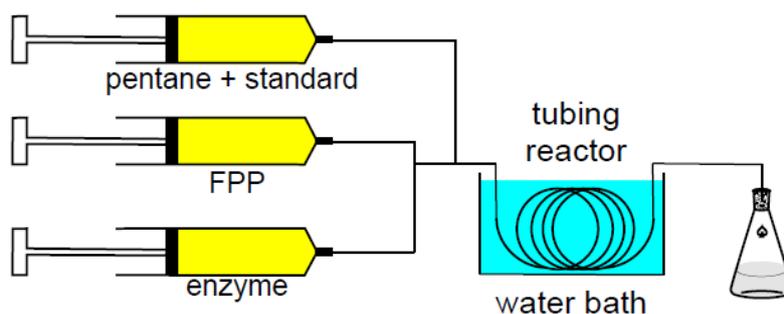
Figure 24. Flow reactor set-up with separate addition of enzyme and FDP.
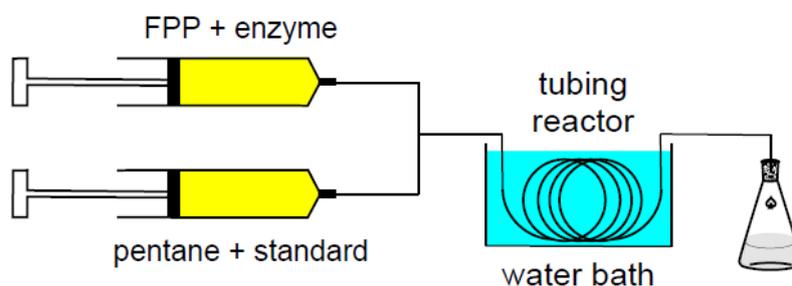
Figure 25. Flow reactor set-up with premixing of the FDP and enzyme.

3.5.2 Preliminary studies of GDS in a flow reactor

The first set of reactions was performed at constant GDS concentration of 9 μM and 30 min residence time (see Table 7 for conditions *vide infra*). The first experiment using a flow reactor with a small internal diameter (0.175 mm) generated a white precipitate upon mixing, suggesting enzyme inactivation by aggregation or denaturation of the protein (Table 5, entry 1). This observation indicated that the vortex generated inside the reactor was too intense, and

milder conditions must be use to avoid enzyme inactivation. Thus, the experiment was repeated with a 0.45 mm internal reactor diameter and this proceeded without precipitation, achieving 17% conversion (Table 5, entry 2).

It has been demonstrated that a combination of a flow reactor with ultrasound is a powerful combination to increase mass transfer in a biphasic system. This improvement arises from the cavitation collapse of bubbles in a sonicated liquid. The bubbles formed in the interface of the biphasic mixture increase the contact area between phases.[259] For instance, Jolhe *et al.* reported a dramatical reduction in the reaction time (less than ten minutes compared to batch reactor) for the preparation of peracetic acid in an ultrasound irradiated flow reactor, compared to analogous reaction in batch.[260] Hence, for further improving the mass transfer between the organic and aqueous phase, the tube of the reactor was immersed in a sonication bath. Bubbles were observed inside the tube, which confirms that cavitation is occurring inside the tube. Consequently, the conversion of the reaction increased to 48% (Table 5, entry 3). A control experiment in batch under the same reaction conditions (temperature, time, reaction volume, organic solvent, etc) led to conversions of about 24% (Table 5, entry 4). The control experiment show that the conversion of incubations can be increased a 100% by performing the experiment in a flow reactor under sonication.

Entry	Internal reactor diameter [mm]	Sonication	Conversion [%]
1	0.175	NO	<1
2	0.45	NO	17
3	0.45	YES	48
4	Batch (Control)	YES	24

Table 5. GDS ([GDS] = 9 µM) preliminary studies with different internal reactor diameters (0.175 and 0.45 mm) with and without sonication for comparison of conversion in batch and in a flow reactor.

3.5.3 Test of enzymatic aggregation in flow

Experiments in batch have demonstrated that enzyme aggregation severely impairs the conversion rate when enzyme concentration was increased (see Sections 3.3.1 and 3.3.2). In order to investigate enzymatic aggregation in flow reactors and see if this problem can be overcome by the use of a flow reactor, three different concentrations of GDS (6, 9 and 12 mM) were tested in flow with optimised conditions (0.45 internal reactor diameter, under sonication). Enzymatic aggregation is best evidenced by the non-linear increase in conversion values upon progressive enzyme concentration increments (Table 6, entries 1, 2 and 3).

Entry	[GDS] µM	Conversion [%]
1	6	27
2	9	48
3	12	52

Table 6. Conversion *vs* GDS concentration studies in flow (0.45 mm internal reactor diameter with sonication).

Previous experiments have demonstrated that enzymatic aggregation can be overcome by adding detergent to the incubation buffer. Additionally, Jensen *et al.* demonstrate that the formation of emulsions derived from the addition of detergents in a flow reactor can increase the contact surface between phases with a consequent increase in the conversion.[261] This possibility was explored by inclusion of 1% CHAPS in the buffer to prevent aggregation but, unfortunately, this experiment led to the blockage of the reactor due to the formation of emulsions, thus demonstrating that this detergent is incompatible with the design and the enzymatic aggregation can not be overcome with this method.

3.5.4 Reaction time optimisation

The optimal reaction time in the flow reactor under sonication conditions was also evaluated by decreasing the flow rate while keeping both the enzyme concentration (9 μM) and the internal reactor diameter (0.45 mm) constant. Surprisingly, increasing the reaction time up to 1 h led to a substantial (ca 5-fold) decrease in conversion. More puzzling, a control experiment in batch (1 h) led to a modest 1.6-fold increase in conversion. The decrease in the turbulences generated inside the tube resulting from the decrease in the flow rate worsened the extraction of the product, thus explaining the greatly diminished conversion of the 1 h run.

3.5.5 Internal reactor diameter optimisation

Convective forces inside the flow reactor can be modulated by changing the internal reactor diameter while retention time is kept constant by adjusting the flow rate and tube length. A smaller internal reactor diameter increases the interactions of the fluid with the wall, thus increasing the mixing between organic and aqueous phase. On the other hand, bigger internal reactor diameters decrease the vortex effect generating milder mixing conditions.[262] This effect was tested by Claus *et al.* in the multiphase hydrogenation of α, β-unsaturated aldehydes. In this experiment, the catalyst was dissolved in aqueous solution and the aldehyde was dissolved in organic solvent. The biphasic mixture was then treated with hydrogen gas to carry on the hydrogenation. Using these conditions, Claus *et al.* found that a change from 1000 μm to 500 μm internal reactor diameter had a dramatic effect, increasing the reaction rate over 260%.[263] Bearing in mind this previous example of optimisation, a second set of reactions was performed to optimise the diameter of the tube used in the flow reactor. The internal reactor diameter was changed while the tube length and the flow rate were adjusted to 30 min reaction time, as shown in Table 7.

Internal reactor diameter [mm]	Length [m]	Flow reactor volume [mL]	Flow rate [μLmin^{-1}]	Residence time [min]
0.175	5	0.12	4	30
0.45	5	0.8	26	30
0.8	4	2	66	30
1.6	1	2	66	30

Table 7. Different flow rates used with different internal reactor diameters to obtain a 30 min residence time.

Initial experiment with a 0.175 mm diameter was repeated using optimised conditions ([GDS] = 12 μM, sonication), resulting in a 12% conversion (Table 8, entry 1). As expected, the increase in diameter of 0.45 mm led to a larger conversion (51%) (Table 8, entry 2). However, a further increase of the diameter (0.8 mm) did not result in a further increase in conversion (47%) (Table 8, entry 3). Moreover, an increase of the diameter to 1.6 mm resulted in a less efficient extraction and only a modest conversion of 22% (Table 8, entry 4) was observed. This demonstrate that internal reactor diameter bigger than 0.8 mm create too mild vortex mixing thus decrease in conversion is observed.

Entry	Internal reactor diameter [mm]	Conversion [%]
1	0.175	12
2	0.45	51
3	0.8	47
4	1.6	22

Table 8. Optimisation of the internal reactor diameter. This set of experiments were performed at [GDS] = 12 μM under sonication.

3.5.6 Conclusion

Incubations of GDS in a flow reactor were best performed in a internal reactor diameter of 0.45 mm under sonication at an enzyme concentration of 12 μM for 30 min reaction time. Under these conditions, a control experiment in batch revealed that incubations in the flow reactor doubled the conversion achieved. Enzyme aggregation was observed upon increasing enzyme concentration. In contrast with previous experiments in batch, this problem could not be overcome by addition of detergents due to the formation of emulsion that blocks the flow reactor.

3.5.7 General application flow reactors with AS and DCS

The general applicability of this methodology was demonstrated by extending the study to DCS and AS. These two enzymes were tested at different concentrations in the flow reactor and batch for 30 min reaction time. All experiments were performed first without sonication for their comparison between batch and flow reactions. As with GDS, the conversion of FDP into the corresponding products by AS and DCS was improved under flow reactor conditions. Furthermore, experiments performed with sonication in a flow reactor resulted in better conversion for both enzymes.

Conversions obtained in batch indicated that AS does not aggregate at concentrations of up to 12 μM, judging by the linear increase of the conversion with enzyme concentration (Table 9, entries 1-3). Comparison of the conversion values obtained in batch and in flow without sonication, revealed a rather modest increment in conversion at any enzyme concentration (Table 9, entries 1-3). Further experiments were performed at an enzyme concentration of 12 μM with different internal reactor diameters under sonication. In this case, an internal reactor diameter of 0.45-0.8 mm increased the conversion to 66-88%, respectively. In contrast, otherwise identical batch experiments under sonication achieved a conversion of 43% (Table 9, entries 4 and 5). Remarkably, sonication seems to have negligible effect in batch reactions (Table 9, entries 3 and 4-5).

Entry	[Enz] µM	Internal reactor diameter [mm]	Sonication	Conversion in flow [%]	Conversion in batch [%]
1	6	0.45	NO	21	15
2	9	0.45	NO	34	27
3	12	0.45	NO	49	43
4	12	0.45	YES	66	
5	12	0.8	YES	88	43

Table 9. Flow and batch experiments with AS performed with and without sonication in 0.8 and 0.45 mm internal flow reactor diameter (30 min).

Unfortunately, an increase of the reaction temperature (40 ℃) with the conditions shown in Table 9 entry 5 resulted in the denaturation of the protein and a decrease in the conversion (21%).

Values obtained in batch experiments indicated that DCS does not aggregate at concentrations of up to 12 µM (30 min reaction), judging by the linear increase of the conversion with enzyme concentration (Table 10, entries 1-3). In contrast, with previous experiments in batch, where aggregation was observed during the 24 h incubation period, enzymatic aggregation seems to take place only during prolonged incubations periods. By comparing the corresponding batch with flow experiments (Table 10, entries 1-3), the production of (+)-δ-cadinene in continuous extraction in flow was double the efficiency of the equivalent batch process. Further experiments at 12 µM enzyme concentration, with sonication and internal reactor diameter of 0.45 mm, gave the highest conversion of 56% (Table 10, entry 4). In contrast with AS, experiments with DCS in an internal reactor diameter of 0.8 mm led to a decrease in the conversion (27%, Table 10, entry 5).

Entry	[Enz] µM	Internal reactor diameter [mm]	Sonication	Conversion in flow [%]	Conversion in batch [%]
1	6	0.45	NO	14	6
2	9	0.45	NO	26	11
3	12	0.45	NO	42	15
4	12	0.45	YES	56	
					30
5	12	0.8	YES	27	

Table 10. Flow and batch experiments with DCS performed with and without sonication in 0.8 and 0.45 mm internal flow reactor diameter (30 min).

These experiments outline that incubations with DCS benefit from incubation in a flow system, even without sonication. Nevertheless, the best results were obtained with sonication achieving double conversion in comparison with batch experiments.

3.5.8 Conclusion

The conversions obtained with both AS and DCS were increased in both cases over a 100% by performing reactions in flow reactor under sonication, but using different tubing diameter for each case. These results demonstrate that this methodology can be applied to other sesquiterpene synthesis. However, an optimisation of the conditions used in each case needs to be performed.

3.5.9 Enzyme recycling study

The vortex process used to extract the enzymatic products from batch experiments, results in enzyme inactivation. However, continuous extraction in a flow system is performed in milder mechanical conditions that can keep the enzyme active after the incubation period. In order to test this possibility, the aqueous phase was recovered for a previous run with AS (61%) and assayed twice for residual activity by exposure to additional FDP (1 eq) under identical flow conditions. The conversion dropped to 16% (2nd cycle) and 6% (3rd cycle).

These experiments show that the enzyme is losing activity in each catalytic cycle and after the third use, the enzyme is almost inactive.

3.5.10 Production of unnatural terpenes

Metabolic engineering and synthetic biology protocols have recently shown innovative and more sustainable large-scale production of valuable terpenes. However, these approaches cannot yet be used for the generation of modified-FDP and, therefore, for the production of unnatural terpenoids.[264]

In vitro experiments have shown that some unnatural FDP analogues acted as substrates of terpene synthases, generating a range of unnatural terpenes (Chapter 4). Three alkyl analogues of FDP (12Me-FDP (**136**), 14Me-FDP (**137**), and 15Me-FDP (**138**)) were used under flow conditions generating a set of potentially bioactive unnatural compounds. By using the reaction conditions identified above, DCS and AS were used to transform these three FDP analogues into their corresponding sesquiterpene derivatives.

The incubation of AS in flow with analogues showed a clear improvement of the reactions performed in flow in comparison with the corresponding reactions in batch (Table 11, entry 1-3), achieving 1.6 to 2.9 fold higher conversion. In addition, two sesquiterpene derivatives that has been characterised in Chapter 4 were obtained: 14Me-germacrene A (**287**) and 15Me-aristolochene (**288**) (Figure 26, Table 11, entries 2-3, see Chapter 4, Section 4.2.3 and 4.2.5 respectively for their characterisation).

14Me-gemacrene A
287

15Me-aristolochene
288
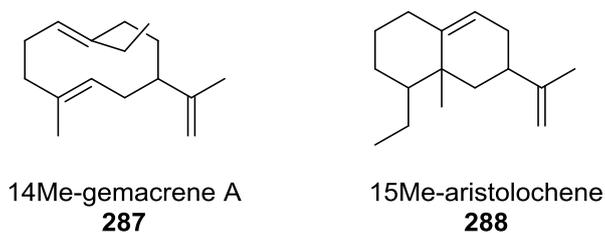
Figure 26. Structures of products obtained from the incubation of 14Me-FDP (**137**) and 15MeFDP (**288**) with AS.

The incubation of DCS in flow also shows an improvement in the conversion, in this case, between 3.5 to 1.5 times higher than the analogous reaction performed in batch (Table 11, entry 4-6).

Entry	Enzyme	Analogue	Product	Conversion (batch)	Conversion (flow)
1	AS	12Me-FDP (**136**)	Mixture	22	63
2	AS	14Me-FDP (**137**)	14Me-germacrene A (**287**)	6	11
3	AS	15Me-FDP (**138**)	15Me-aristolochene (**288**)	9	15
4	DCS	12Me-FDP (**136**)	Mixture	2.5	5
5	DCS	14Me-FDP (**137**)	Unknown	9	14
6	DCS	15Me-FDP (**138**)	Mixture	1	3.5

Table 11. Result from the incubation FDP analogues and AS or DCS. AS was incubated with FDP analogues in a 0.45 mm internal reactor diameter under sonication. DCS was incubated with FDP analogues in a 0.8 mm internal reactor diameter under sonication.

The results show that the methodology is also applicable to the conversion of FDP analogues into aristolochene and δ-cadinene derivatives. The difficulty with such FDP analogues in batch reactions is largely overcome given that the reactions are about two to three times more efficient when performed in flow.

3.5.11   Conclusion

These experiments demonstrate that the use of a biphasic continuous flow system increases the enzymatic conversion of terpene synthases through the improved extraction of the hydrophobic cyclic reaction products. This effect is not limited to a specific enzyme and is especially useful when the overall conversion is low, as in the case of substrate analogues. In general, all the enzymes examinated in this chapter performing the incubations in a flow reactor improved conversion at least 100%, in comparison with the reactions performed in batch. In the future, efforts can be focused in combined flow reactors with immobilised enzymes to improve enzyme stability to allow several catalytic cycles.

CHAPTER 4

Enzymatic production and NMR characterisation of unnatural sesquiterpenoids

4. Introduction

The FDP analogues used in this study can be classified into four classes: alkyl substituted, frame shifted, fluorinated analogues and hydroxyl substituted analogues. All farnesyl diphosphate (FDP) analogues described in Chapter 2 and some other previously synthesised within our group were tested with (+)-germacrene A (GAS), aristolochene (AS), (-)-germacrene D (GDS) and δ-cadinene (DCS) synthases.

FDP analogues with alkyl modifications of the universal C15 farnesyl chain such as 12Me-FDP (**136**), 14Me-FDP (**137**), 15Me-FDP (**138**) or 14,15diMe-FDP (**139**) have been prepared before and shown to be substrates of protein-farnesyltransferases.[265] However, to the best of our knowledge, these CH_3-alkylated variants had never been tested with terpene cyclases prior to this study.

Frame-shifted FDP analogues are generally defined as those possessing either one extra or one less [eg. 8nor-FDP (**140**)] CH_2 units (or spacer) between the double bonds of native FDP. The removal of one spacer will modify the interaction of the FDPs with the enzyme, likely changing the interactions between the key amino acids of the active site and the analogue. These interactions are responsible for the stabilisation of reaction intermediates. Previous studies have demonstrated that some of these analogues are substrates of mammalian FTase[266] but no report has ever been published describing reactions with terpene cyclases.

Historically, fluorinated FDP analogues have been used in mechanistic investigations of sesquiterpene synthases because the electronegativity of the fluorine atom can interfere with the carbocationic intermediates, whereas changes in the binding to the active site of the enzyme are minimised due to the small size of the fluorine atom.[267] More often, fluorinated analogues have been used as inhibitors of these enzymes, but in some instances, they have been shown to act as substrates surrogates of terpene synthases[268] producing valuable novel fluoro-containing terpenoids. The fluorinated FDP analogues chosen for the present study were 6F-FDP (**289**) (analogue provided by Dr. D. J. Miller), 10F-FDP (**290**) (10F-farnesol provided by Dr. J. A. Faraldos) and 14F-FDP (**291**) (analogue provided by Dr. V. Gonzalez).

Hydroxyl FDP analogues have not been tested with terpene synthases. The electron π-donating properties of the hydroxyl group are expected to stabilise α-cationic species, however the strong electronegativity of oxygen can destabilised positively charged

intermediates place at β-positions. Hydroxyl group can quench the intermediate carbocation and consequently, this can result in the formation of either a ketone or an aldehyde. Therefore, these novel mechanistic probes are expected to act as carbocationic traps. To investigate this possibility, 8OH-FDP (**141**) was prepared (Dr. J. A. Faraldos) and tested with (+)-δ-cadinene synthase.
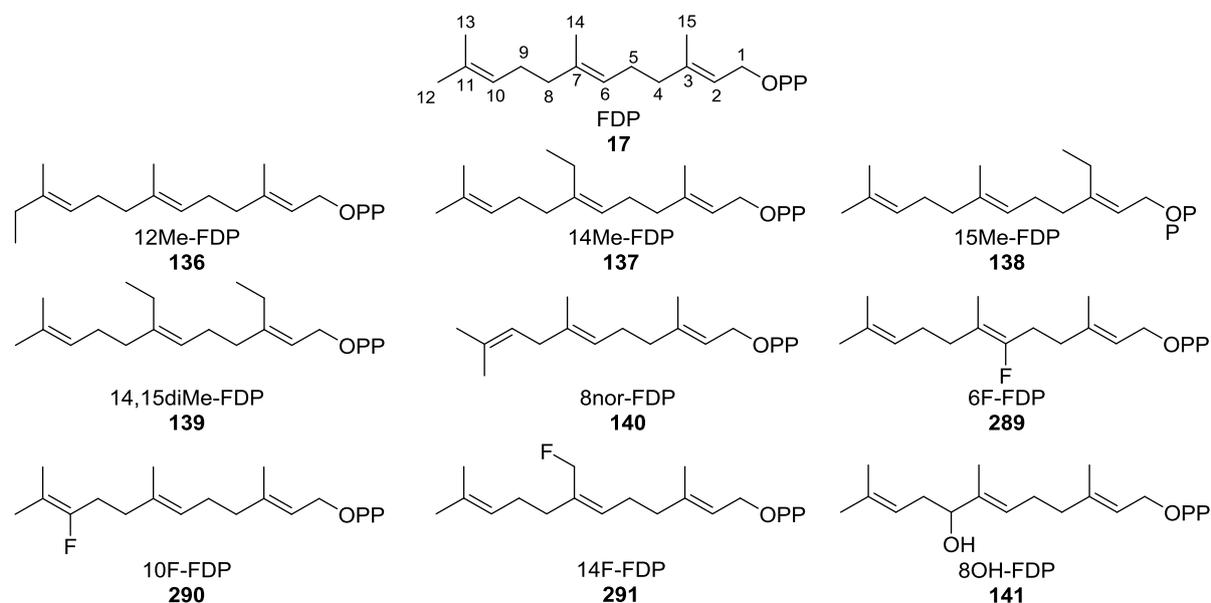
Scheme 78. FDP analogues used in this study.

4.1 (+)-**Germacrene A synthase from** *Solidago canadensis*

4.1.1 Incubation of FDP (17) with GAS

The incubation of FDP (**17**) with GAS gave one major pentane extractable product as judged by GC-MS analysis. The GC chromatogram displays a broad peak at lower retention time (Figure 27). The signal has been noticed before for all-*trans* configured germacrenoids and it is believed to represent the thermally-induced Cope rearrangement of (+)-germacrene A to the corresponding β-elemene (Chapter 1, Scheme 10) isomers, hence suggesting a (+)-germacrene A analogue. Its mass spectrum displayed a peak at *m/z* = 162, which could indicate the loss of an isopropenyl group as reported for the parent (+)-germacrene A.[269]
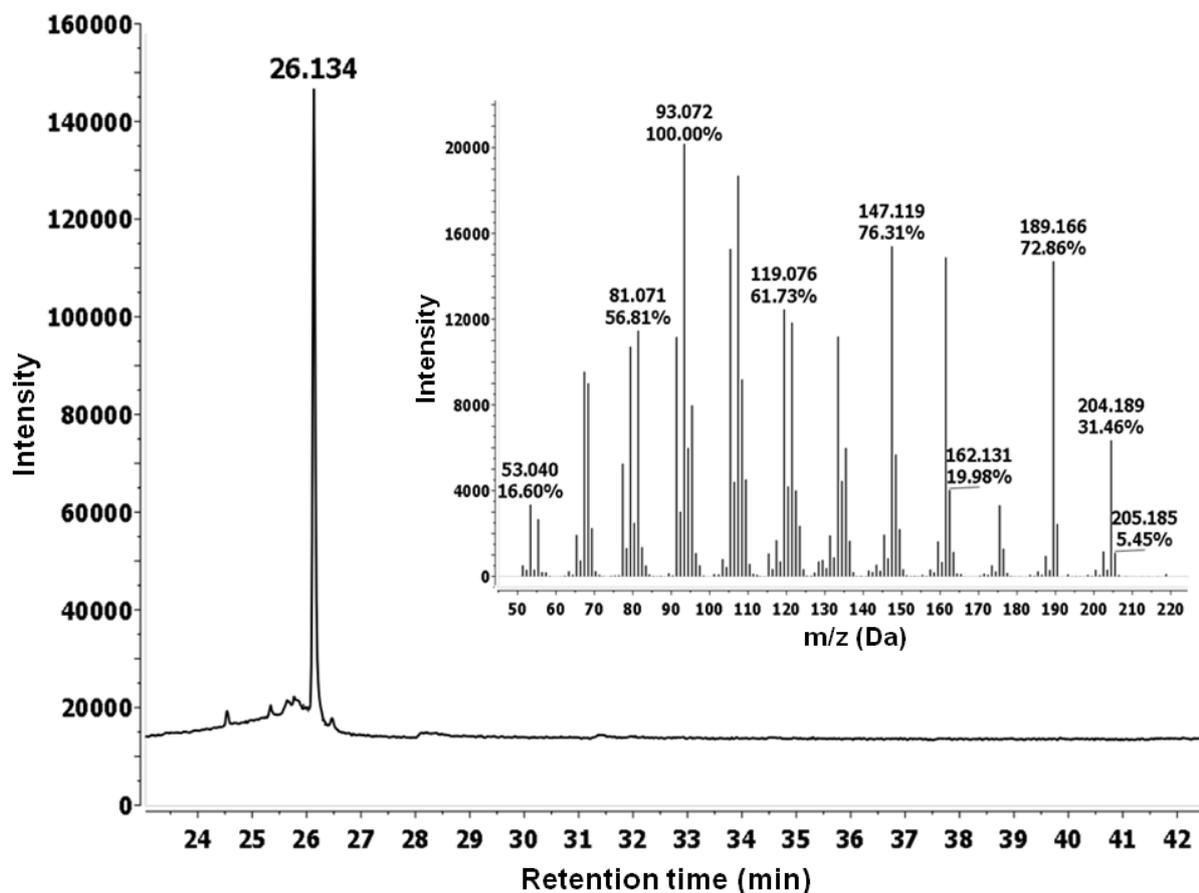
Figure 27. Gas chromatogram of the pentane extractable products obtained from incubation of FDP (**17**) and GAS. Inset, mass spectrum (+)-germacrene A (**50**) eluting at 25.134 min.

The Cope rearrangement of (+)-germacrene A (**50**) into (-)-β-elemene (**51**) is a [3,3] sigmatropic reaction that takes place with retention of configuration at C10 (Chapter 1, Scheme 10).[270] The Cope rearrangement has been used for the identification of (+)-germacrene A (**50**). The simple increase of the temperature injection port of the GC-MS is enough to increase the amount of β-elemene (**51**) (lower t_r) and consequently decrease of the amount of (+)-germacrene A (**50**).[271]

4.1.2   Incubation of 14Me-FDP (137) with GAS

The GC-MS analysis of the pentane extractable products obtained from the incubation of 14Me-FDP (**137**) with GAS revealed the presence of one major product, which GC trace displays the characteristic broad peak previously observed for (+)-germacrene A (Figure

28A). The presence of the broad peak suggests that the product obtained is a (+)-germacrene A derivative containing an extra methyl group on C14 (**292**, Scheme 79). Confirmation of the identity of the compound was obtained by increasing the temperature of the injector port to 250 ℃. The new chromatogram obtained shows that the peak previously centred at 28.5 min has shifted to 25.7 min. This observation is in good agreement with the formation of the 14Me-β-elemene (**297**, Scheme 79) (Figure 28B).
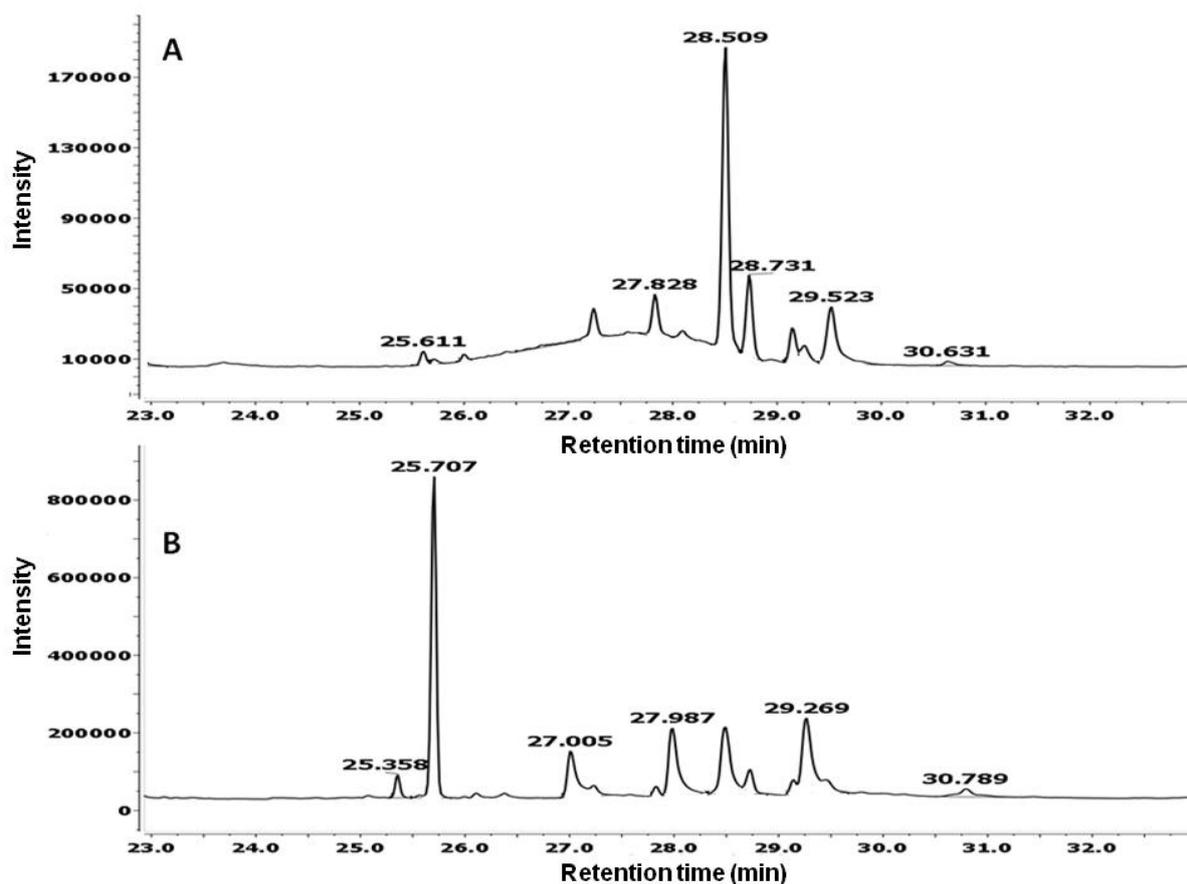
Figure 28. A) Gas chromatogram of the pentane extractable products obtained from incubation of 14Me-FDP (**137**) and GAS using an injector port temperature of 50 ℃ B) Gas chromatogram of the pentane extractable products obtained from incubation of 14Me-FDP (**137**) and GAS using an injector port temperature of 250 ℃

In addition, the chromatogram shows small peaks that persist when the Cope rearrangement is induced by increasing the temperature injection port of the GC-MS. The peaks are attributed to the presence of acid in the GC column coming from previous samples. The acid

transformation of (+)-germacrene A into bicyclic selenines (**294**, **295** and **296**) has been previously observed (Scheme 79).[272]
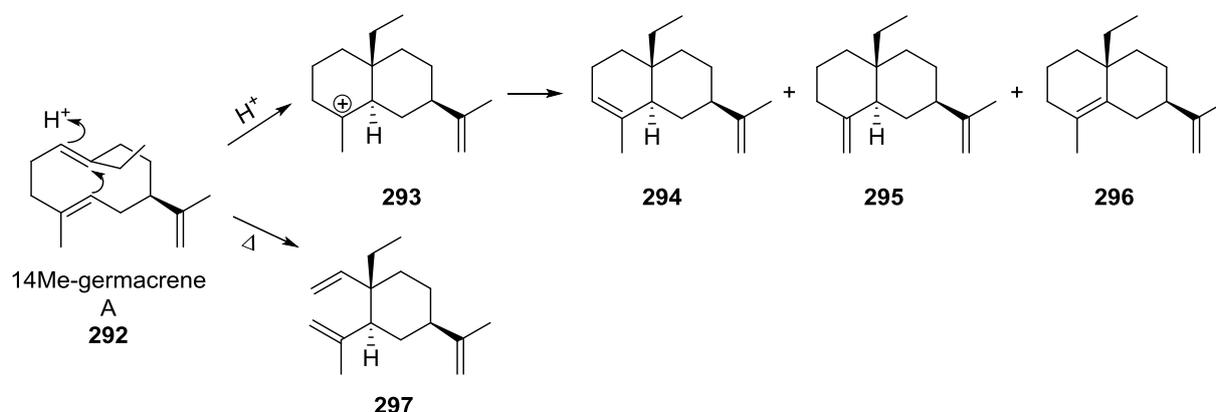
Scheme 79. Acid catalysed formation of selenines and thermal rearrangement of 14Me-germacrene A.

Variable temperature ^{1}H NMR spectroscopy (from RT to -50 °C) with this new product revealed the temperature dependent ^{1}H NMR spectrum shape that is characteristic of the interconversion of different conformers of germacrene A. This evidence confirms that the obtained product from the incubation of 14Me-FDP with GAS is 14Me-germacrene A (**292**). For a more detailed interpretation of ^{1}H NMR spectrum see section 6.19, table 15. Additionally, the spectrum mass spectrum obtained of **292** resembles mass spectrum obtained for the parent (+)-germacrene A (**50**, Figure 27).

4.1.3   Incubation of 15Me-FDP (138) with GAS

In contrast to 14Me-FDP (**137**), 15Me-FDP (**138**) was inactive with GAS as judged by the absence of products in the pentane extracts. The extra methyl group in this case seems to prevent the initial 1,10-cyclisation normally mediated by GAS. Hence, this observation suggests the formation of nerolidyl diphosphate (NDP, **95**) during the native cyclisation with FDP (Chapter 1, Scheme 20). This isomerisation is impeded in the in the case of 15Me-FDP (**138**) due to the size of the extra methyl group (Scheme 80).
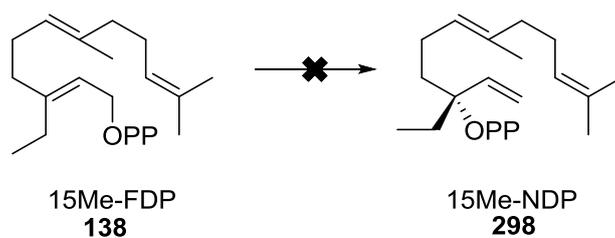
Scheme 80. Isomerisation of 15Me-FDP (**138**) to 15Me-NDP (**298**).

4.1.4 Incubation of 10F-FDP (290) with GAS

The fluorine substituent in 10F-FDP (**290**) should prevent the 1,10-cyclisation of GAS, due to the relatively reduced electron density at C10 caused by F-substitution. In addition, the destabilising effect of the fluorine atom should suppress the formation of the germacrenyl cation **301** and hence behave as an inhibitor for GAS. However, GC-MS analysis revealed the presence of a product that display a molecular ion with $m/z = 222$ eluting at 25.6 min, which appears to confirm the unexpected formation of a fluoro-containing sesquiterpenoid.
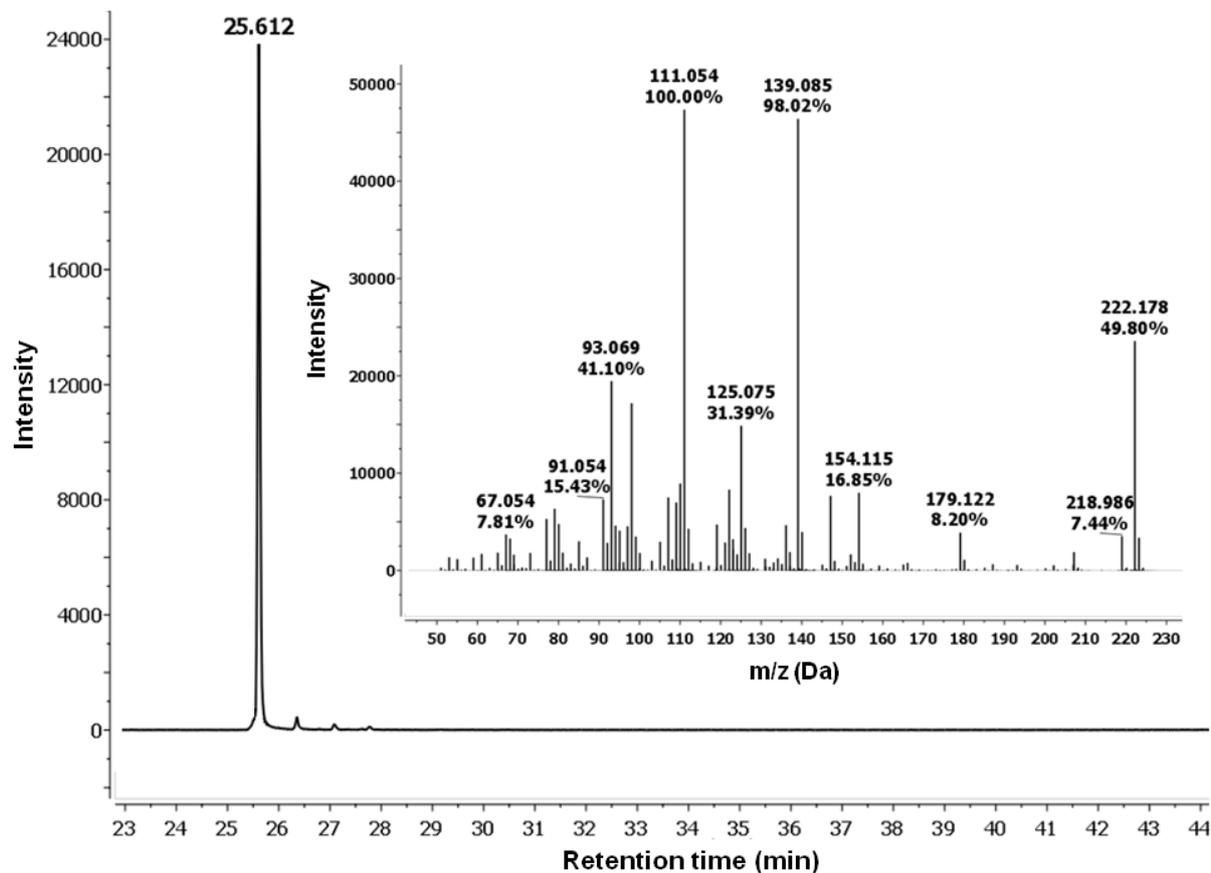
Figure 29. Gas chromatogram of the pentane extractable products obtained from incubation of 10F-FDP (**290**) and GAS. Inset, mass spectrum of the compound eluting at 25.612 min.

Fluorinated germacrene A (**302**, Scheme 81) obtained from a 1,10-cyclisation is expected to undergo thermally induced Cope rearrangement to obtain the corresponding fluorinated, β-elemene. However, sesquiterpenoid obtained failed to undergo facile thermal Cope rearrangement, thus ruling out unambiguously a fluoro-germacrene as the putative enzymatic product.

Other possible products are the linear farnesenes obtained after the elimination of diphosphate. Co-elution and spiking experiments using GC-MS with an authentic sample of a mixture of linear 10F-farnesenes (**303**, **304** and **305** Scheme 81)[273] did not show any similarity in the retention time nor mass spectrum with the obtained sesquiterpene. Farnesene products were ruled out thus indicating that the new sesquiterpene was most likely cyclic.

GAS is known to produce small amounts of α-humulene (2%) with the native substrate FDP.[274] The new fluorinated sesquiterpene obtained was most likely a 1,11-cyclisied humulene product. The reaction is driven by the change in the reactivity of the double bond that favour this 1,11-cyclisation pathway by fluorine donation of π-electrons to the distal 10,11-double bond of FDP (Scheme 81).
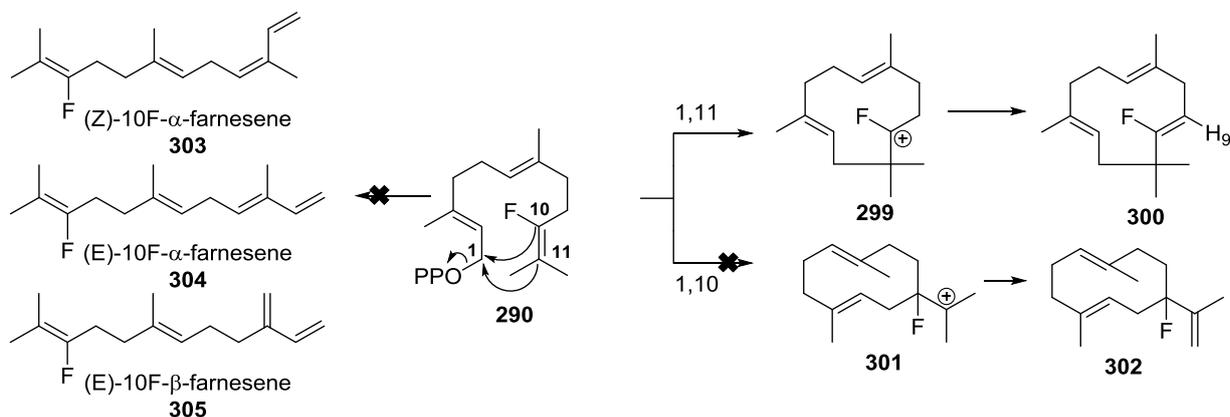
Scheme 81. Reaction mechanism accounting for the formation of 10F-α-humulene (**300**), and excluding formation of other possible products.

Alternatively, it has been proposed in a mechanistic study recently published by Faraldos *et al.* that GAS can transform FDP into (+)-germacrene A through a cyclopropyl carbocationic intermediate.[75] In this alternative mechanism, the formation of 10F-α-humelene (**300**) is explained by the formation of the intermediate **307**. In this scenario, path a is very slow because the energetic barrier for the formation of **301** is high whereas path b is more favourable because the lonely pairs of the F can give extra stabilisation to carbocation **299** reducing the energetic barrier for this transformation (Scheme 82).
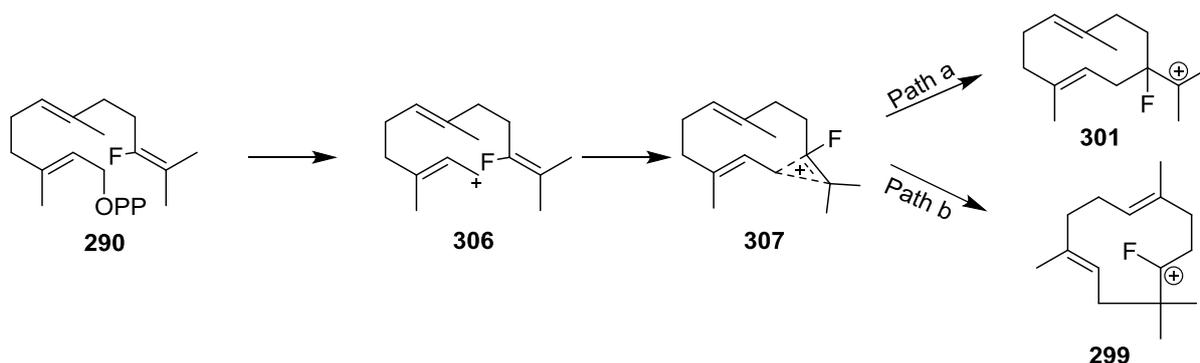
Scheme 82. Alternative mechanism for the formation of 10F-α-humulene (**300**).

^{1}H and ^{19}F NMR spectroscopy analyses supported the formation of **300** by the observation of a downfield doublet of triplets signal (J_{H-F} = 40.4 Hz and J_{H-H} = 8.1 Hz) at 4.89 ppm, which strongly resembles the characteristic doublet of triplets absorbance at 5.38 ppm (J_{H-H} = 15.8. Hz, and J_{H-H} = 7.3 Hz) assigned to the H-9 olefinic proton of α-humulene. The relative upfield H-9 shift (ca. 0.5 ppm) observed for **300** is consistent with the γ-shielding effect of the vinylic fluoro substituent. Moreover, ^{19}F NMR spectrum of the reaction product generated displayed a doublet (J_{H-F} = 41.6. Hz) centred at −109.9 ppm, which further supports the identification of this enzymatic hydrocarbon as 10F-α-humulene (**300**).[136]
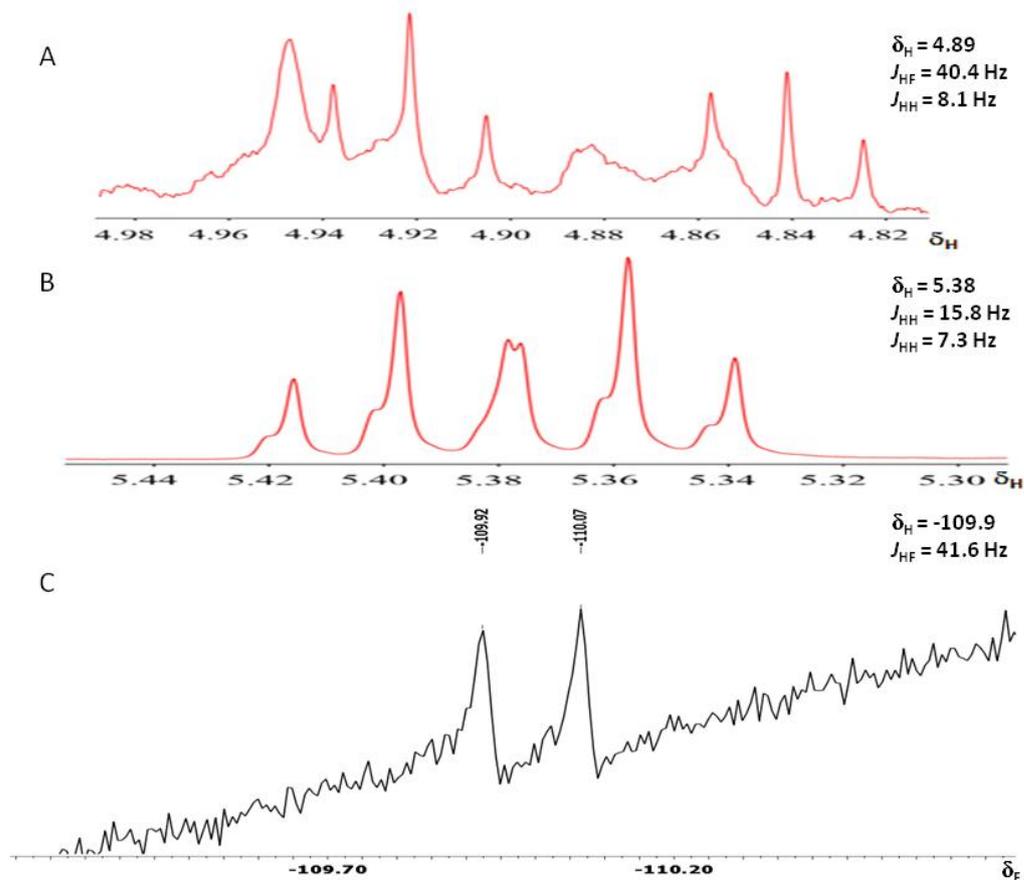
Figure 30. A) doublet of triplets of 10F-α-humulene (**300**). B) Doublet of triplets of α-humulene. C) ^{19}F NMR spectrum of 10F-α-humulene (**300**).

4.1.5 Incubation of 8nor-FDP (140) with GAS

The incubation of 8nor-FDP (**140**) gave one major compound eluting at 17.8 min (Figure 31) as judged by GC-MS. The comparison of the GC-MS trace of a non-enzymatic preparation of 8-nor-farnesene derivatives, prepared by the dehydration of the corresponding alcohol using the procedure reported by Coates,[275] rules out any of three 8-nor-farnesene derivatives (**228**, **229**, **230**) as a possible product.
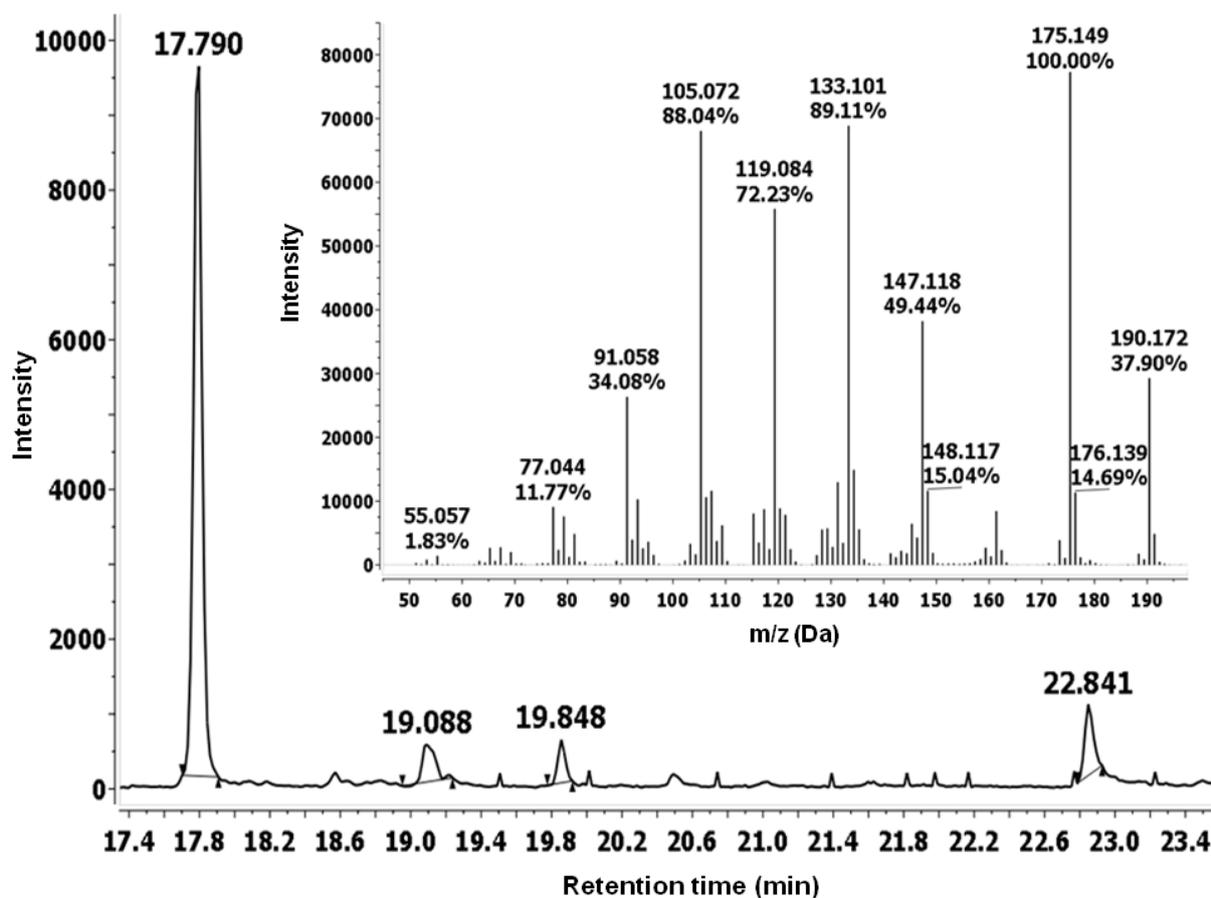
Figure 31. Gas chromatogram of the pentane extractable products obtained from incubation of 8nor-FDP (**140**) and GAS. Inset, mass spectrum of the compound eluting at 17.790 min.

8-nor-*E*-β-farnesene
228

8-nor-*E*-α-farnesene
229

8-nor-*E*-α-farnesene
230
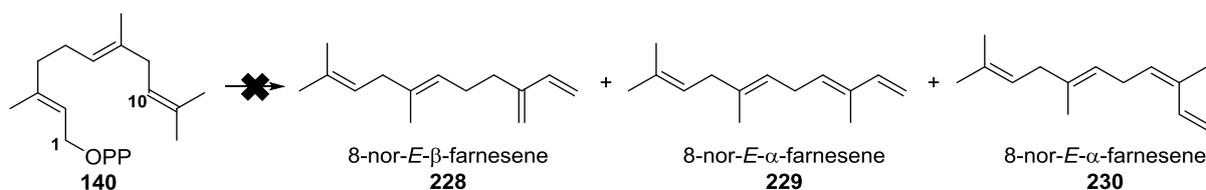
Scheme 83. Mixture of 8nor-farnesenes.

If 8nor-FDP (**140**) followed the natural reaction mechanism catalysed by GAS, it should afford a nine member ring carbocation **306**, which could then undergo either a proton removal to yield **308**, or a hydride shift from C1 to C11 to give the allylic carbocation **307** and then **309** (Scheme 84) after proton loss from C15.

Another possible scenario of the incubation with 8nor-FDP (**140**) involves an inverse attack of the distal double bond at C10 on C1 to afford **310**. The enzyme performs an inverse attack of the distal double bond with the native substrate to give a small amount of α-humelene. Stable allylic carbocation **312** is achieved form **310** by hydride shift and subsequent deprotonation yields in this case **313** resembling γ-humulene **314**. Alternatively, **311** can be formed from carbocation **310** by deprotonation (Scheme 84).
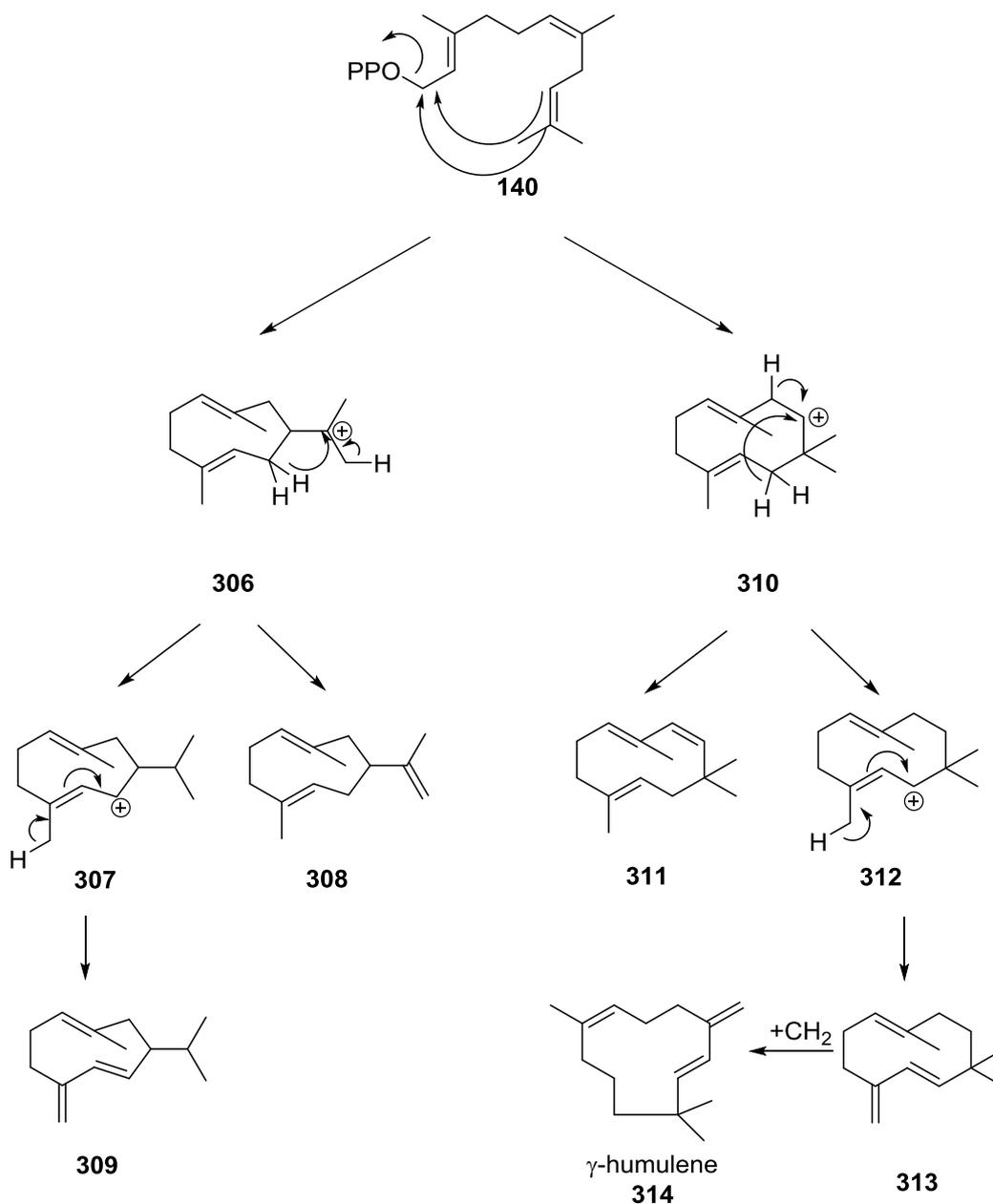
Scheme 84. Mechanisms of formation and possible products of the incubation of 8nor-FDP and GAS.

Comparison of the published mass spectrum of γ-humulene (**314**)[276] with the one obtained in the incubation of the 8nor-FDP (**140**) revealed a very similar fragmentation pattern. The overall shape of the spectrum is comparable with the one published. The mechanism proposed for the formation of **313** is very similar to the mechanism proposed for the conversion of FDP into (-)-germacrene D by GDS and remarkably GDS produce the same product as GAS when incubated with 8nor-FDP (**140**) (*vide infra*). GAS and GDS enzymes can operate using an inverse attack of the distal double bond, when incubated with some FDP analogues.[243] This evidence points to **313** as the product of the incubation but unfortunately, all the efforts to confirm the proposed structure using ^{1}H NMR spectroscopy were unsuccessful. The preparative scale incubations did not afford sufficient material for full characterisation by NMR spectroscopy. Therefore, this proposal could not be confirmed during this study.

4.1.6 Quantification of enzymatic products of GAS

In order to estimate how good each FDP analogue performs as substrate surrogates, incubations harbouring an internal standard (α-humulene) of known concentration were repeated for each FDP analogue under optimal conditions (Chapter 3). These experiments were performed in an analytical scale for 24 h and conversion was measured using a GC-FID apparatus.

As shown in Table 12, FDP analogues were in all cases worse substrates than FDP (**17**, Table 12, entry 1). 6F-FDP (**298**, Table 12, entry 2) was the substrate that displayed the highest activity and 10F-FDP (**290**, Table 12, entry 3) displayed lower substrate activity. This result explains the poor quality of the NMR spectrum obtained in the incubation of 10F-FDP. 14Me-FDP (**137**, Table 12, entry 4) has a moderate substrate activity retaining around 60% of activity compared with FDP (**17**, Table 12, entry 1).

Entry	Substrate	Conversion (%)	Conversion relative to FDP (%)[a]
1	FDP (**17**)	40	100
2	6F-FDP(**289**)[b]	30	74
3	10F-FDP (**290**)	12	29
4	14Me-FDP (**137**)	23	57
5	15Me-FDP (**138**)	-	-

a) Conversion normalised to 100% conversion with FDP. b) Product identified as 6F-germacrene A.[243]

Table 12. Conversions of FDP (**17**) and FDP analogues with GAS.

4.1.7 Conclusions

As expected, the incubation of 14Me-FDP (**137**) with GAS gave 14Me-germacrene A (**292**) as the only pentane extractable product, whereas 15Me-FDP (**138**) has no substrate activity. This observation can be explained by the formation of NDP in the mechanism of GAS. The isomerisation of 15Me-FDP (**138**) to 15Me-NDP (**298**) is prevented by the steric impediment of the methyl group on C15 (Scheme 80).

The product obtained from the incubation of 10F-FDP (**290**) was identified as 10F-α-humulene (**300**). This product is obtained from an unexpected 1,11-cyclisation and provides evidence that GAS retains a certain character of α-humulene synthase.

The same unexpected 1,11-cyclisation was proposed for the formation of the 8nor-γ-humulene (**313**) from the incubation of 8nor-FDP (**140**). This result again showed the dual ability of the enzyme to operate two different pathways (1,11-and 1,10-cyclisation).

4.2 Aristolochene synthase from *Penicillium roqueforti*

4.2.1 Incubation of FDP (17) with AS

The incubation of FDP (**17**) with AS gave one major product as judged by GC-MS, with a product peak at 25.8 min and other two minor products were observed at 26.2 and 26.5 min (Figure 32). The two minor products were previously identified as valencene and (-)-germacrene A, respectively.[277]
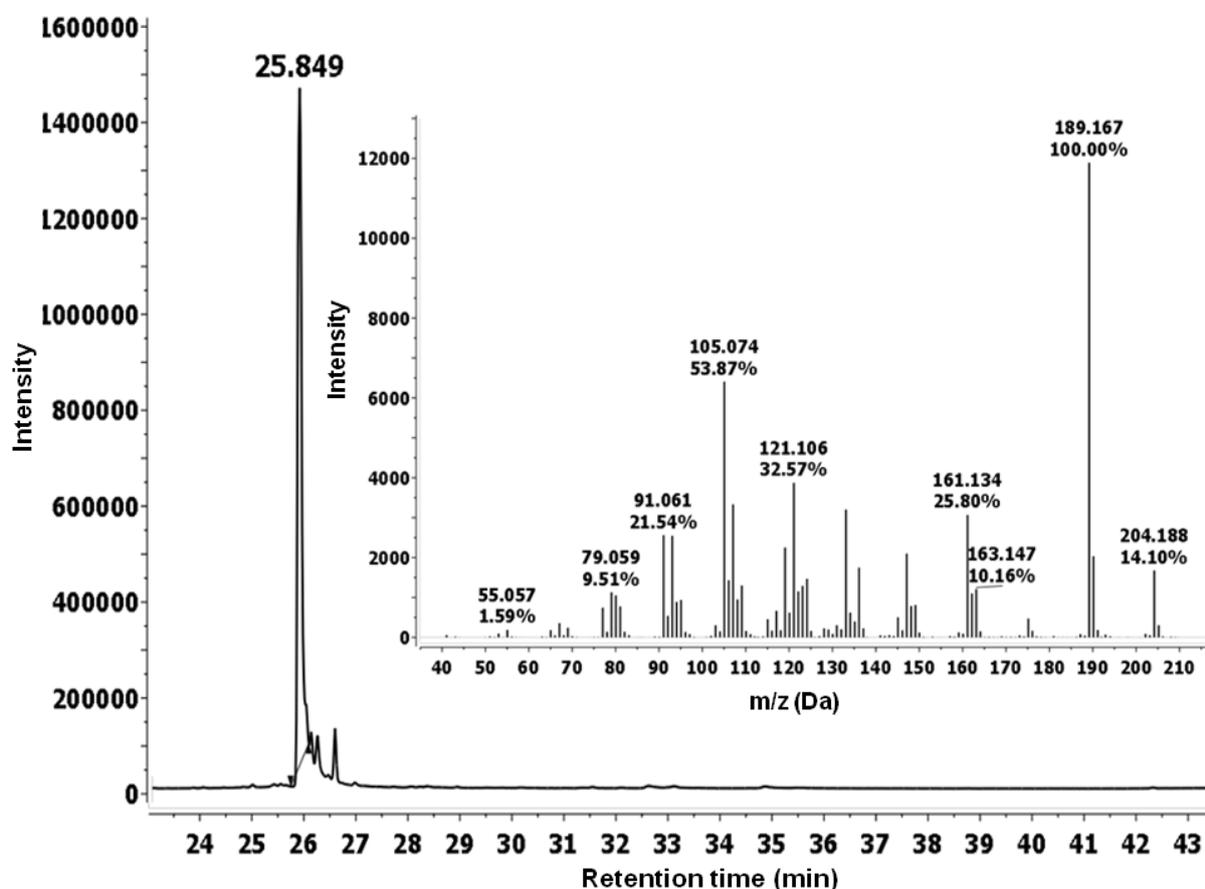
Figure 32. Gas chromatogram of the pentane extractable products obtained from incubation of FDP (**17**) and AS. Inset, mass spectrum of (+)-Aristolochene (**56**) eluting at 25.849 min.

A preparative scale incubation afforded enough aristolochene for characterisation by ^{1}H NMR and ^{13}C NMR spectroscopy. The data obtained from the ^{1}H NMR and ^{13}C NMR spectra are in agreement with the structure of the expected product (**56**, Scheme 85). The

spectroscopic data are in agreement with the NMR spectrum published for the synthetically prepared (-)-aristolochene (**56**, Scheme 13) (see section 6.20.1 for full interpretation).[278]
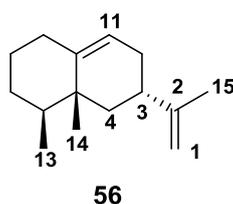
Scheme 85. (+)-Aristolochene (**56**).

4.2.2 Incubation of 12Me-FDP (136) with AS

The incubation of 12Me-FDP (**136**) with AS gave four different compounds in the pentane extracts as indicated by GC-MS analysis. By increasing the injection temperature port to 250 ºC a new peak at 19.6 min appeared while the intensity of the peaks at 29.6 and 29.4 min decreased (Figure 33). This behaviour is indicative of an all-*trans* germacrene analogue.
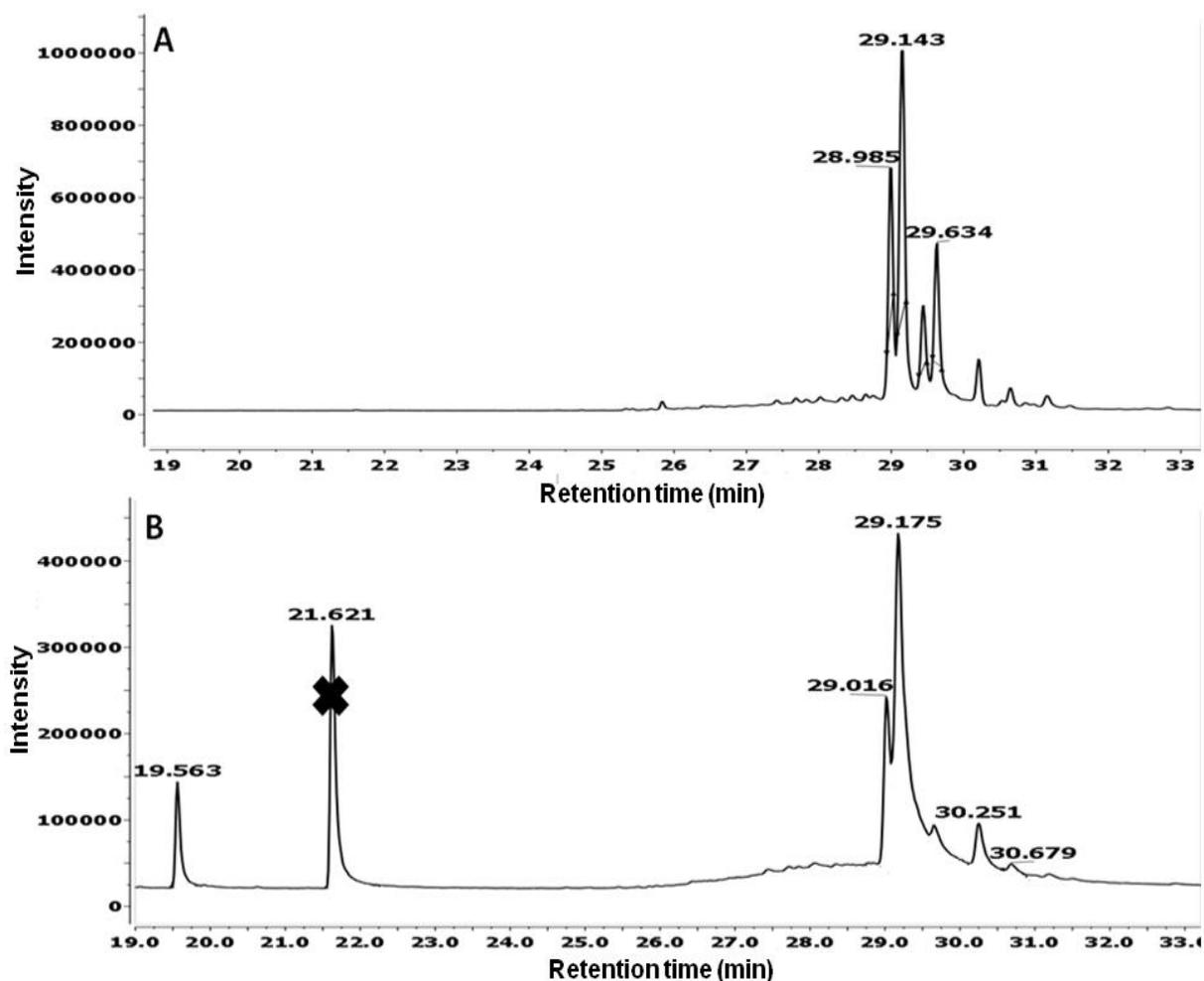
Figure 33. Gas chromatogram of the pentane extractable products obtained from incubation of 12Me-FDP (**136**) and AS using an injector port temperature of 50 ℃ B) Gas chromatogram of the pentane extractable products obtained from incubation of 12Me-FDP (**136**) and AS using an injector port temperature of 250 ℃ (peak at 21.6 min is an impurity that does not display sesquiterpene molecular ion mass).

The mass spectrum of the products eluting at 29.0 and 29.1 min display a molecular ion with $m/z = 218$. The shape and the fragmentation pattern of both compounds (Figure 34) strongly resemble the mass spectrum of aristolochene (Figure 32). The two compounds could be isomers of aristolochene achieved by deprotonation at C12 from the intermediate 12Me-germacradienyl cation (**315**). The intermediate 12Me-germacrene A (**316** or **318**) can undergo the same reaction as the natural intermediate (+)-germacrene A to afford the final mixture of products **317** and **319** (Scheme 86).
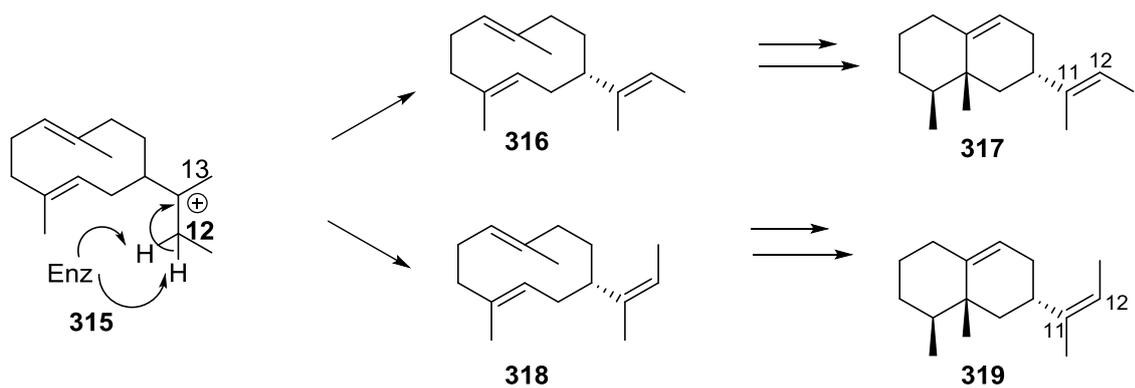
Scheme 86. Possible mechanism of product formation.

Both mass spectrums indicate formation of a fragment with $m/z = 203$ that correspond to the loss of a methyl group. The difference in abundance of this peak in each spectrum (96% in Figure 34A and 25% in Figure 34B) may be due to the different geometry of double bond between C11 and C12 of **317** and **319** (Scheme 86). Different geometry of the double bond makes methyl group of one isomer easier to fragment than the methyl group of the other isomer and therefore a difference in abundance is observed in the mass spectrum.
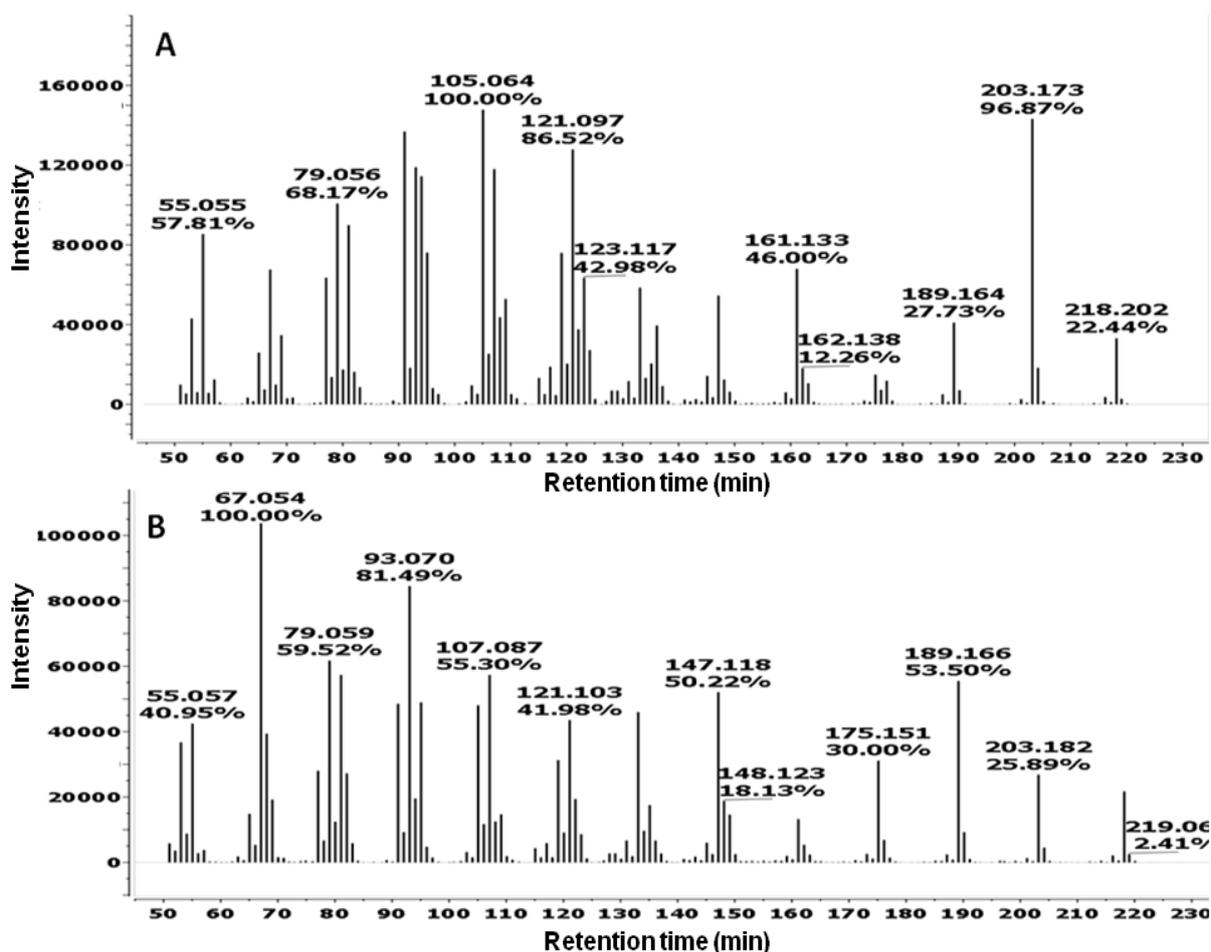
Figure 34. A) mass spectrum of the product eluting at 29.1 min from Figure 33A B) mass spectrum of the product eluting at 29.0 min from Figure 33A.

4.2.3 Incubation of 14Me-FDP (137) with AS

The incubation of 14Me-FDP (**137**) with AS gave a mixture of at least 7 different products with the expected molecular ion with $m/z = 218$ as indicated by GC-MS. This complex mixture of compounds can be produced by the acidic decomposition of 14Me-germacrene A, as shown in the case of GAS. The major compound eluting at 28.5 min has the characteristic broad peak attributed to the Cope rearrangement that occurs in the injection port (Figure 35A). Indeed, the elevation of the injection port temperature to 250 ℃ resulted in a shift to shorter retention time of the major peak, and a much clearer chromatogram (Figure 35B) was obtained. The new peak is the β-elemene derivative (Scheme 87, **321**) obtained as the product

of the Cope rearrangement: therefore, the most probable major product of the incubation of 14Me-FDP (**137**) with AS is 14Me-germacrene A (Scheme 87, **320**).

(-)-14Me-germacrene A
320

β-elemene
321
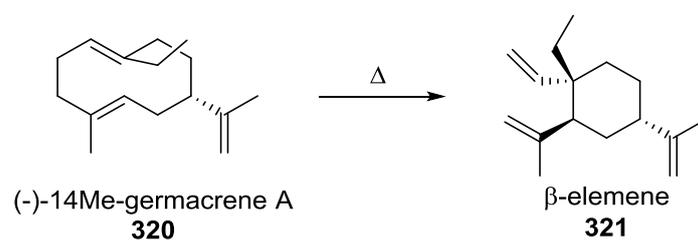
Scheme **87.** Cope rearrangement of 14Me-FDP (**137**).

Figure 35. Gas chromatogram of the pentane extractable products obtained from incubation of 14Me-FDP (**137**) and AS using an injector port temperature of 50 ℃ B) Gas chromatogram of the pentane extractable products obtained from incubation of 14Me-FDP (**137**) and AS using an injector port temperature of 250 ℃.

(*E, E*)-germacrenes are known to exist as a mixture of slowly interconverting conformers at ambient temperatures. The population of these different conformers in bulk solution has been extensively studied. For instance, the cyclodecadiene ring of hedycaryol can theoretically adopt four different conformations that are denoted as UD (**322**), DU (**323**), DD (**324**) and UU (**325**). This denomination is referred to up (U) or down (D) orientation of the methyl groups in C2 and C6 of the cyclodecadiene ring (Scheme 88). The two double bonds in the cyclodecadiene ring, in the conformations denoted as UU (**325**) and DD (**324**), have a crossed disposition between them whereas double bonds in the conformations denoted as DU (**323**) and UD (**322**) have a parallel disposition in the cyclodecadiene ring. In all the conformers, the substituent on C7 is preferable in equatorial position or pseudoequatorial.[279] Studies of hedycaryol using ^{1}H NMR spectroscopy have concluded that in solution, hedycaryol exists only as two parallel UD (**322**) and DU (**323**) and one crossed conformation UU (**325**) (scheme 88).[280]
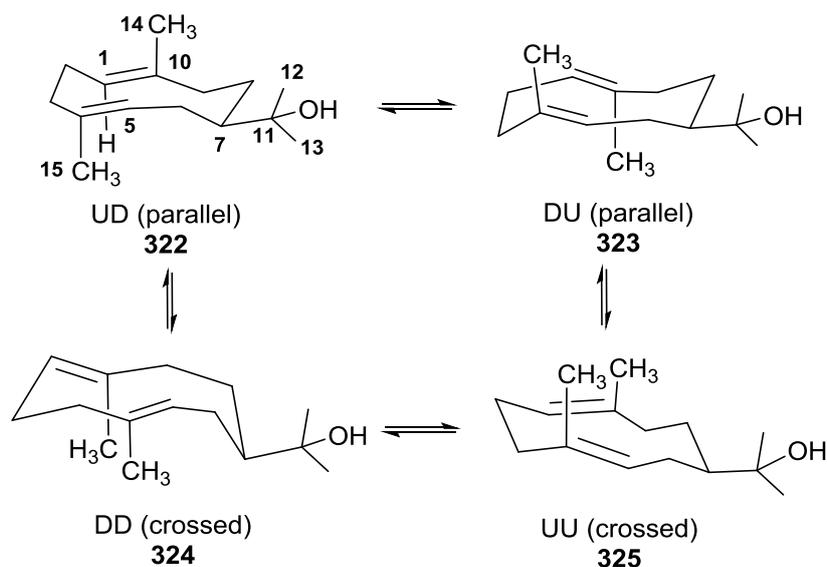
Scheme 88. Four possible conformations of hedycaryol.

In solution, the fast interconversion of the three different conformers (DU, UD and UU) of germacrene A in equilibrium gives the appearance of the ^{1}H NMR spectrum a strong temperature dependence.[281] As a result, the ^{1}H NMR spectrum signals are broad at room

temperature, but they get sharper at lower temperatures at slower interconversion rates and the different conformers can be separately characterised.[282]

Some examples are found in literature where NOE experiments in combination with variable temperature NMR spectroscopy are used to determine the most stable conformation of simple all *trans*-germacrenes such as germacrene A.[283] The relative populations of each conformer of germacrene A were difficult to determine due to the small amounts of impure germacrene A isolated from plants. Purification was also difficult due to the thermal and photo-lability of germacrene A.[284] Recently, the Coates group was able to obtain large amounts of pure (+)-germacrene A (**50**), using genetically-engineered yeast for high level production of farnesyl phosphate[285] combined with DNA encoding (+)-germacrene A (**50**) synthase. Full characterisation was performed. NOE experiments at variable temperature allowed the assignment of each ^{1}H NMR signal to the corresponding proton in each conformation. The conformer population was calculated from the integrals of the individual olefinic protons.

The conformer of (+)-germacrene A (**50**) named as UU (crossed, **328**) was the major conformation the population of this conformer accounts for 52%. The signals corresponding to the two olefinic protons were identified as a doublet of doublets at 4.78 ppm (H1) and doublet at 4.51 ppm (H5) as previously suggested for one conformer of hedycaryol and dihydropregeijerene.[286] Additionally, the two protons at C12 were assigned to singlets at 4.65 and 4.54 ppm overlapping with the same protons from the conformation **326** and **327**. The assignments of the methyl groups were established by Sathe *et al.*[287] by comparison with data obtained from deuterated dihydropregeijerenes which identified the olefinic methyl singlets of **328** at 1.37 (H14) and 1.52 (H15) ppm. In addition, the saturation of the frequency corresponding to H1 (4.78 ppm) of **328** produced a small NOE enhancement (1%) of signal at 4.51 (H5, **328**) ppm, thus, corroborating the syn relationship of the vinylic hydrogens of **328**. The saturation of this frequency was transferred to the signals at δ_H 5.01 (**326**) and 4.94 ppm (**327**) by an exchange process verifying the H1 chemical shift position of the less populated conformers **326** and **327**.

The conformer of (+)-germacrene A (**50**) named as UD (parallel, **326**), accounts for 29%. The signals corresponding to the two olefinic protons were identified as a triplet at 5.01 ppm (H1) and doublet of doublets at 5.07 ppm (H5). Additionally the two protons of double bond on C12 were assigned to singlets at 4.65 ppm, overlapping with the same protons from the conformation UU (**328**) and 4.54 ppm. NOE enhancement (3%) obtained from vinylic methyl

signals at 1.46 (H15) and 1.54 (H14) ppm when signals of vinyl protons at 5.01 (H1) and 5.07 (H5) ppm are irradiated, corroborate the anti conformation and assignation of the methyl groups

The population of the conformer of (+)-germacrene A (**50**) named as DU (parallel, **327**) accounts for 19% of the population of the three conformers. The signals corresponding to the two olefinic protons were identified as a doublet at 4.94 ppm (proton on C1) and triplet at 5.21 ppm (proton on C5). Additionally, the two protons of double bond on C12 were assigned to singlets at 4.65 ppm, overlapping with the same protons from the conformations UD (**326**) and UU (**328**), and 4.51 ppm. NOE enhancement (2%) obtained from vinylic methyl signals at 1.48 (H15) and 1.68 (H14) ppm when signal of vinyl protons at 4.94 (H1) and 5.21 (H5) ppm are irradiated, corroborate the anti conformation and assignment of the methyl groups.[288]
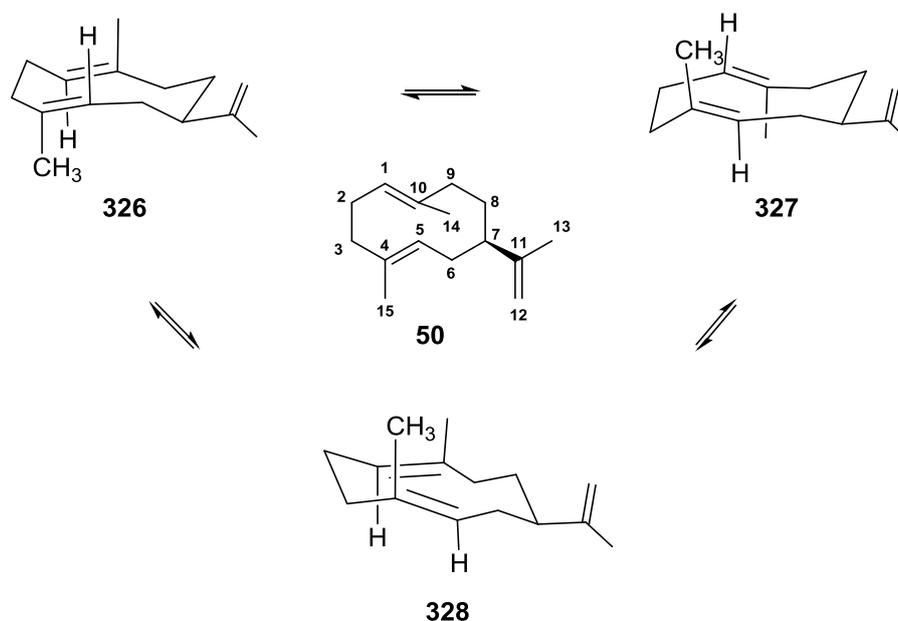
Scheme 89. Conformers of (+)-germacrene A (**50**).

The population of the corresponding conformations of 14Me-germacrene A (**320**) are different to the parent germacrene A (**50**) due to the bulky methyl group. The additional methyl group in the structure can increase the steric interactions between the pendant methyl groups, increasing the energy of the conformation that places the bulkiest groups close together, in this case the UU conformation (**320a**, Figure 36). As a result, the population of

this conformer is expected to be reduced with a consequential increase in the populations of the UD (**320b**, Figure 36) and DU (**320c**, Figure 36).

Preparative incubation of 14Me-FDP (**137**) with AS afforded 14Me-germacrene A (**320**) in sufficient quantity (6 mg) to be studied by ^{1}H NMR spectroscopy. Direct comparison of the ^{1}H NMR spectrum of **320** with that of the parent (+)-germacrene A and analysis according to Faraldos *et al.*[288] allowed the assignment of signals to each individual conformation of 14-Me germacrene A (**320**).

Although the UU conformer of 14Me-germacrene A (**320a**, Figure 36) was still dominant, its relative population dropped to 39% when compared to the 52% observed for UU conformer of the parent (+)-germacrene A. In the ^{1}H NMR spectrum, the doublet of doublets corresponding to proton on C1 is shifted upfield to 4.71 ppm. The doublet at 4.51 ppm observed for the parent (+)-germacrene A (**50**) corresponding to the proton on C5 is shifted downfield at 4.53 ppm and overlaps with the signal of the protons on C12. The signal for one of the protons on C12 is approximately constant for the three conformers at 4.64 ppm and so appears as a broad singlet.

Interestingly, the population of the UD conformer of 14Me-germacrene A (**320b**, Figure 36), accounts for 29%, as it was observed for the parent (+)-germacrene A (**50**). The triplet at 5.01 ppm for (+)-germacrene A has shifted to 4.91 ppm (proton on C1) and now overlaps with the signal of the conformation DU (**320c**, Figure 36) (proton on C1), making the signal appear as an asymmetric doublet of doublets. The doublet of doublets at 5.07 ppm, in 14Me-germacrene A (**320**), is shifted to 5.01 ppm (proton on C5). Additionally the two protons of double bond on C12 were assigned to singlets at 4.64 ppm and overlaps with the same protons from the conformations UU (**320a**, Figure 36) and DU (**320c**, Figure 36), and 4.60 ppm.

The DU conformer (**320c**, Figure 36), increased its population to 33% of the total population of conformers. The triplet at 5.21 ppm for (+)-germacrene A (**50**) is shifted to 5.22 ppm in 14Me-germacrene A (**320**) (proton on C2).

The signals for the CH_3 of the ethyl group corresponding to each conformation are three triplets overlap and are centred at 0.9 ppm (Figure 36). For a more detailed analysis of ^{1}H NMR signals, see Chapter 6, Section 6.20.2, Table 16.

As for the parent (+)-germacrene A the most abundant conformation is UU (**320a**, Figure 36), in contrast, the other two conformations DU (**320c**, Figure 36) and UD (**320b**, Figure 36) dominate over the UU (**320a**, Figure 36) conformation. The increasing bulkiness reduces the energy gaps between different conformations.
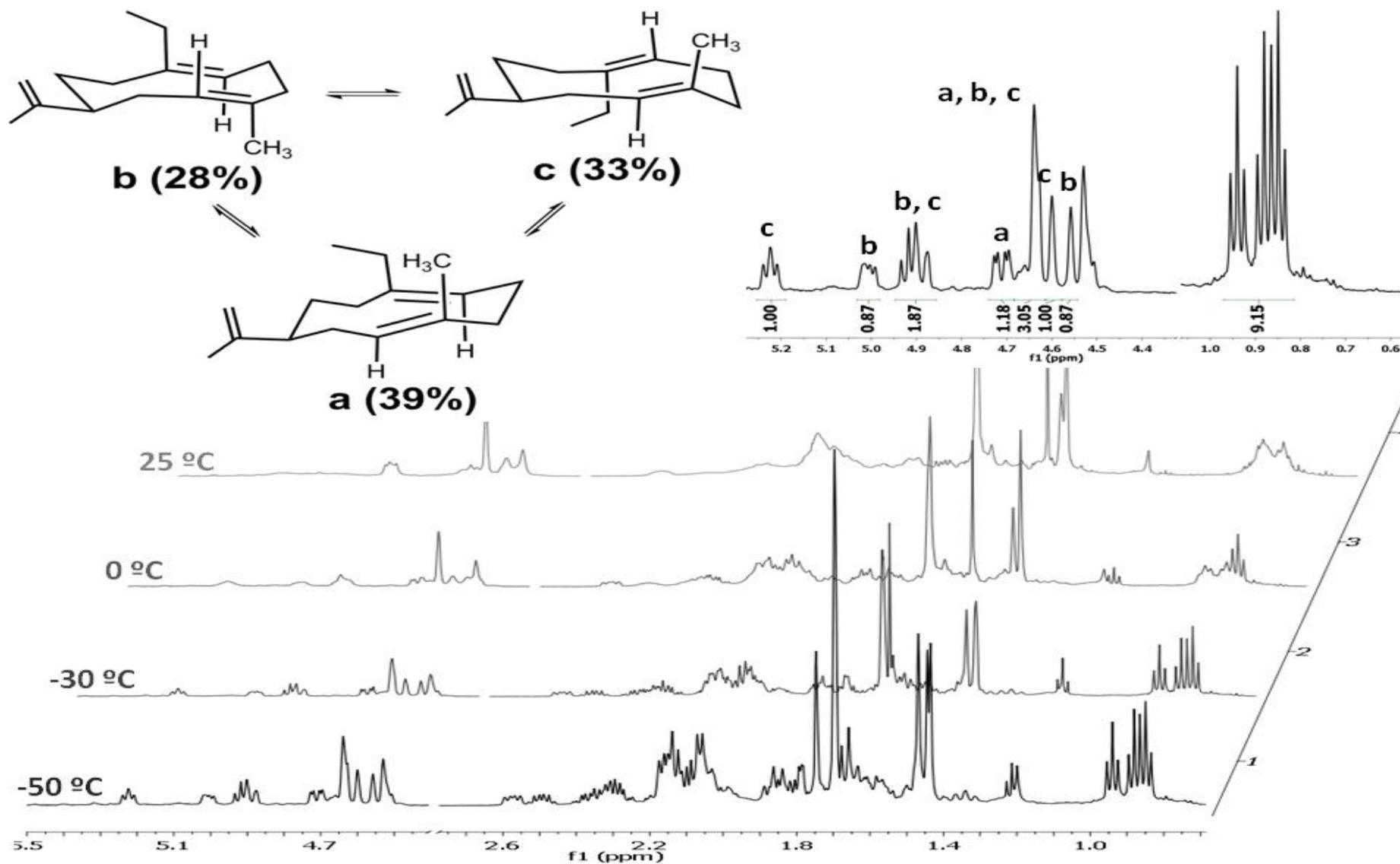
Figure 36. Variable temperature ^{1}H NMR (500 MHz, CDCl$_3$) spectra of 14Me-germacrene A (**320**) and conformational study.

4.2.4 Incubation of 14,15diMe-FDP (139) with AS

The incubation of 14,15diMe-FDP (**139**) with AS gave a complex mixture of compounds in the pentane extracts. However, the big broad peak present in the chromatogram (Figure 37A) suggested that the major product could be 14,15diMe-germacrene A (**329**, Scheme 90). Increasing the injector port temperature to 250 ℃ led to a cleaner chromatogram displaying only one major product at 26.29 min (Figure 37B). The experiment strongly implies that the compound was 14,15diMe-germacrene A (**329**) and the product of the Cope rearrangement as the β-elemene derivative **330**.

14,15diMe-germacrene A
329

14,15diMe-β-elemene
330
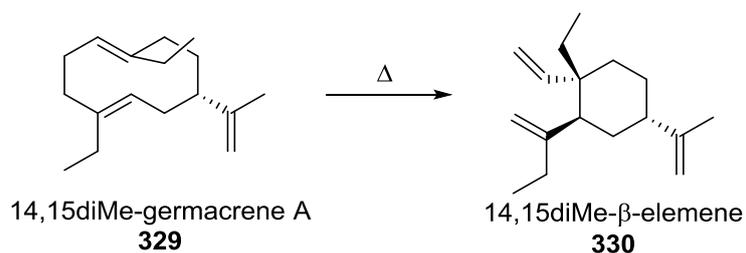
Scheme 90. Cope rearrangement of 14,15diMe-germacrene A (**329**) into **330**.
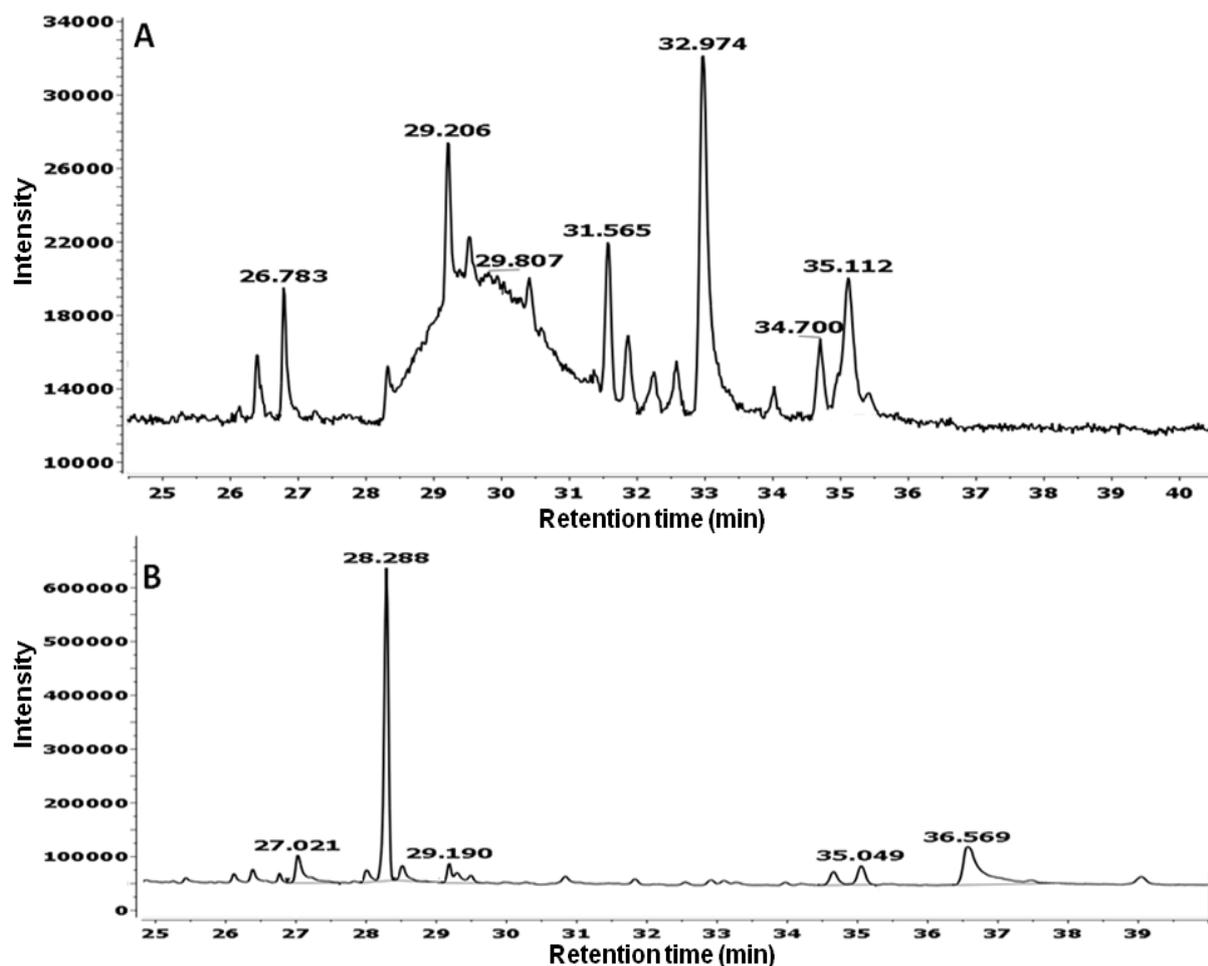
Figure 37. Gas chromatogram of the pentane extractable products obtained from incubation of 14,15diMe-FDP (**139**) and AS using an injector port temperature of 50 ℃ B) Gas chromatogram of the pentane extractable products obtained from incubation of 14,15diMe-FDP (**139**) and AS using an injector port temperature of 250 ℃.

Preparative incubations were performed in order to determine the relative population of each conformation of 14,15diMe-germacrene A (**329**) by NMR spectroscopy. The increasing bulkiness of an additional methyl group would be expected to increase the energy of the UU (**329a**, Figure 38) conformation, and, therefore, a reduction on its population in favour of the UD conformation (**329b**, Figure 38) was expected.

The ^{1}H NMR spectrum displayed the diagnostic temperature dependence of flexible germacrenes. The signal pattern was very similar to the one shown by 14Me-germacrene A

150

(**320**). The complexity of the multiplet at 0.85 ppm is indicative of the presence of the additional methyl group. Chapter 6, Section 6.20.3, Table 17.

Surprisingly, the conformational distribution was found to be identical to the conformational distribution observed for 14Methyl germacrene A (**320**, Figure 36). This result suggests that the bulkiness of the substituents on C15 had no incremental effect on the relative energy of each conformer over and above that exerted by the C14 methyl group and hence on the population of the conformations.
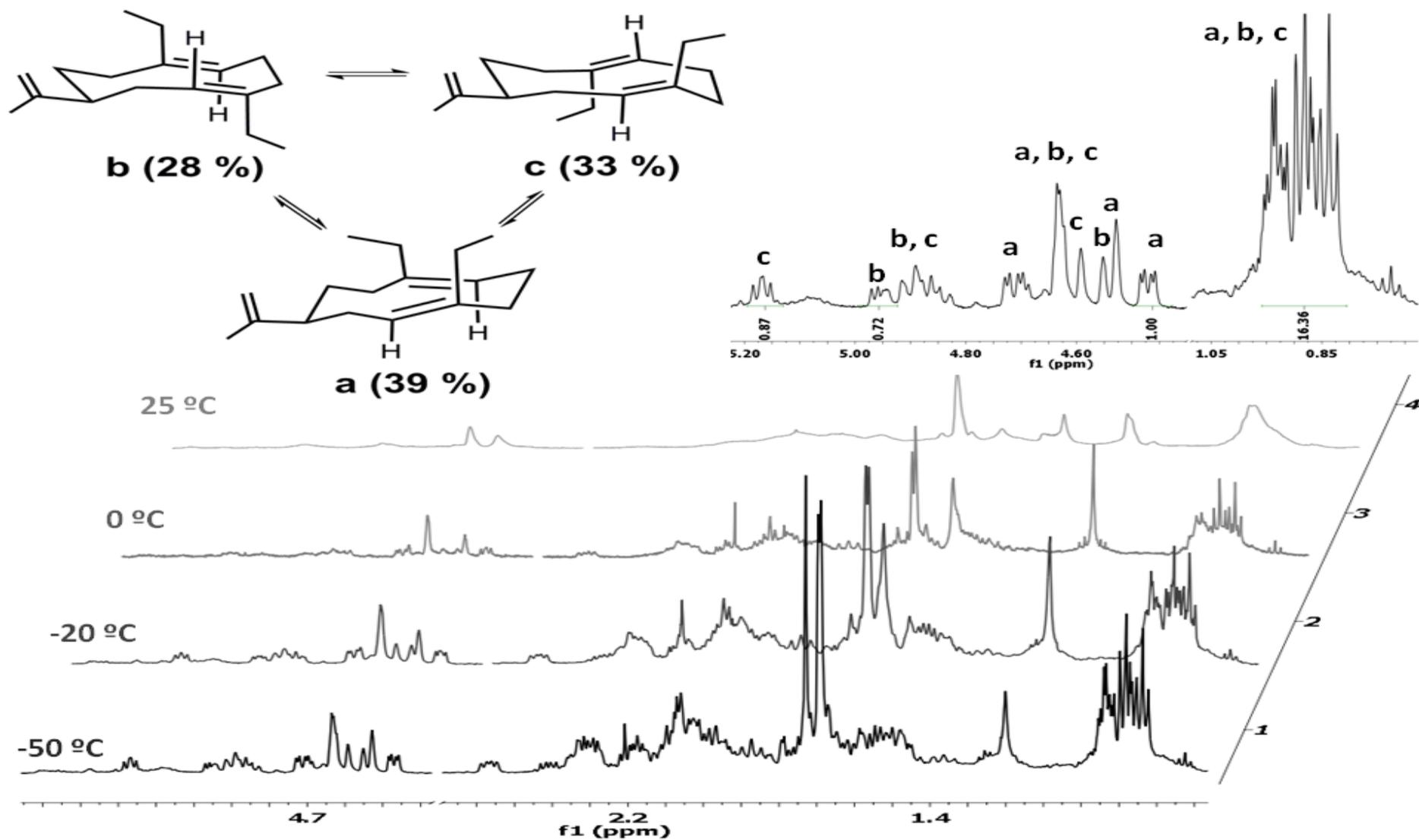
Figure 38. Variable temperature ^{1}H NMR (500 MHz, CDCl$_3$) spectra of 14,15diMe-germacrene A (**329**).

4.2.5 Incubation of 15Me-FDP (138) with AS

The incubation of 15Me-FDP (**138**) with AS gave only one compound in the pentane extracts with *m/z* = 218 eluting at 28.5 min. The remarkable similarity of its mass spectrum with aristolochene (Figure 32) strongly suggested that the product was the 15Me-aristolochene (**331**) (Figure 39).

15Me-aristolochene
331
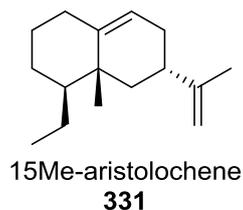
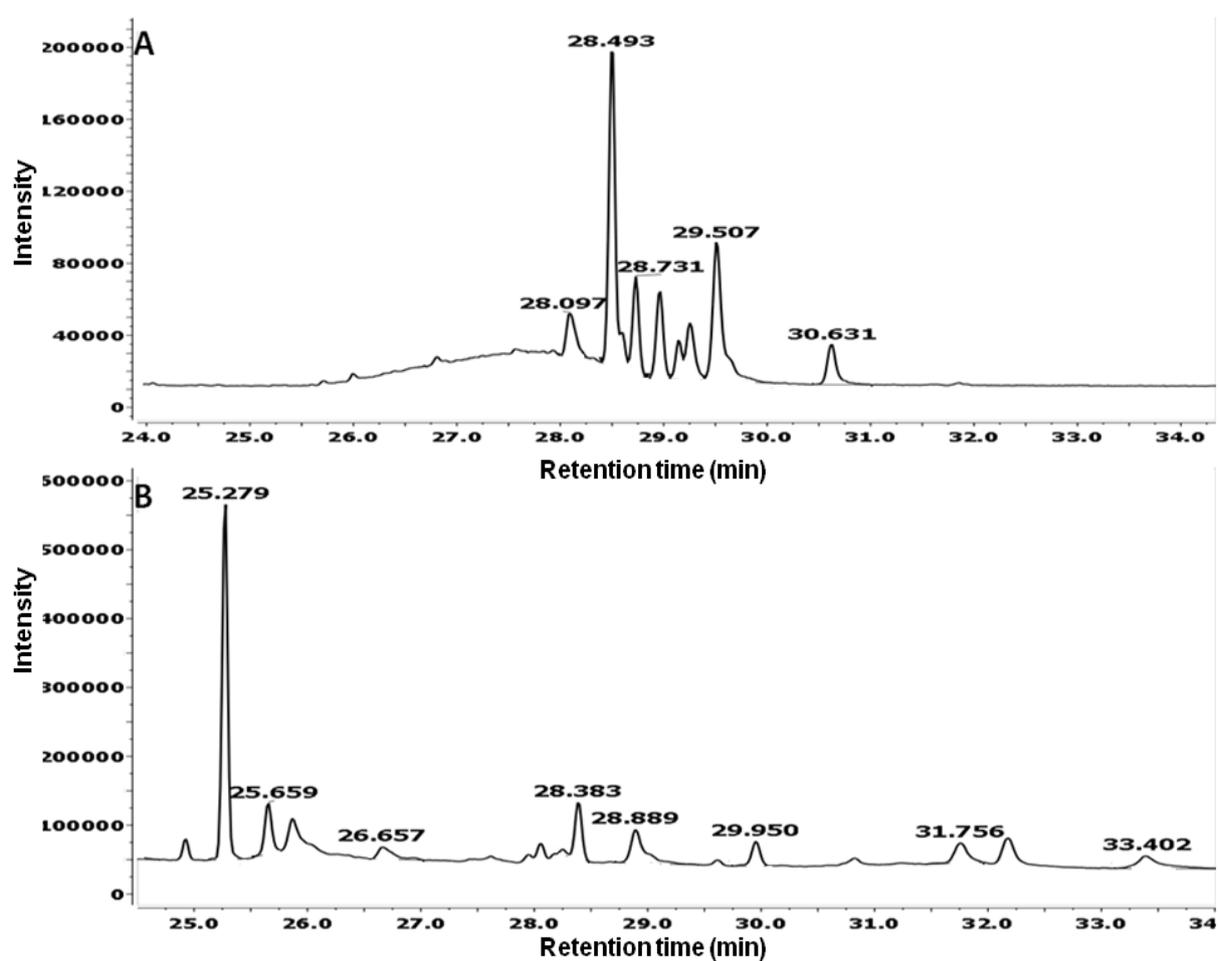**Scheme 91.** Product of the incubation of 15Me-FDP (**138**) with AS.

Figure 39. Gas chromatogram of the pentane extractable products obtained from incubation of 15Me-FDP (**138**) and AS. Inset, mass spectrum of the compound eluting at 28.493 min.

4.2.6 Incubation of 8nor-FDP (140) with AS

The incubation of 8nor-FDP (**140**) with AS gave a complex mixture of compounds in the pentane extracts as indicated by GC-MS analysis with a major peak at 21.5 min (Figure 40A). The sample was compared with an authentic sample of 8-nor-farnesene prepared by dehydration of 8-nor-farnesol (Figure 40B).[289] The comparison of the chromatograms revealed that the major product of the incubation was 8-nor-β–farnesene (Scheme 83, **228**) and the peak eluting at 22.8 min was the mixture of (*E*)-α–farnesene (Scheme 83, **229**) and (*Z*)-α–farnesene (Scheme 83, **230**) (Figure **40**).

AS was not able to produce cyclic products with 8nor-FDP (**140**). However, the diphosphate analogue appears to be bound to the active site and the ionisation of the diphosphate generates a mixture of 8-nor-farnesenes (Scheme 83).

154

Figure 40. A) Gas chromatogram of the pentane extractable products obtained from incubation of 8nor-FDP (**140**) and AS. B) Gas chromatogram of mixture of 8nor-farnesenes generated by dehydration of 8-nor-farnesol.

4.2.7 Conclusions

The incubation of 12Me-FDP (**136**) with AS gave a mixture of different geometrical isomers of 12Me-germacrene A (**316** and **318**, Scheme 86) and 12-Me-aristolochene (**317** and **319**, Scheme 86) derivatives. The complex mixture of compounds is due to the presence of geometrical isomers formed during the reaction.

The incubation of 14Me-FDP (**137**) and 14,15diMe-FDP (**139**) afforded in both cases the corresponding germacrene A derivative and remarkably, both germacrene A derivatives displayed the same conformer distribution (Figures 36 and 38). These results suggest that the increasing steric impediment has a limited impact on the population of conformers.

In contrast to GAS, AS appears not to have the ability of triggering 1,11-cyclisations, judging by the incubation of 8nor-FDP (**140**) with AS that resulted in a mixture of 8-nor-farnesenes.

4.3 **(-)-Germacrene D synthase from** *Solidago canadensis*

4.3.1   Incubation of FDP (17) with GDS

The incubation of (-)-germacrene D synthase with FDP gave, as expected a single, product eluting at 25.77 min that has a molecular ion with m/z = 204. The major fragment at m/z = 161 corresponds to a loss of 43 units of mass, which corresponds with the loss of an isopropyl group (Figure 41). Previously published mass spectral data for (-)-germacrene D (**78**) were found to be identical to those obtained in this case.[290]
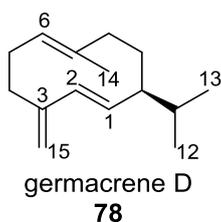
germacrene D
78

Scheme 92. Structure of **(-)-**germacrene D (**78**).
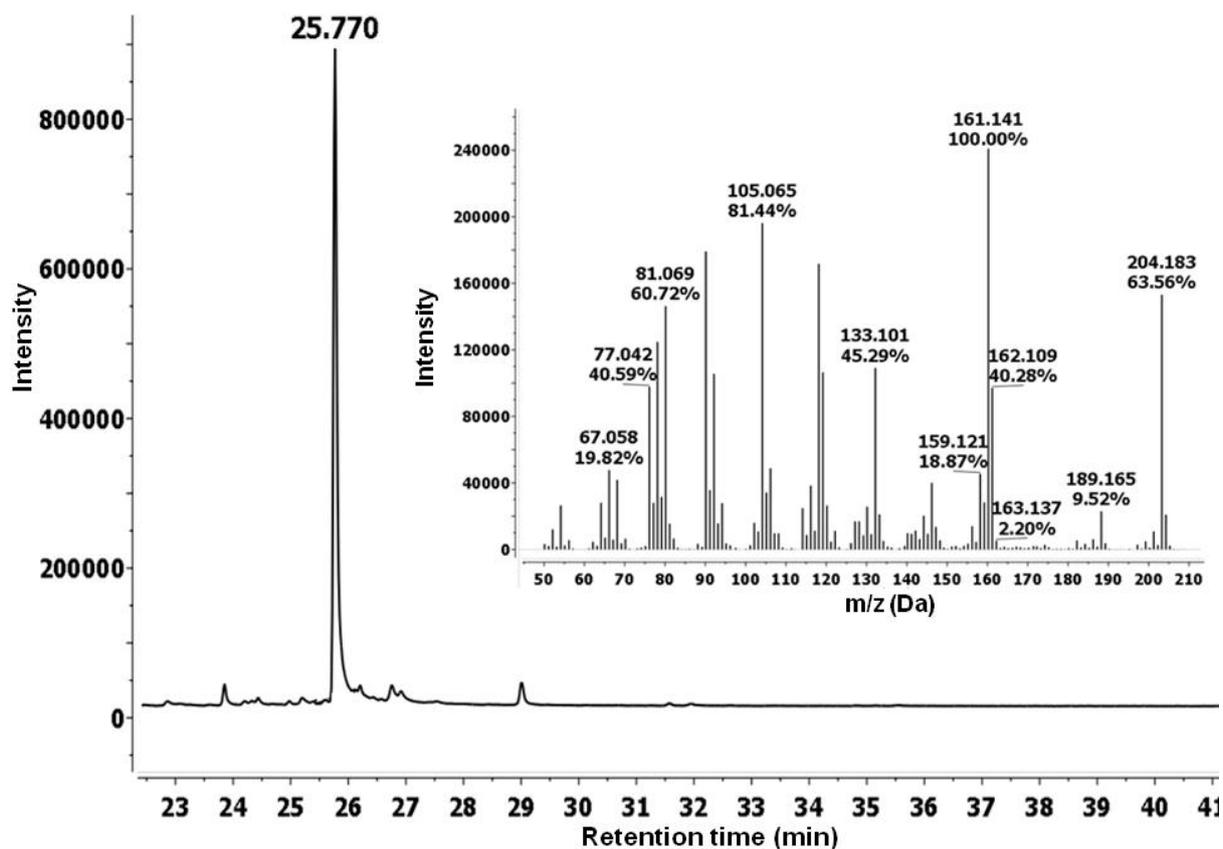
Figure 41. Gas chromatogram of the pentane extractable products obtained from incubation of FDP (**17**) and GDS. Inset, mass spectrum of (-)-germacrene D (**78**) eluting at 25.770 min.

For characterisation purposes, a preparative incubation was performed using optimised conditions (Chapter 3). The sample obtained from FDP (**17**) was sufficient (1.7 mg) to allow full characterisation. The ^{1}H NMR spectrum displays characteristic signals that agree with previously published NMR spectroscopy data for (-)-germacrene D.[291] The doublet at 5.78 ppm is assigned to the protons H_b (Figure 42). The coupling constant of the doublet at 5.78 (*J* = 16 Hz) matches the minor coupling constant of the doublet of doublets centred at 5.25 ppm which supports the assignation of the signal to H_c (Figure 42). The two remaining doublet of doublets were assigned through their integration: therefore, the doublet of doublets centred at 5.13 ppm is assigned to H_a (Figure 42) and 4.76 ppm is assigned to H_d (Figure 42). Another pair of characteristic signals are the two upfield doublets at 0.86 and 0.81 ppm corresponding to the methyl groups from the pendant isopropyl group (Figure 42).
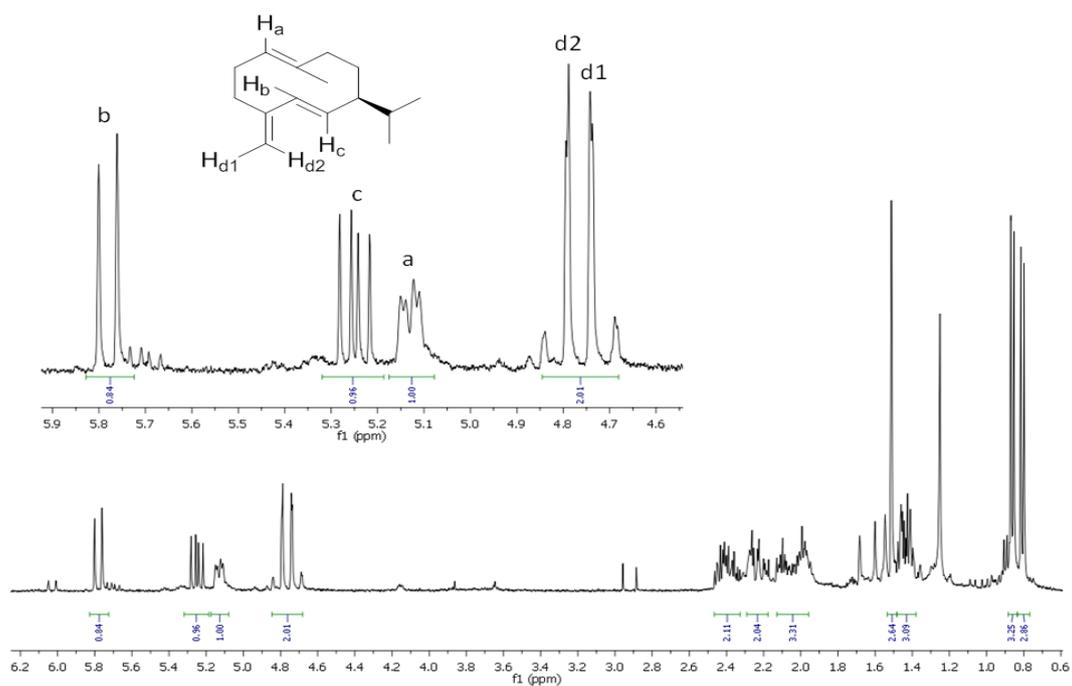
Figure 42. ^{1}H NMR (400 MHz, CDCl$_3$) spectrum of (-)-germacrene D (**78**).

This sample was sufficiently concentrated to record a ^{13}C NMR spectrum that further confirmed the identity of (-)-germacrene D (**78**).
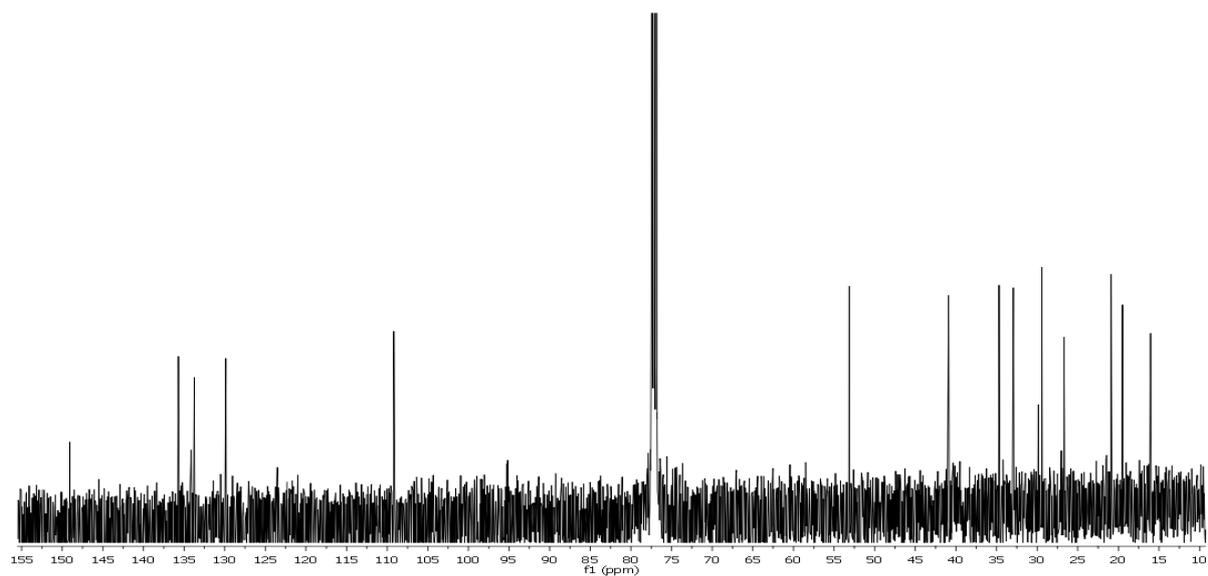
Figure 43. ^{13}C NMR (126 MHz, CDCl$_3$) spectrum of (-)-germacrene D (**78**).

4.3.2 Incubation of 12Me-FDP (136) with GDS

The additional methyl group on 12Me-FDP (**136**) was not expected to interfere in the reaction mechanism of GDS. Hence, a (-)-germacrene D derivative was expected from the incubations with GDS. Nevertheless, a new chiral centre at C11 will be created due to the presence of the additional methyl group that will desymmetrise the isopropyl group of native (-)-germacrene D. The hydride shift from C1 to C11 should not be impeded by the bulkiness of the additional methyl group and the stereochemistry of the shift should not be affected. In the case of FDP, the rotation of the isopropyl group has no effect on the stereochemistry of the final product because of the symmetry of the isopropyl group. However, in the case of the 12Me-FDP (**136**), the stereochemistry of the final product is determined by the relative position of the ethyl group when the hydride shift takes place (**332** or **333**). Free rotation of carbocation **332** would lead to two diastereoisomers (**337** and **335**, Scheme 93).
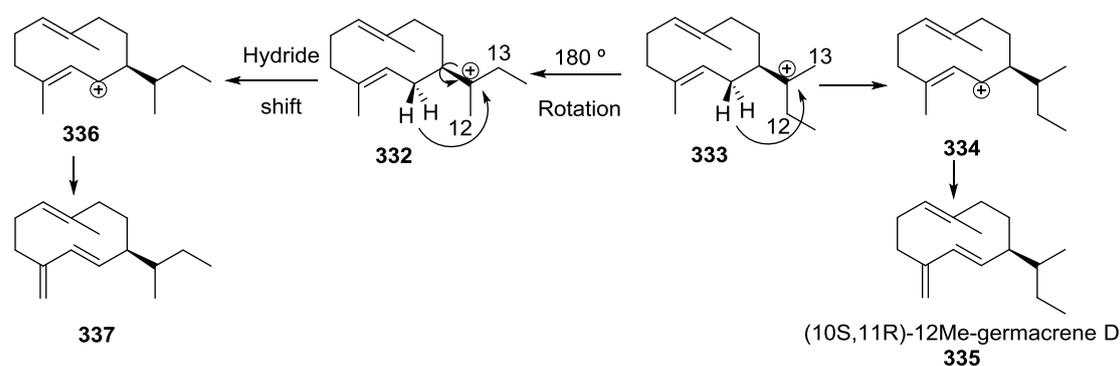
Scheme 93. Possible products formation of the incubation of 12Me-FDP (**136**) and GDS.

The incubation of 12Me-FDP (**136**) with GDS led to the formation of two main and four minor products as judged by GC-MS (Figure 44). Comparison of the mass spectrum of the two new enzymatic sesquiterpenoids eluting at 27.4 and 28.9 min and spectrum of (-)-germacrene D (**78**, Figure 41) revealed that these products were not the expected 12-Me derivatives of **78**. Nevertheless, these two compounds share an almost identical mass spectrum, suggesting that they are most likely diastereoisomers. On the other hand, the mass spectrum of one of the minor products eluting at 29.3 min displays the distinctive (-)-germacrene D peak at $m/z = 161$ and the overall fragmentation pattern that strongly resembles (-)-germacrene D (**78**, Scheme 32).
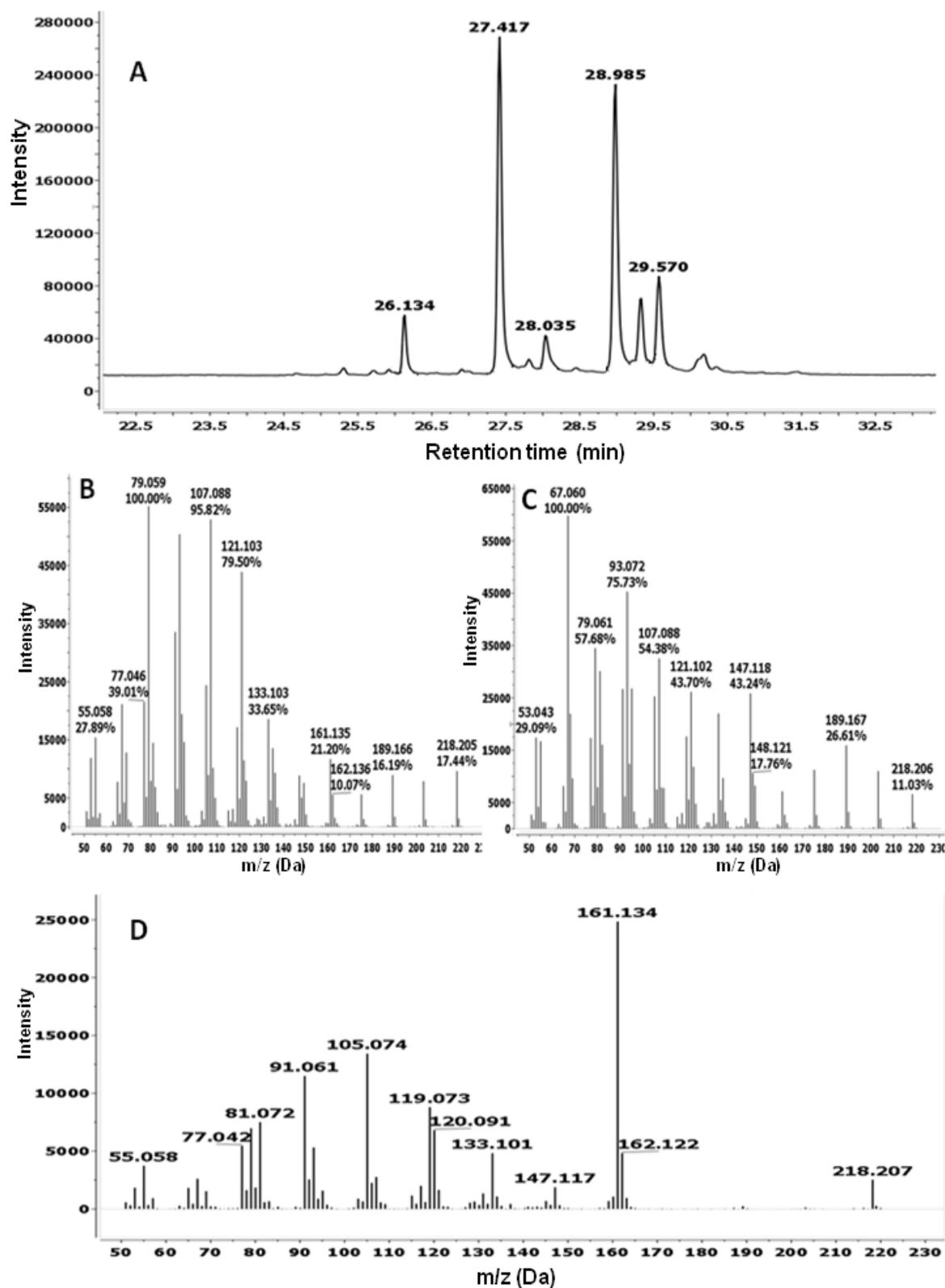
Figure 44. A) Gas chromatogram of the pentane extractable products obtained from incubation of 12Me-FDP (**136**) and GDS. B) Mass spectrum of the peak eluting at 27.4 min. C) Mass spectrum of the peak eluting at 28.9 min D) Mass spectrum of the peak eluting at 29.3 min.

The mixture of compounds made difficult the identification of the two main products by ^{1}H NMR spectroscopy. The sample obtained from a preparative incubation was not a sufficient amount to obtain a clear ^{13}C NMR spectrum. The ^{1}H NMR spectrum of the crude displays a doublet of doublets centred at 4.72 ppm (Figure 45) that resembles the doublet of doublets observed for (-)-germacrene D (**78**, Figure 42) corresponding to the protons on C15. The assignment is supported by the HSQC correlation of each of the doublets with the same carbon signal. The measured coupling constant for this doublet of doublets is much higher than the values of the ones observed for (-)-germacrene D. However, the signals have the same chemical shift as olefinic protons on C1 and C6 for (-)-germacrene D. The doublet at 5.78 ppm is almost identical to the signal observed in (-)-germacrene D (Figure 42). Despite of these similarities, the identity of these two compounds could not be established and is still unknown.
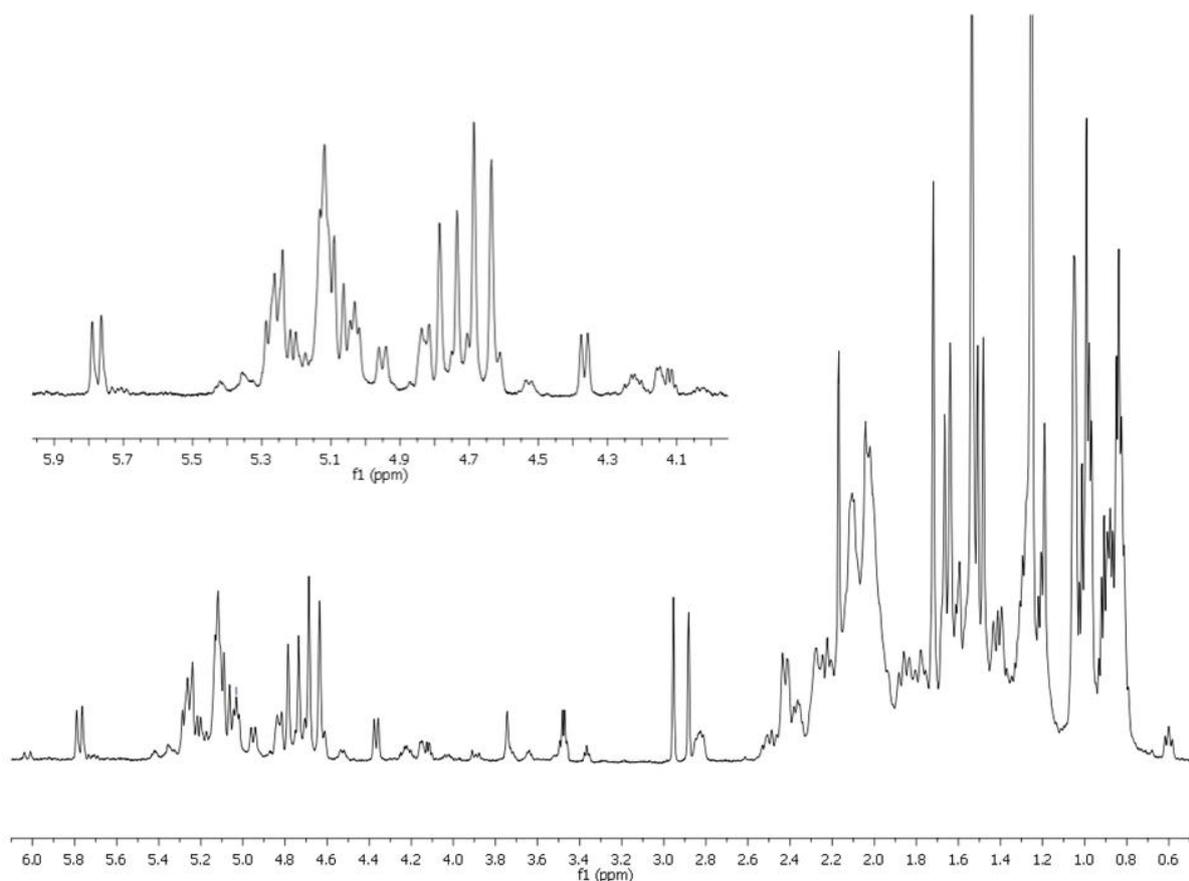
Figure 45. ^{1}H NMR (600 MHz, CDCl$_3$) spectrum of the products from the incubation of 12Me-FDP (**136**) and GDS.

4.3.3 Incubation of 14Me-FDP (137) with GDS

The methyl group in C14 is separated from any carbon that is involved in the reaction mechanism of GDS. Therefore, 14Me-FDP was expected to produce 14Me-germacrene D (**338**) upon incubation with GDS. While the mass spectrum displays a single $m/z = 218$ peak, the mass spectrum displays a mass and the base peak at $m/z = 175$ indicating a loss of an isopropyl group which is a diagnostic peak for (-)-germacrene D. The mass spectrum of the new compound has a fragmentation pattern almost identical to (-)-germacrene D (Figure 46).
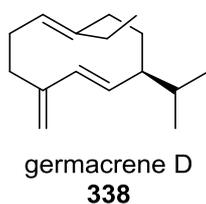
germacrene D
338

Scheme 94. Structure of 14Me-germacrene D (**338**).
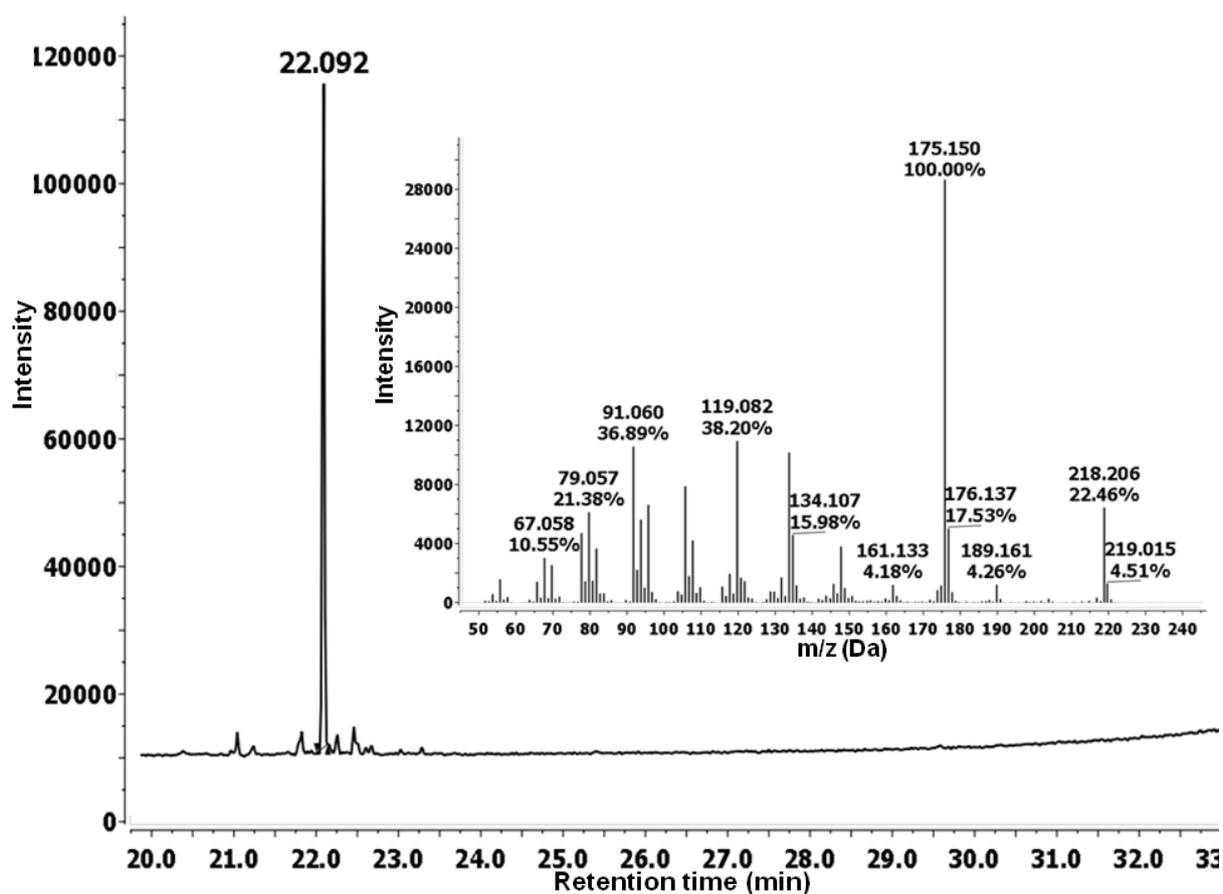
Figure 46. Gas chromatogram of the pentane extractable products obtained from incubation of 14Me-FDP (**137**) and GDS. Inset, mass spectrum of 14Me-germacrene D (**338**) eluting at 22.092 min.

Preparative incubation allowed ^{1}H NMR and ^{13}C NMR spectroscopic analysis. The comparison with previously obtained NMR spectrum of (-)-germacrene D (Figure 47) revealed the expected differences that confirm the identity of 14Me-germacrene D (**338**). Indeed, the region in the ^{1}H NMR spectrum between 5.1-4.7 ppm was identical for both compounds. In the downfield region, a new triplet is observed at 0.87 ppm with a coupling constant of $J = 7.5$ Hz which is assigned to the presence of the ethyl group. On the other hand, the singlet observed at 1.51 ppm for germacrene D (Figure 42) has now disappeared which is consistent with the expected product. The signal for the new methylene group at C14 was identified at 2.30 ppm as doublet of triplets and assigned to this carbon through its coupling constant of $J = 7.5$ Hz indicating that these protons are coupled with the triplet observed at 0.87 ppm (Figure 47).
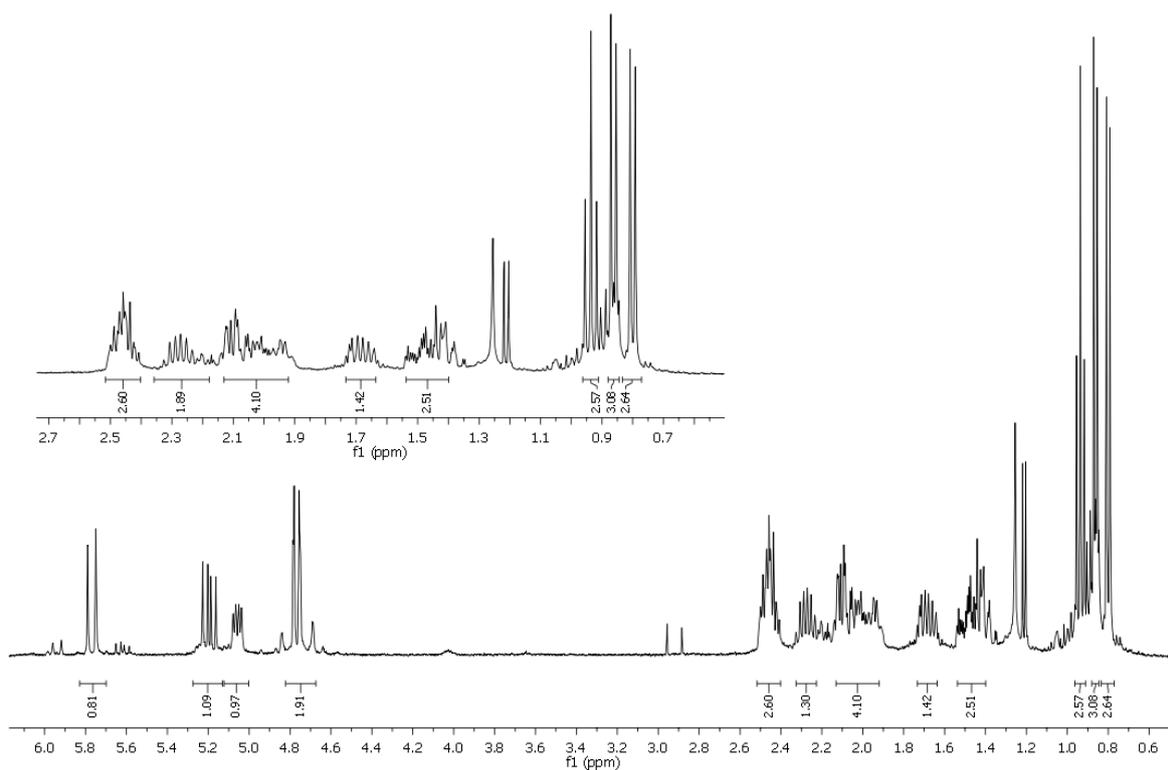
Figure 47. ^{1}H NMR (500 MHz, CDCl$_3$) spectrum of 14Me-germacrene D (**338**).

^{13}C NMR spectrum revealing the presence of an additional signal upfield at 21.4 ppm, which confirms the presence of an additional methyl group (Figure 48).
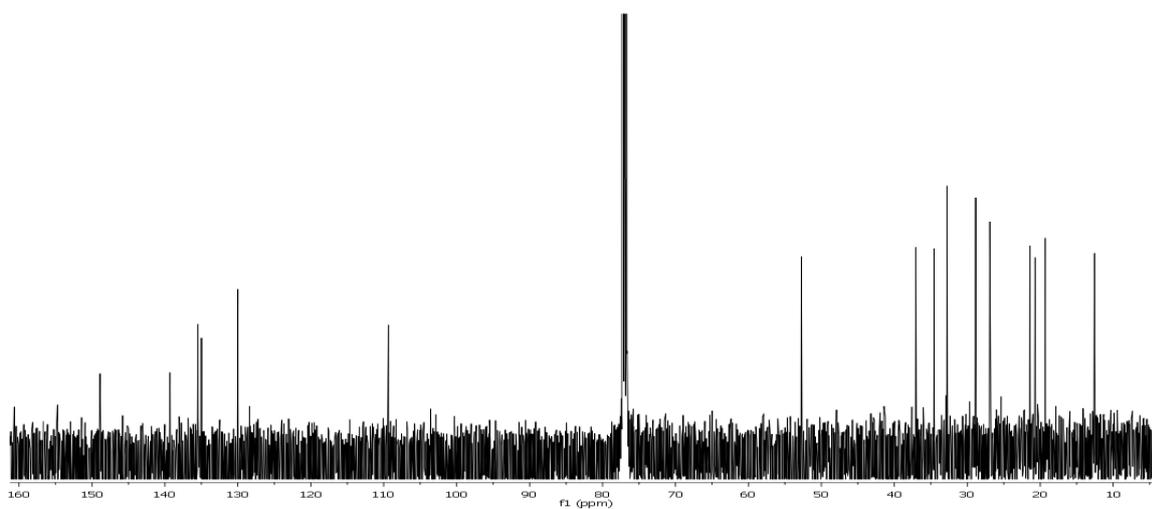
Figure 48. ^{13}C NMR (126 MHz, CDCl$_3$) spectrum of 14Me-germacrene D (**338**).

4.3.4 Incubation of 14,15 diMe-FDP (139) with GDS

The GC-MS analysis revealed the presence of at least nine different products with $m/z = 232$ (Figure 49). The products eluting at 29.5 min (Figure 49A) and 33.2 min (Figure 49B) were the two major fractions. The mass of the product A was similar to the mass spectrum of the compound obtained from the incubation of 15Me-FDP (138) with GDS (*vide infra*). The mass spectrum for the peak B (Figure 49B) was very similar to the mass spectrum of (-)-germacrene D, displaying the characteristic peak at $m/z = 189$ corresponding to the loss of an isopropyl group. The mass spectrum (Figure 49B) suggests 14,15 diMe-germacrene D (339) as possible product.

14,15diMe germacrene D
339
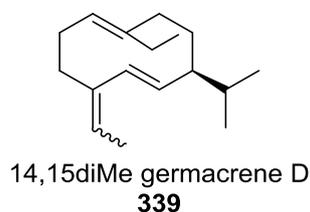
Scheme 95. Structure of 14,15diMe germacrene D (**339**).

Figure 49. Gas chromatogram of the pentane extractable products obtained from incubation of 14,15diMe-FDP (**139**) and GDS. A) Mass spectrum of the peak eluting at 29.491 min. B) Mass spectrum of the peak eluting at 33.180 min.

Unfortunately, all the attempts to perform a preparative scale incubation of 14,15diMe-FDP (**139**) with GDS to confirm the identity of the product B and solve the structure of A were unsuccessful. The inability to obtain a ^{1}H NMR spectrum is probably due to the low conversion rate with 14,15diMe-FDP (**139**) and the mixture of products obtained.

4.3.5   Incubation of 15Me-FDP (138) with GDS

The GC-MS analysis revealed the presence of at least six different products with the expected m/z = 218 (Figure 50). The mass spectrum of the product eluting at 26.9 min (Figure 50A) has remarkable similarities with the product obtained in the incubation of 14,15- diMe-FDP (**139**) (*vide supra*), but the identity of both products remains unknown. The mass spectrum of the product eluting at 29.8 min displays great similarities with the mass spectrum of (-)-germacrene D (Figure 41) including the diagnostic loss of the isopropyl group. Therefore, the identity of this product can be assigned to the corresponding germacrene D derivative. The identity of the product eluting at 29.1 min is unknown.
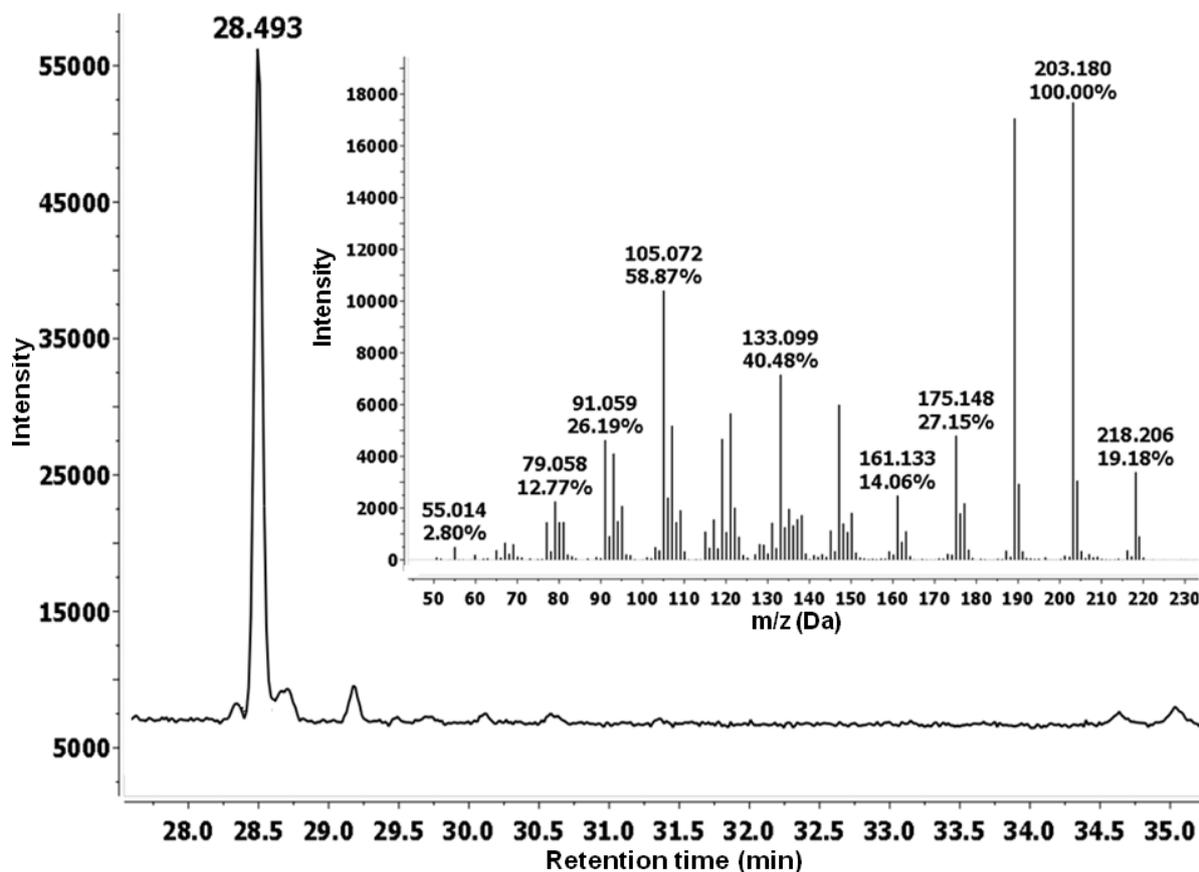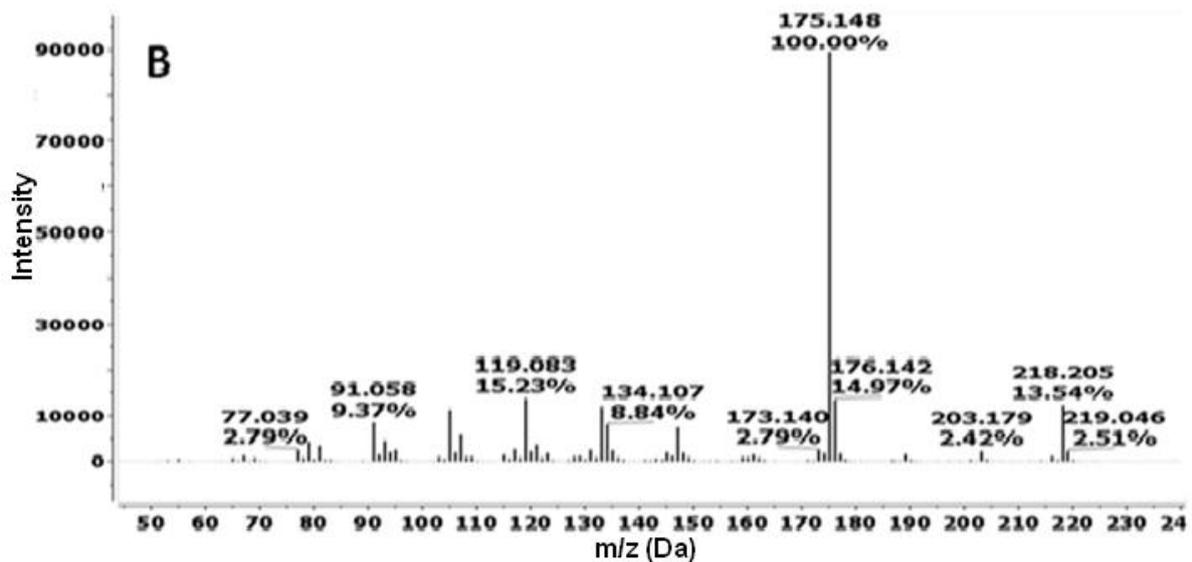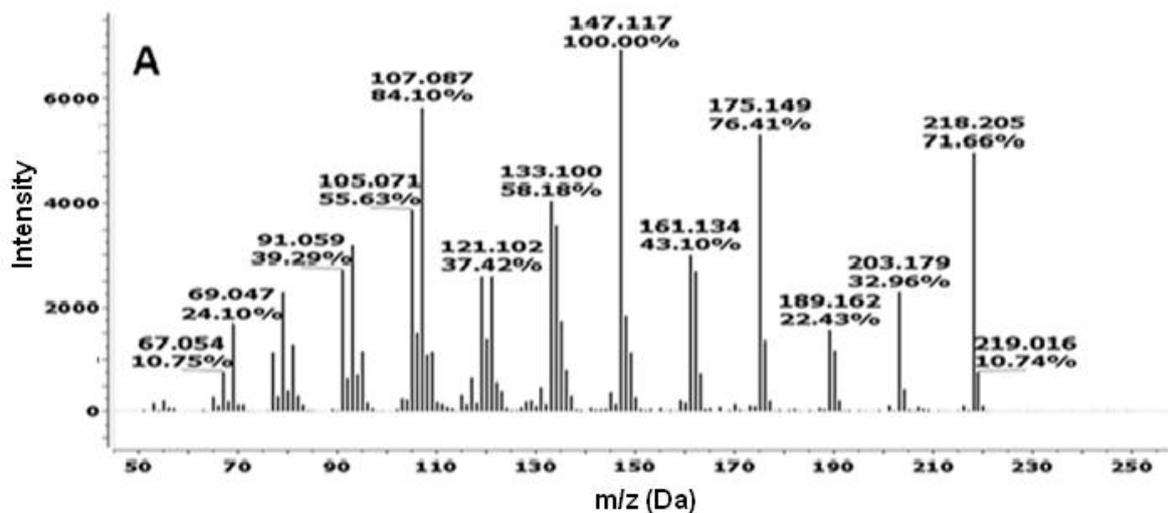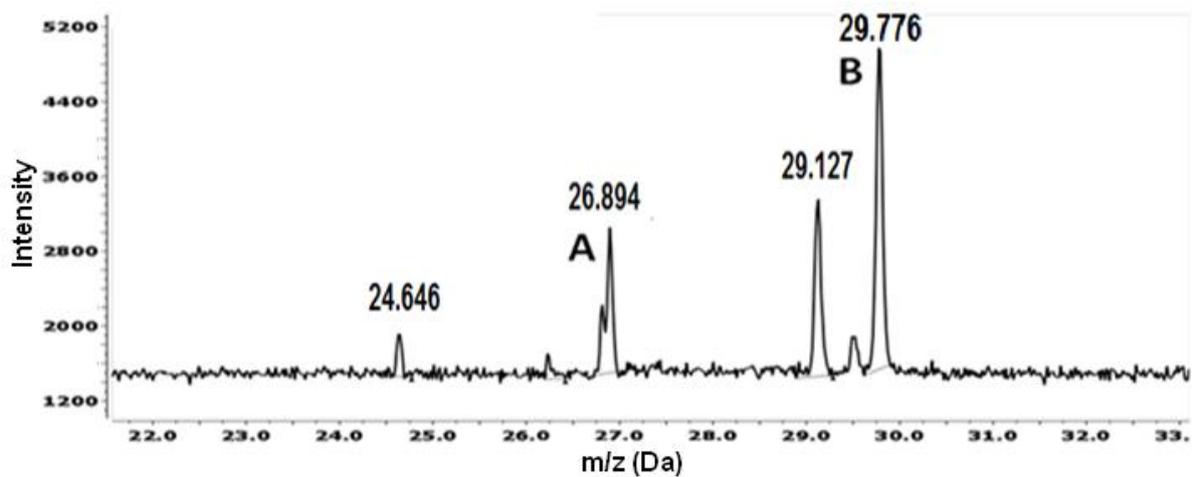
Figure 50. Gas chromatogram of the pentane extractable products obtained from incubation of 15Me-FDP (**138**) and GDS. A) Mass spectrum of the peak eluting at 26.894 min. B) Mass spectrum of the peak eluting at 29.776 min.

Again, in this case, it was not possible to obtain a clear ^{1}H NMR spectrum from the preparative incubation of 15Me-FDP (**138**) with GDS due to a mixture of compounds obtained and the low yield of the enzymatic conversion (*vide infra*).

4.3.6 Incubation of 8nor-FDP (140) with GDS

GC-MS analysis products obtained from the incubation of 8nor-FDP (**140**) and GDS revealed the presence of at least eight products in trace quantities and one major product eluting at 17.8 min. The retention time and the mass spectrum (Figure 42) are identical to the ones obtained for the major product of the incubation of 8nor-FDP (**140**) and GAS (Figure 31). The identity of this product was discussed in section 4.1.5 and **313** (Scheme 84) was suggested as product. In addition the mechanism previously proposed for the formation of this product is similar to the mechanism of formation of (-)-germacrene D (*vide supra*).
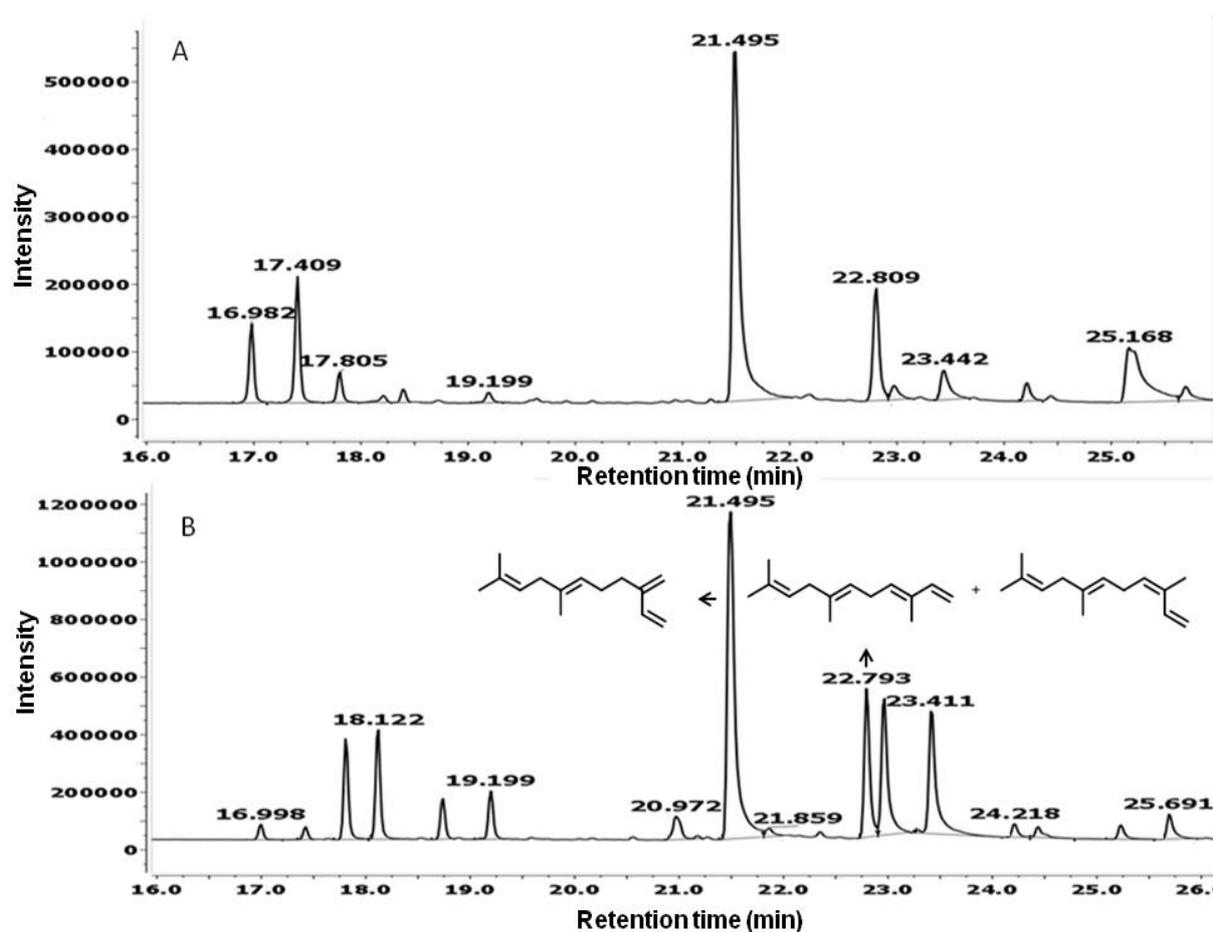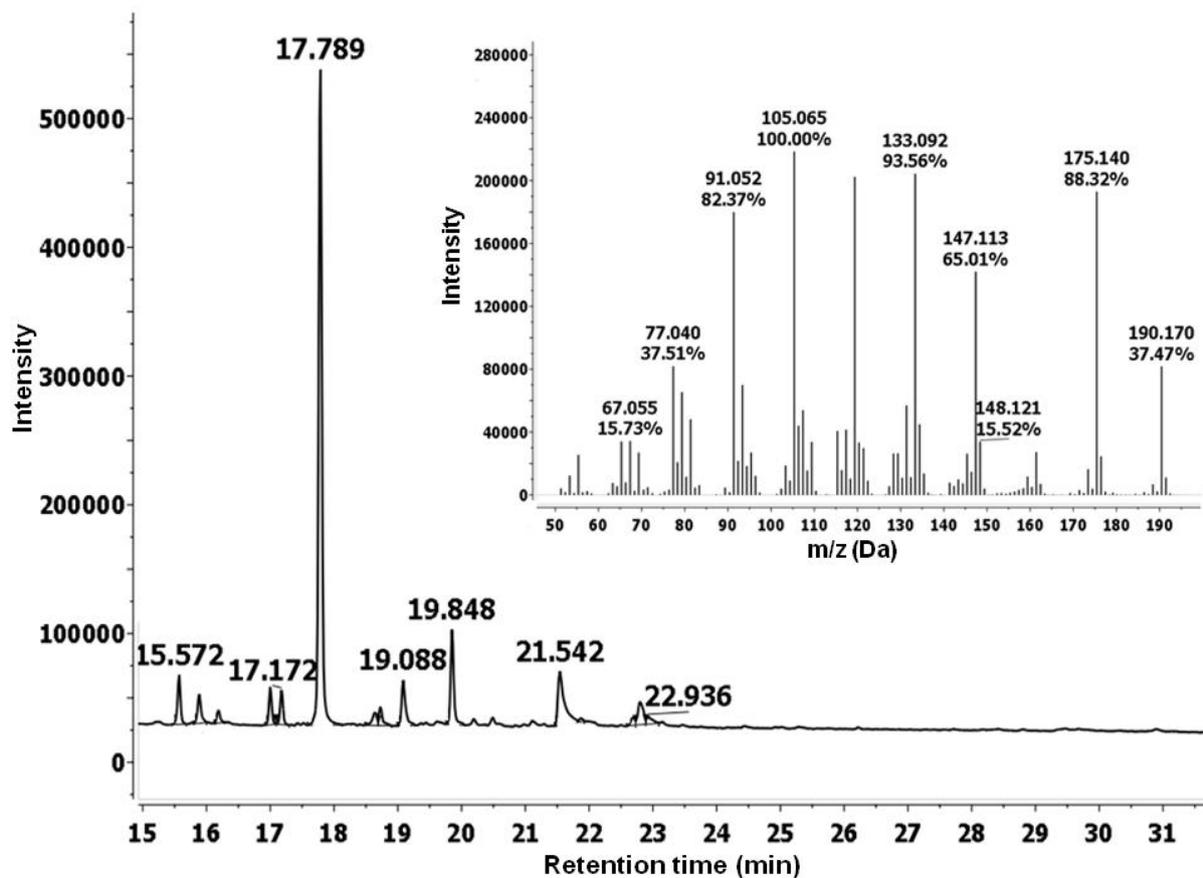
Figure 51. Gas chromatogram of the pentane extractable products obtained from incubation of 8nor-FDP (**140**) and GDS. Inset, mass spectrum of product eluting at 17.789 min.

4.3.7   Incubation of 6F-FDP (289) with GDS

The double bond between C5 and C6 (FDP, **17**) is not playing any role in the catalysis of GDS. Therefore, the fluorine atom at C6 should not interfere in the GDS mechanism and 6F-germacrene D is the expected product of the enzymatic reaction.

The GC-MS analysis revealed that GDS was indeed able to turn over 6F-FPP (**289**) to give one single fluorine containing sesquiterpenoid. The mass spectrum of the product has a signal at $m/z = 222$, which is in agreement with the presence of a fluorinated sesquiterpene. In addition, a peak at $m/z = 179$ indicates the characteristic loss of an isopropyl group. The molecular ion at $m/z = 159$ is generated by further fragmentation of the peak at $m/z = 179$ by losing the fluorine atom (Figure 52).
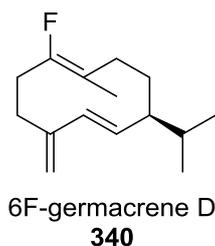
Scheme 96. Structure of 6F-germacrene D (**340**).
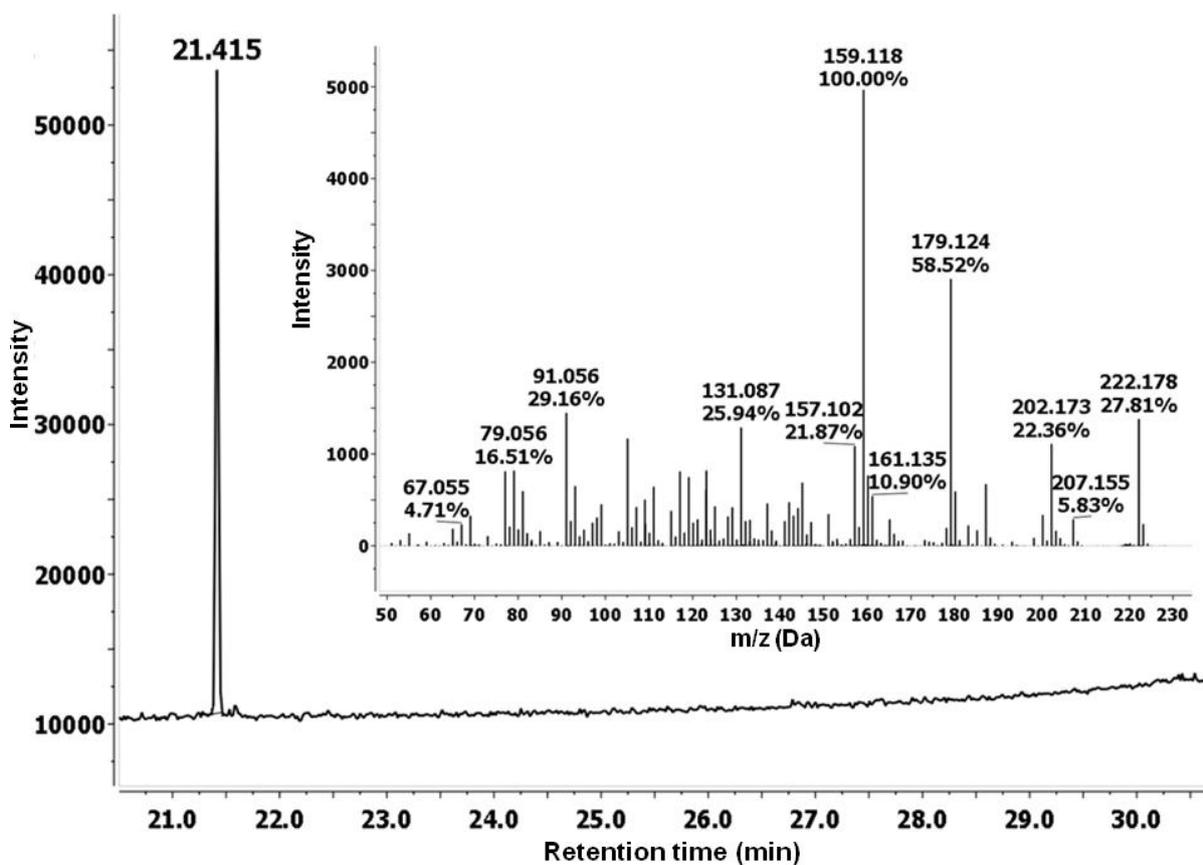
Figure 52. Gas chromatogram of the pentane extractable products obtained from incubation of 6F-FDP (**289**) and GDS. Inset, mass spectrum of product eluting at 21.415 min.

Preparative scale incubation allowed the characterisation by ^{1}H NMR spectroscopy of 6F-germacrene D (**340**). The downfield region of the spectrum displayed a similar pattern of signals as (-)-germacrene D. Although in this case one of the signals is missing, this is due to substitution of one of the olefinic protons with a fluorine atom. In addition, all the signals at

5.8 and 5.25 have shifted downfield to 6.15 and 5.83, respectively, due to the presence of the shielding effect of the fluorine atom in the molecule. In the upfield region, the characteristic doublets at 0.8 ppm confirm the presence of the isopropyl group. The rest of the signals are unfolded in a different manner from (-)-germacrene D due to the presence of the fluorine atom. The ^{19}F NMR spectrum displays an apparent broad doublet at -106.0 ppm. The signal can be a doublet of doublets form by the separate coupling of the fluorine atom with each of the protons of the neighbouring methylene moiety (Figure 53).
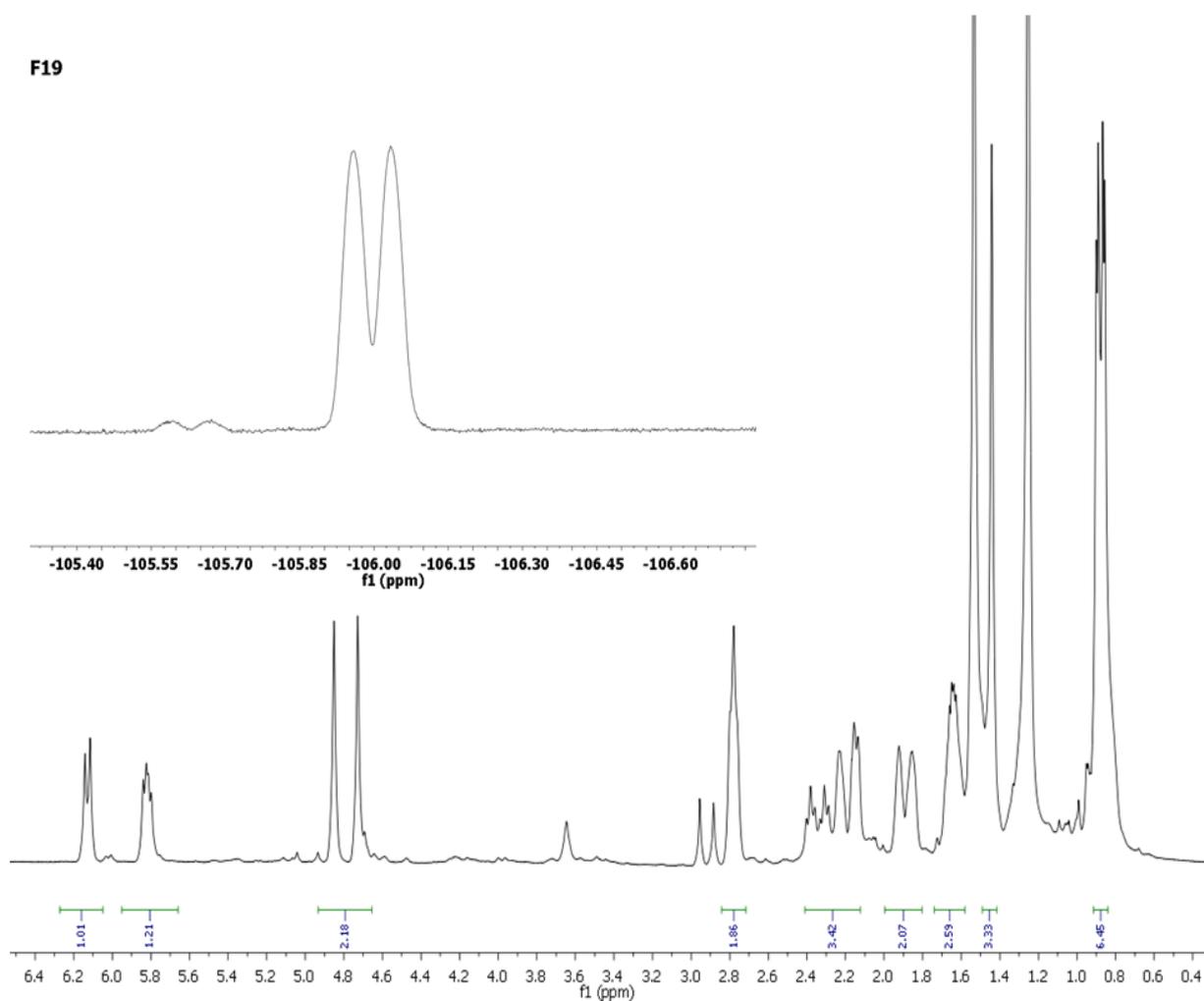
Figure 53. ^{1}H NMR and ^{19}F NMR (565 MHz, CDCl$_3$) spectrum of 6F-germacrene D (**340**).

4.3.8 Incubation of 14F-FDP (291) with GDS

Three different products were obtained from the incubation of 14F-FDP (**291**) with GDS as indicated by the GC-MS analysis. However, previous studies have shown that with short incubation periods only two products were observed.[292] This observation can be attributed to the instability of the resulting product that is slowly decomposing. Another eight secondary products in trace amounts are observed in the chromatogram (Figure 54).
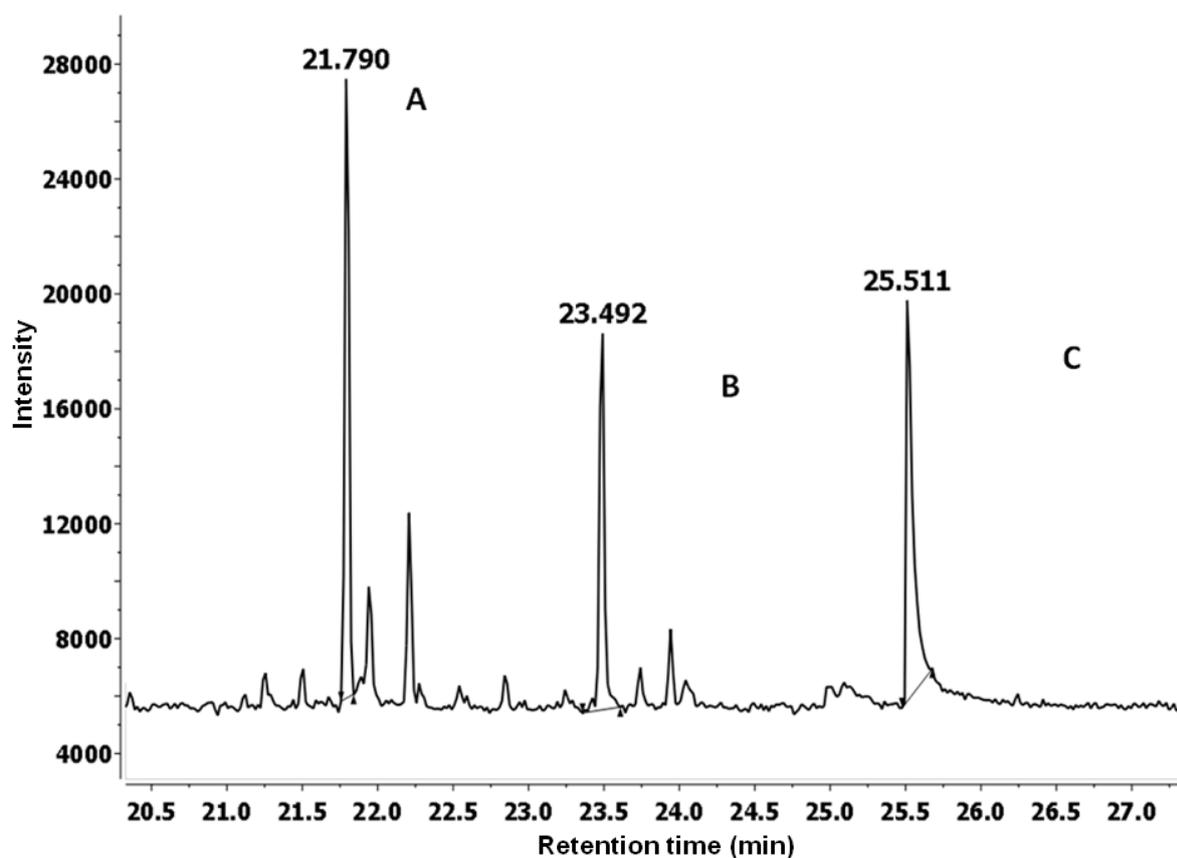
Figure 54. Gas chromatogram of the pentane extractable products obtained from incubation of 14F-FDP (**291**) and GDS.

The mass spectrum of the peak eluting at 25.5 min (Figure 54C) displays a signal at $m/z = 220$ (Figure 55C), the mass matches the presence of fluorinated dehydrogermacrene D. It has been suggested that the formation of the new double bond occurs during the ionisation of the sample in the chamber of the mass spectrometer.[243] The molecular ion is $m/z = 159$ and

corresponds to the loss of the isopropyl group and the loss of HF at the same time. The overall shape of the mass spectrum resembles the shape of (-)-germacrene D hence most probable product is 14F-germacrene D (**341**). The mass spectrum of the two peaks eluting at 21.8 min and 23.5 min is very similar and both display a molecular mass of $m/z = 202$, this molecular mass denotes loss of HF (Figures 55A and 55B).

14F-germacrene D
341
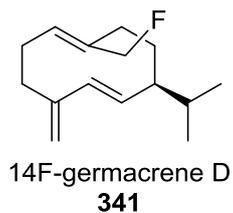
Scheme 97. Structure of 14F-germacrene D (**341**).
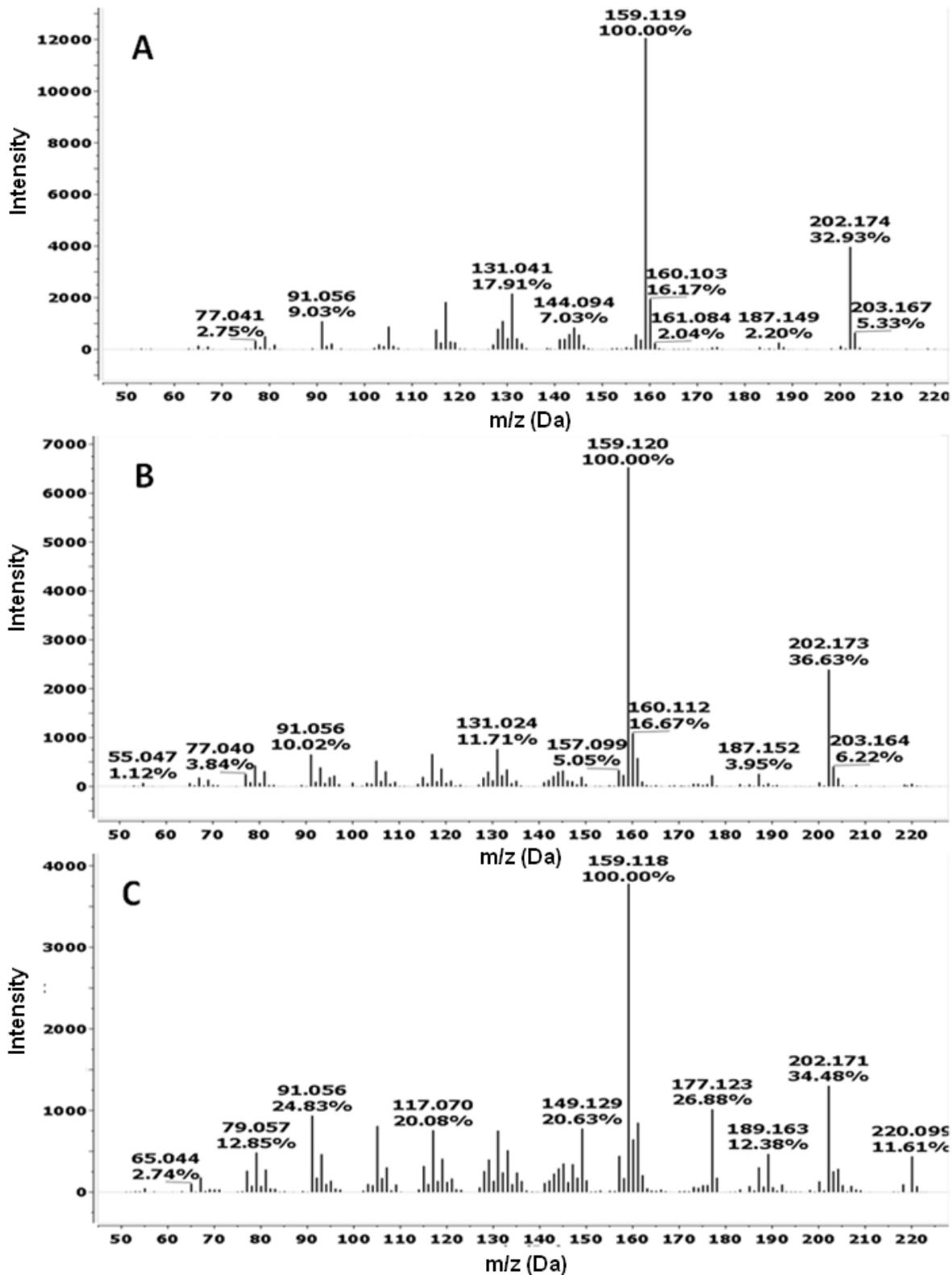
Figure 55. A) Mass spectrum of the product eluting at 21.8 min. B) Mass spectrum of the product eluting at 23.5 min. C) Mass spectrum of the product eluting at 25.5 min.

Preparative incubation allowed analysis by ^{1}H NMR and ^{19}F NMR spectroscopy of the products. The ^{19}F NMR spectrum displays three absorptions at -73.45 (t, J = 9.5 Hz), -178.9 (d, J = 48.5 Hz) and -183.3 (d, J = 49.7 Hz) ppm. The three absorptions observed by ^{19}F NMR spectroscopy confirm that the three products obtained from the incubation contain a fluorine atom and the loss of HF observed in the mass spectrum happened in the mass spectrometer.

Unfortunately, the three major products of the incubation could not be separated and the complexity of the spectrum made the full identification of each product difficult. However, the comparison of this spectrum with ^{1}H NMR spectrum of pure (-)-germacrene D (**78**, Figure 41) revealed two set of signals that could correspond to the expected germacrene D derivative. The doublet at 5.80 ppm (d, J = 15.6 Hz, H_b') appears at the same chemical shift as in the (-)-germacrene D spectrum (Figures 41 and 56). The doublet at 5.43 ppm (H_a, Figure 56) is the equivalent to the doublet of doublet that is found at 5.15 ppm for (-)-germacrene D (Figure 41), the signal has been shifted downfield due to the presence of the fluorine atom, which also broadens the signals and the final shape is an apparent doublet. The doublet of doublets at 5.26 ppm (dd, J = 16.0, 9.9 Hz, H_c, Figure 56) has also a similar chemical shift as for (-)-germacrene D. Another set of signals that can be attributed to 14F-germacrene D is displayed as a doublet at 5.90 ppm (d, J = 31.8 Hz, H_b), doublet of doublets at 4.80 ppm (dd, J = 38.6, 20.0 Hz, H_c) and doublet at 4.58 ppm (d, J = 17.3 Hz H_d) (Figure 56).

In addition, two signals are observed that correspond to the CH_2F, appearing as singlets at 2.20 and 1.56 ppm. Both signals are shifted down field in comparison with the parent (-)-germacrene D, which is in agreement with the presence of the fluorine (Figure 56).
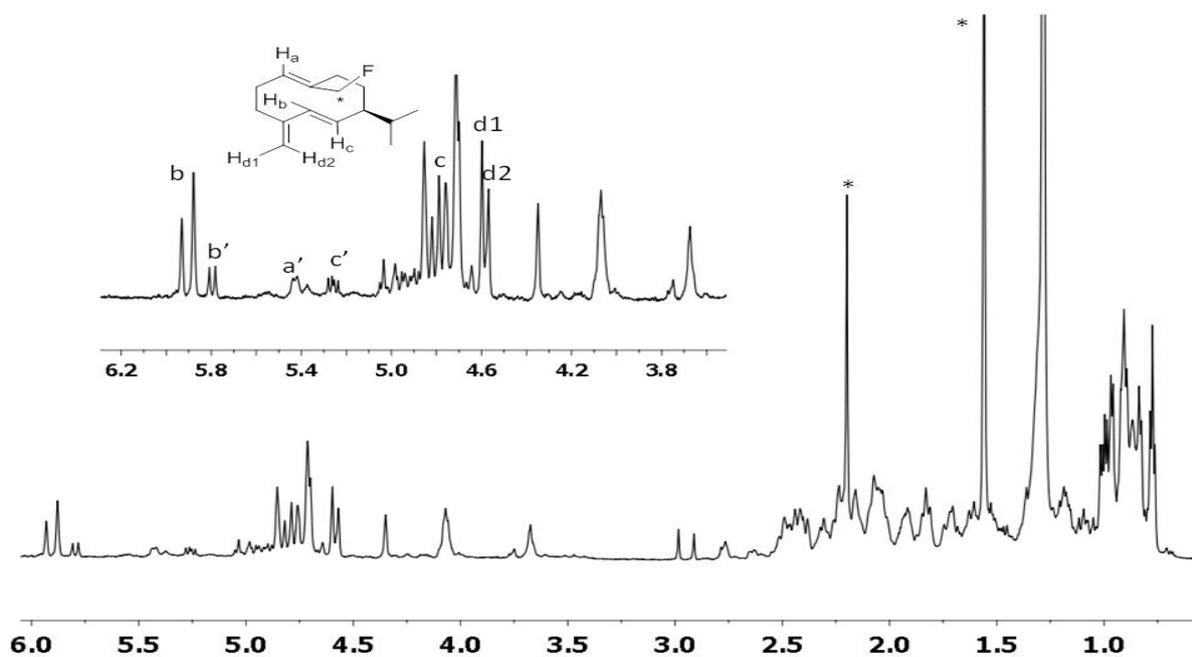
Figure 56. ^{1}H NMR (600 MHz, CDCl$_3$) spectrum obtained from the preparative incubation of 14F-FDP (**291**) and GDS.

4.3.9 Quantification of enzymatic products of GDS

As it has been mentioned for GAS, all products obtained from the incubation of GDS and FDP analogues were quantified using GC-FID by comparison with internal standards (α-humelene). These experiments allowed an estimation of the enzymatic conversion and allow comparison with FDP (**17**).

6F-FDP (**289**, Table 13, entry 2) and 14Me-FDP (**137**, Table 13, entry 3) present approximately half of the conversion in comparison with FDP (**17**, Table 13, entry 1). These two analogues present the highest capability as substrates, which is linked to the fact that they are the only two analogues, which give a single product. 14F-FDP (**291**, Table 13, entry 4) displays high conversion but lower than 6F-FDP (**289**, Table 13, entry 2) and 14Me-FDP because of the instability of the obtained product. 15Me-FDP (**138**, Table 13, entry 6) and 14,15diMe-FDP (**139**, Table 13, entry 7) gave complex mixtures of compounds and in consequence the efficiency of the enzyme is low, which explains why was not possible to obtain the NMR spectrum.

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Entry	Substrate	Conversion (%)	Conversion relative to FDP (%)[a]
1	FDP (**17**)	76	100
2	6F-FDP (**289**)	38	50
3	14Me-FDP (**137**)	45	59
4	14F-FDP (**291**)	33	43
5	14,15-diMe-FDP (**139**)	2.3	3
6	15Me-FDP (**138**)	2.4	3.2

a) Conversion normalised to 100% conversion with FDP.

Table 13. Conversions of FDP (**17**) and FDP analogues with GDS.

4.3.10 Conclusions

The GDS incubation of 14Me-FDP (**137**) and 6F-FDP (**289**) gave single products characterised as 14Me-germacrene D (**338**, Scheme 94) and 6F-germacrene D (**340**, Scheme 97), respectively. As in the case of GAS, these two analogues 14Me-FDP (**137**) and 6F-FDP (**289**) display the highest substrate activities.

12Me-FDP (**136**) gave two major products: one of them was identified as a 12Me-germacrene D (**335**, Scheme 93) by its mass spectrum (Figure 44) and the identity of the second major product is unknown but it is suggested as a possible isomer of 12Me-germacrene D. The minor products are still unidentified.

14,15-DiMe-FDP (**139**) and 15Me-FDP (**138**) gave in both cases one product that can be identified by its mass spectrum as a 14,15-diMe-germacrene D (**339**, Scheme 95) and 15Me-germacrene D, respectively, and secondary products display with very similar mass spectrum between the two analogues (Figures 49 and 50). The identity of the secondary products is still unknown.

14F-FDP (**291**) gave a mixture of three compounds: one of them has been previously identified as a decomposition product. The major product was identified as the 14F-germacrene D (**341**, Scheme 97).

8nor-FDP (**140**) proved the ability of GDS to induce 1,11 cyclisation giving the same product as GAS when incubated with 8nor-FDP (**140**).

4.4 (+)-δ-Cadinene synthase from *Gossypium arboreum*

4.4.1 Incubation of 10F-FDP (290) with DCS

The single product obtained from the incubation of 10F-FDP (**290**) and DCS was the same product previously characterised from the incubation of GAS with 10F-FDP (**290**) namely 10F-α-humelene (**300**). 10F-α-humulene (**300**) arose from a 1,11-macrocyclisation triggered by the presence of the fluorine atom. The fact that the DCS generates the same product as GAS (enzyme that operates through 1,10-cyclisation) supports the proposed 1,10-cyclisation catalytic mechanism for this enzyme.

4.4.2 Incubation with 8nor-FDP (140) with DCS

The GC-MS analysis of the pentane extractable products of the incubation of 8nor-FDP (**140**) with DCS indicated a complex mixture of products, with the major product eluting at 19.8 min. The rest of the products were found in trace amounts and their identity is unknown (Figure 57). The comparison of the gas chromatogram with the corresponding gas chromatogram of the authentic sample of 8-nor-farnesene (Figure 40B) ruled out any of the 8-nor-farnesenes.

The mass spectrum of the major product shows similarities with the mass spectrum obtained from the incubation of GAS and GDS (Figure 31 and 51) with 8nor-FDP (**140**). Indeed, the fragmentation pattern is identical and the only difference observed is the intensity of some peaks (Figure 57). The different retention time and the similarities in the mass spectrum could indicate a isomeric relationship between the products.
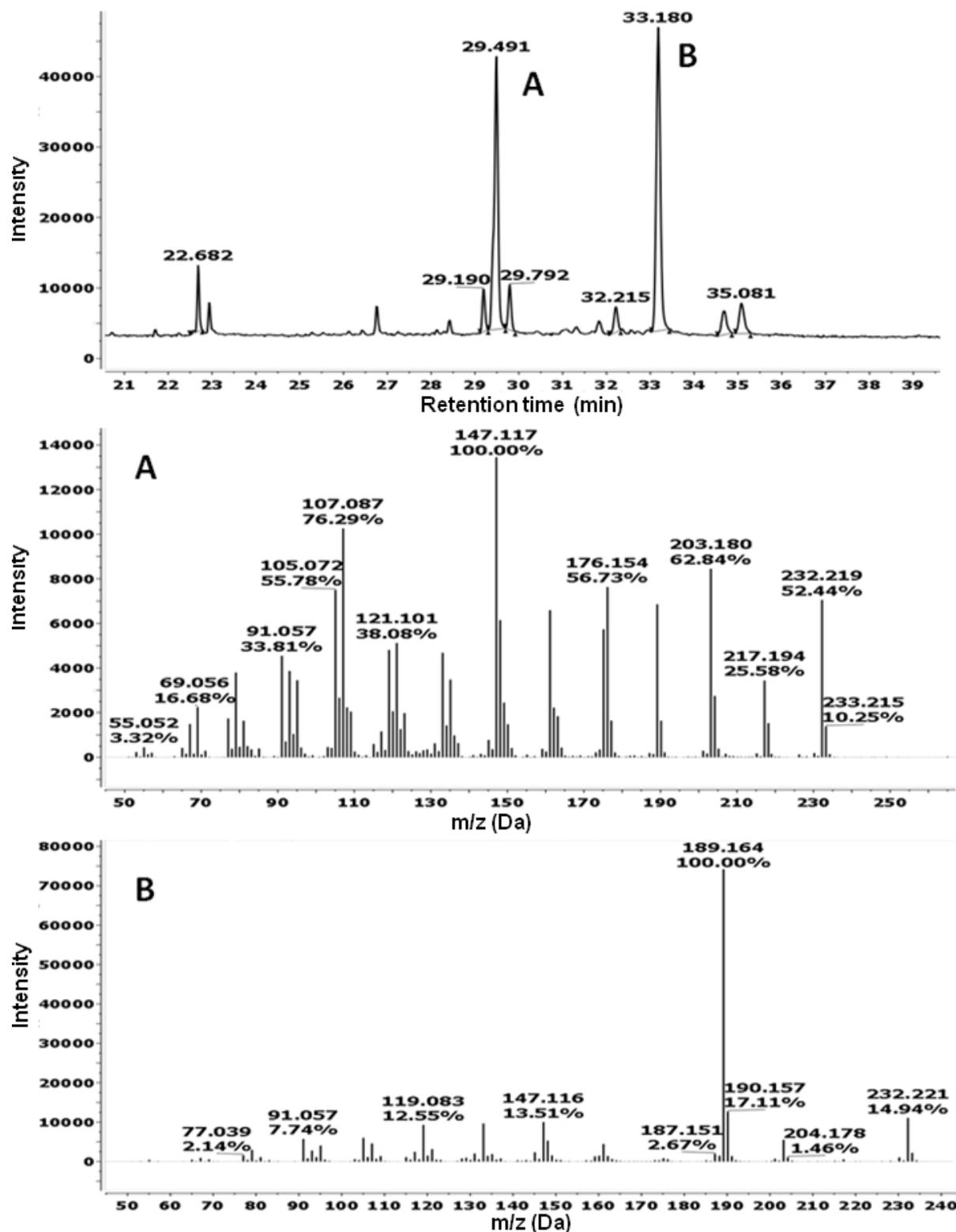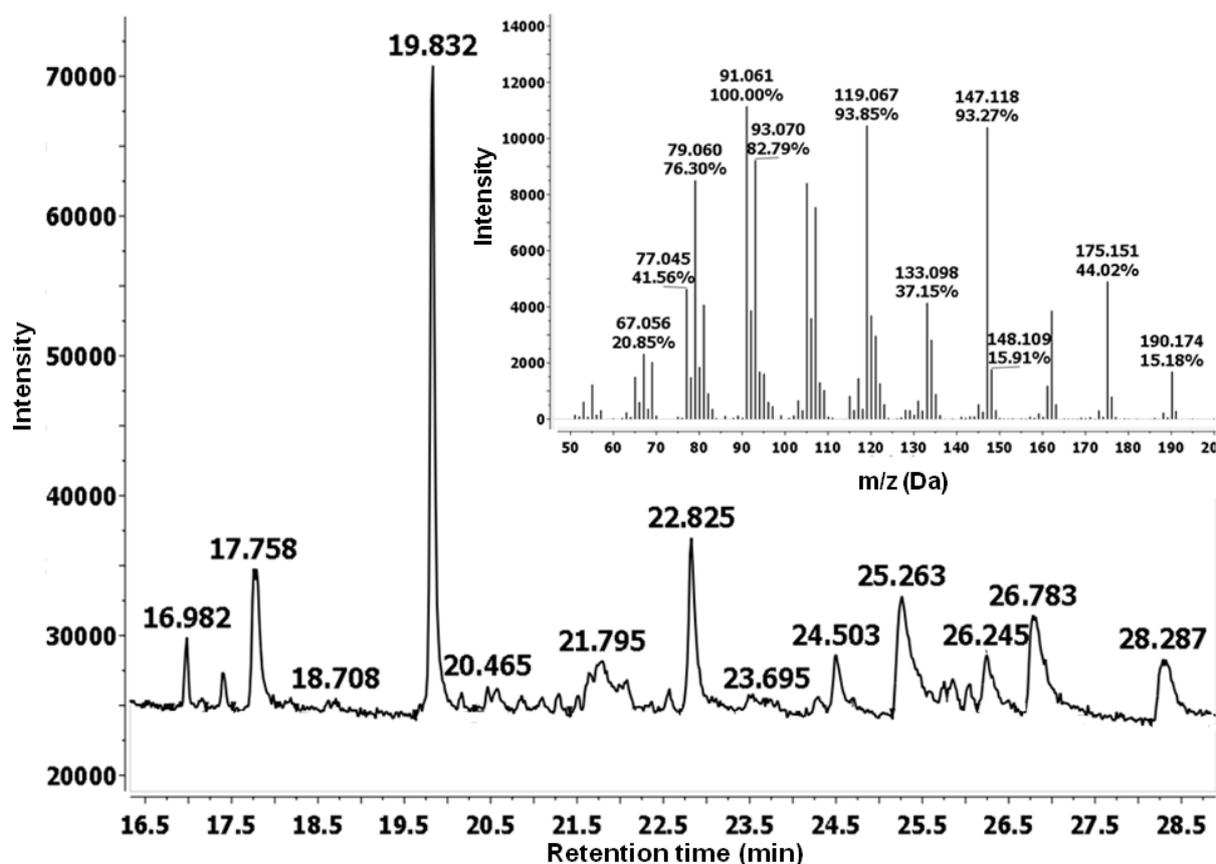
Figure 57. Gas chromatogram of the pentane extractable products obtained from incubation of 8nor-FDP (**140**) and GDS. Inset, mass spectrum of product eluting at 19.832 min.

The first steps of the mechanism proposed for DCS (involving a 1,10-cyclisation) led to germacradienyl cation (Chapter 1, Scheme 21, **97**). The same intermediate has been proposed for the formation of (+)-germacrene A and (-)-germacrene D by GAS and GDS respectively (Chapter 1, Scheme 9 and 16, **49** and **76**). However, the germacradienyl cation generated by DCS is proposed to have a *cis* conformation of the C2,C3-double bond due to a previous isomerisation of the FDP (**17**) into NDP (Scheme 20, **95**).[293] The isomerisation of 8nor-FDP (**140**) will give the intermediate 8nor-NDP (**342**), the 1,10-attack of the distal double bond will afford the unstable intermediate **343**. Subsequent hydride shift affords the stable carbocation **344**. Quenching of this carbocation by the loss of the proton on C13 will yield the transoid 8nor-γ-humulene (**345**) which is in agreement with the observed mass spectrum and the retention time.
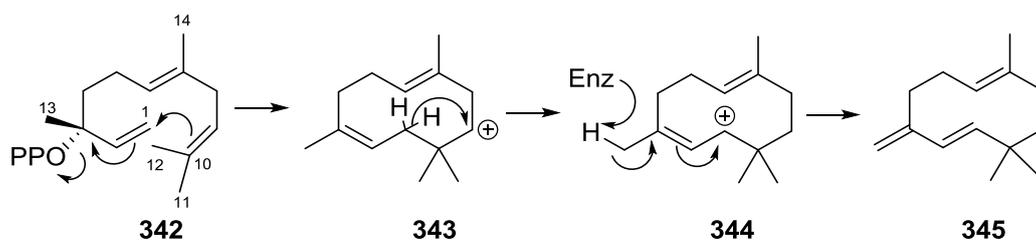
Scheme 98. Mechanism for the formation of the proposed product for the incubation of 8nor-FDP (**140**) and DCS.

The formation of product **345**, which arises from a macrocyclisation of the distal double bond and is similar to the products obtained with enzymes that operates using the 1,10 cyclisation, agrees with the proposed mechanism involving a 1,10 cyclisation for DCS (Chapter 1, Scheme 21).

4.4.3   Incubation of 8OH-FDP (141) with DCS

The incubation of the 8OH-FDP (**141**) led to one major compound with signal at $m/z = 218.9$, and minor compounds with same m/z. Two different compounds were isolated from the mixture by preparative TLC and chemical structures that were in agreement with the NMR spectroscopic studies for each compound were proposed.

The less polar compound (major product) was analysed by ^{1}H NMR and ^{13}C NMR spectroscopy. The ^{13}C NMR spectrum displays a signal at 214.5 ppm, which strongly suggests that the hydroxyl group has been transform to a ketone. The structure proposed for this compound is the keto δ-cadinene derivative **(346)**. 2D NMR spectroscopic studies were performed to confirm this hypothesis (Table 14).

346
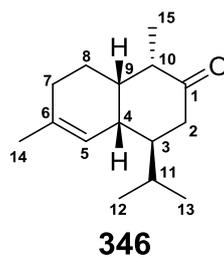
Scheme 99. Structure of the main product obtained from the incubation of DCS with 8OH-FDP (**141**).

^{1}H NMR	^{13}H NMR (HSQC, DEPT-135)	HMBC (Correlation)	COSY (Correlations)	Assignment / C-number (346)
-	214.5	-	-	**1**
-	134.8	7, 8 or/and 14[*]	-	**6**
5.54 (bd, J = 4.0 Hz, 1H)	123.2	8 or/and 14[*]	4, 7 (weak) and 14	**5**
2.75 (dq, $J_1 = J_2$ = 6.5 Hz, 1H)	48.0	2 and 15	9 and 15	**10**
2.31 (m, 1H)	39.2	-	3	**4**
2.22 – 2.12 (m, 2H)	40.2 (CH$_2$) / 43.9	4, 3, 10 (all weak) / 10 and 15	3 / 8 and 10	**2** (1H) / **9**
2.09 (t, J = 13.0 Hz, 1H)	40.2 (CH$_2$)	3, 4(weak), 10, 11(weak)	3	**2** (1H)
2.03 (qd, J = 7.0 3.0 Hz, 1H)	27.2	12 and 13	12 and 13	**11**
1.99 – 1.94 (bd, 2H, J = 6.5 Hz)	31.0 (CH$_2$)	5 (weak), 8	8 and 14 (weak)	**7**
1.83 – 1.75 (m, 1H)	47.5	2, 12 and 13	2, 4	**3**
1.66 (s, 4H)	23.8 / 18.9 (CH$_2$)	5 / 10	4 and 7 / 7 and 9	**14** / **8** (1H)
1.19 – 1.10 (m, 1H)	18.9 (CH$_2$)	10	7, 8 and 9	**8** (1H)
1.01 (d, J = 7.0 Hz, 3H)	11.5	10	10	**15**
0.89 (d, J = 7.0 Hz, 3H)	15.1	12	11	**13**
0.86 (d, J = 7.0 Hz, 3H)	21.4	13	11	**12**

Table 14. Correlation of ^{1}H NMR and ^{13}C NMR spectrum signals observed by HSQC, HMBC and COSY and the assignment of each signal to proton and carbon atoms, respectively.

*The two signals overlap, therefore, it is not possible to distinguish between the effect in one signal from the other.

The isopropyl group is easily recognised from the two doublets up field at 0.86 and 0.89 ppm respectively, also, the same coupling constant suggests that they are coupled to the same proton, which is confirmed by the presence of correlations in the HMBC spectrum. However, the correlation between these two methyl groups and the neighbouring proton on C11 was not observed in the HMBC experiment, it was clearly observed in the COSY. Signal of proton on C3 was assigned through its correlations observed in the HMBC with signals of protons on C12, C13 and COSY correlation with protons on C4 and C2. The resonance in the ^{1}H NMR spectrum of the protons on C2 is splitted in two different signals due to the proximity of the chiral isopropyl group. The signal up field has a strong HMBC correlation with signals of the protons on C3 and C10. In addition, it displays a modest correlation with protons on C11 and C4. This assignation was also confirmed by the single COSY correlation with proton on C3. Only one interaction in the COSY was observed in the case of proton on C4, the strong correlation of this proton with proton on C3 allowed the assignation. The doublet assigned to protons on C15 displays a correlation in both experiments with proton on C10. Signal of proton on C10 correlates in the HMBC with protons on C2 and C15 and in addition COSY correlation with protons on C9 and C15 supports the assignation. Assignation of the proton on C9 was confirmed by HMBC correlation with protons on C10 and C15 COSY correlation with protons on C8 and C10 gives further support for the assignation. The signal in the ^{1}H NMR spectrum of methyl group 14 is easily recognised as being a singlet at 1.66 ppm. The integration of this signal and interactions in the HSQC suggest that another signal is overlapping on in this signal. Protons on C8 are splited in two different signals due to the proximity to the chiral proton on C9. One of these two protons is overlapping with protons on C14. The COSY correlation with protons on C7 and C9 unequivocally supports the assignation. The HMBC correlation of the CH_2 signal of protons on C7 with the methyl group 14 and COSY correlation with protons on C8 supports the assignation.

Figure 58. Expansion of the HMBC spectrum of compound **346** between 2.9 and 0.8 ppm (^{1}H axis) and between 65 and 10 ppm (^{13}C axis).
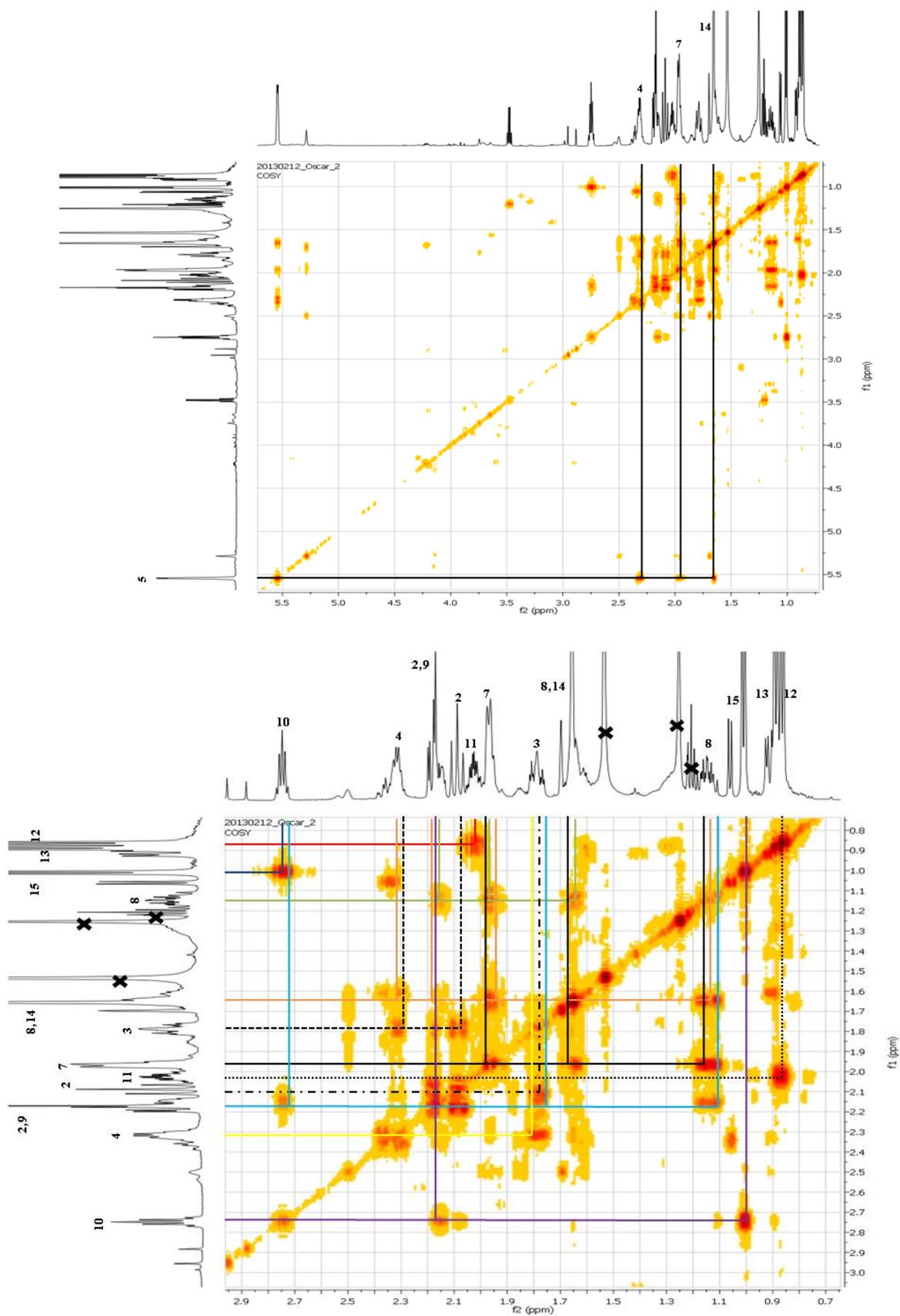
Figure 59. COSY spectrum of compound **346**. Expansion of the COSY spectrum of compound **346** between 2.9 and 0.7 ppm (¹H axis) and between 3.0 and 0.8 ppm (¹H axis).

The relative stereochemistry of ketone **346** was determined by NOE measurements. The saturation of the signal at $\delta_H = 1.01$ ppm, corresponding to the methyl group on C15, led to a modest enhancement on signal at $\delta_H = 1.83$-1.75 ppm corresponding to proton on C3. In addition, the lack of any NOE interaction between the proton on C3 and the methyl on C15, suggest a *syn* geometric relation between these two functional groups. The saturation of the signal at $\delta_H = 2.12$ ppm, corresponding to the proton on C9, led to an enhancement on signals $\delta_H = 0.89$ ppm (4%) and $\delta_H = 5.54$ ppm (1%) corresponding to protons on C12 and C5, respectively. In addition, the saturation of signal at $\delta_H = 2.31$ ppm led to NOE enhancement of signal at $\delta_H = 0.9$ ppm (2%), corresponding to protons on C12. Similarly to the previous case, these interactions indicate the *syn* relation between protons on C9 and C4 of the isopropyl group. Hence, the protons on C9 and C4 as well as the isopropyl group have a *syn* relation, therefore, they have an *anti* geometrical relation with the methyl group on C15. Hence, there are two different possibilities for the absolute configuration of ketone **346**.
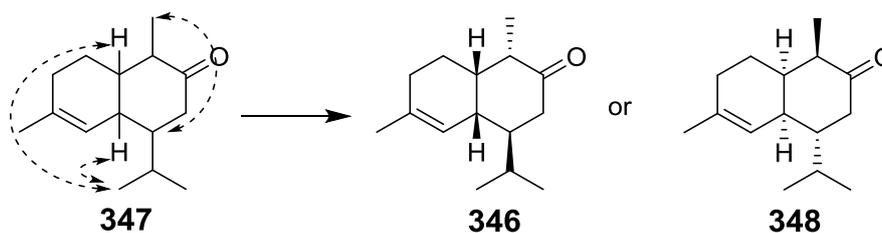
Scheme 100. NOE saturation effect observed and possible stereoisomers.

The stereochemistry of δ-cadinene has been studied and is well established.[294] Assuming that the stereochemistry of the protons on C3 and C4 is the same that the enzyme induces when incubated with FDP, the most probable stereochemical configuration for the product is represented in **346**.

The more polar compound, which is the minor product of the incubation, was isolated in small amounts that were insufficient to study by ^{13}C NMR. The polarity of the product suggests that it could be the corresponding 8-hydroxyl δ-cadinene (**349**) derivative.

349
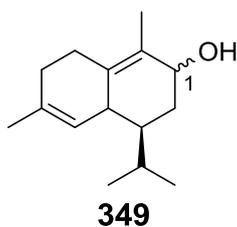
Scheme 101. Secondary product of the incubation of 8OH-FDP (**141**) with DCS.

The product was identified by comparing the recorded spectrum with the previously published ^{1}H NMR spectrum of δ-cadinene.[295, 296] Resonances present in the ^{1}H NMR spectrum of the unknown compound at 5.45, 2.62, 2.46, 2.09, 2.03, 1.68, 0.98 and 0.79 ppm were similar to the corresponding resonances observed for δ-cadinene. In addition, previously published NMR spectroscopic data of a δ-cadinene triol derivative, display a chemical shift of 4.05 ppm for the analogous proton on C1, while a chemical shift of 3.97 ppm was observed in compound **349**.[297] Unfortunately, the signal corresponding to the neighbour CH_2 was not found in the spectrum probably because the signal is overlapped with some of the impurities present in the spectrum. The resonance at 5.30 ppm was the expected signal for the proton on the hydroxyl group of **349**.

In the case of ketone **346**, the proximity of the hydroxyl group to one of the carbocations formed during the catalysis of DCS disrupted the mechanism in the last step by giving a lower energy path through the tertiary carbocation (**351**, Scheme 102) to **346**. However, not all the substrate has been transformed into ketone **346** and part of the compound has been transformed into the corresponding cadinene hydroxyl derivative **349**. A closer look to the ^{1}H NMR spectrum of the ketone **346** reveals that in the aliphatic region signals for the isopropyl have small duplicate signals. This suggests that the two enantiomers of the 8OH-FDP (**141**) are not converted at the same rate, producing different ratios of two diastereoisomers. The hydride shift necessary for the transformation of the hydroxyl group into a ketone (Scheme 102, **351**, path a) can be slow enough for one of the isomers to give time for the removal of the hydrogen to take place (Scheme 102, **349**, path b). This elimination led to the formation of a small amount of the hydroxyl derivative **349** (Scheme 102).
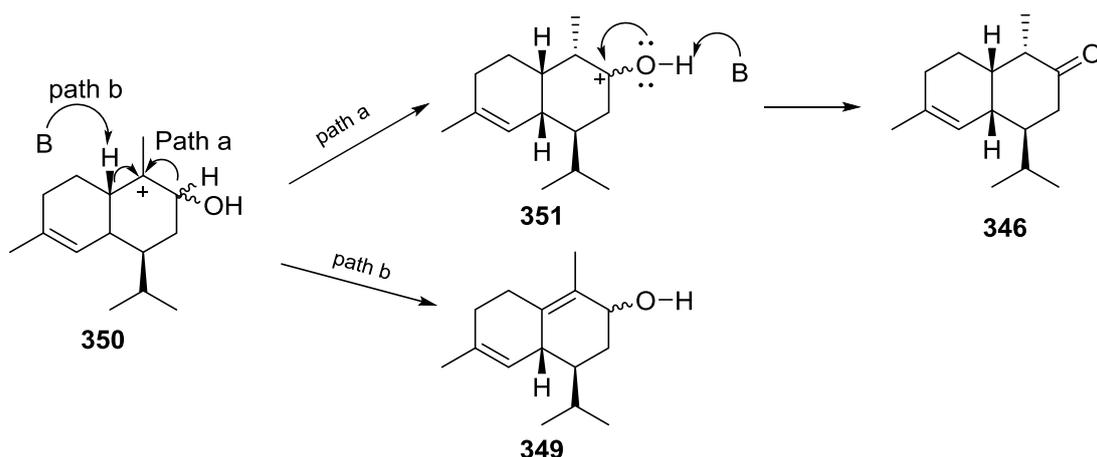
Scheme 102. Proposed mechanism for the formation of the ketone 446 and alcohol 449.

Although the products obtained do not provide any information to distinguish between the two different mechanism proposed (1,10 or 1,11 cyclisation, Scheme 21) for DCS, it reveals the previously unknown stereochemistry of the proton on C9. The stereochemistry of the proton in this position could not be determined because the proton is removed during catalysis and there are no tools to observe the carbocationic intermediate formed within the enzyme. Therefore, the FDP hydroxyl derivative provided us with a new tool to trap these intermediates in the form of carboxyl derivatives, thus allowing us to determine the stereochemistry of the intermediate. Remarkably, this is the first example in which a terpene cyclase is able to convert an alcohol into a ketone, this new ability opens new possibilities for the use of terpene synthases as synthetic tools.

4.4.4 Conclusions

10F-FDP(**290**), 8nor-FDP (**140**) and 8OH-FDP (**141**) were designed to distinguish between the two different mechanisms proposed in the literature for DCS one involving a 1,6 cyclisation and other involving a 1,10 cyclisation mechanism (Chapter 1, Scheme 21).[273]

The incubation of 10F-FDP (**290**) gave 10F-α-humelene (**300**) as single product, as it was obtained when incubated with GAS. The ability of this enzyme to induce a 1,11-cyclisation has been observed before only in enzymes that operate with a 1,10-cyclisation mechanism.

Therefore, this result suggests that this enzyme is more likely to use the 1,10-cyclisation rather than the 1,6-cyclisation pathway.

Similarly, the incubation of 8nor-FDP (**140**) with DCS gave a product containing a ten member ring **345** (Scheme 98), which supports the idea that DCS operates by 1,10 cyclisation.

Ketone **346** and alcohol **349** obtained in the incubation of 8OH-FDP (**141**) do not provide any information about the mechanism of cyclisation by DCS, but they allowed the study of an intermediate that was not studied before uncovering the stereochemistry induced by the enzyme.

Chapter 5

General conclusions

5. General conclusions

A variety of alkyl and fluorine substituted analogues of farnesyl diphosphate (FDP) were synthesised using mostly well-established methodologies. In addition, novel synthetic protocols were developed to prepare hydroxy and nor analogues of FDP, the latter sequence featuring addition of a cuprate to a highly unstable allylic bromide.

Although alkyl-modified FDP analogues have been used as inhibitors and alternative substrates surrogates for class I prenyl transferases, their competency under catalysis by sesquiterpene synthases was little known prior to this study. This research demonstrates for the first time that methylated FDPs are good/excellent substrates of sesquiterpene synthases effecting catalysis *via* initial 1,10-cyclisation of native FDP. The resulting C16 homo germacrenoids were all characterised by NMR spectroscopy and GC-MS spectroscopy. This work was further extended to the production of several fluoro-germacrenoids *via* enzymatic turnover of the corresponding fluorinated FDPs.

Even though terpene synthases catalyse mechanistically complex carbocationic reactions, often leading to polycyclic products with spectacular 3D chemical architectures, their low turnovers numbers together with the instability and volatility of their products have largely prevented their use as biocatalysts in purely synthetic efforts. Recent advances in metabolic engineering and synthetic biology have rendered engineered microbes capable of producing natural sesquiterpenoids (e.g. artemisinin) in industrial scale quantities.[264] However, these sustainable approaches do not yet support the production of non-canonical terpenes. Against this backdrop, a substantial part of this project was devoted to find novel methodologies for the *in vitro* production of unnatural terpenoids. In this project two alternative procedures are developed, (1) batch incubations and extraction with deuterated solvents,[243] and (2) incubation and extraction in continuous flow,[298] that allow production of highly valuable fluorinated and C16 homo sesquiterpenoids in the amounts needed for *in vivo* testing (Rothamsted Research) and NMR spectroscopic characterisation.

In addition, two novel mechanistic probes containing hydroxy groups, were designed to act as active site carbocationic traps, and prepared to re-investigate the reaction mechanism for the putative dual 1,10/1,6-cyclase (+)-δ-cadinene synthase (DCS).[136] This mechanistic study was carried out following the newly developed extraction protocol with deuterated solvents. Direct NMR spectroscopic analysis of the enzymatic product supports a pure 1,10-cyclisation

for DCS. Furthermore, the stereochemistry of the final cadinene cation intermediate was for the first time revealed by NOE and 2D NMR spectroscopic techniques.

The improved methodology in continuous flow has opened the possibly of utilising terpene synthases as biocatalysts to obtain and *in situ* functionalise natural/unnatural terpenes. Nevertheless, issues in scaling up the enzymatic reactions must be addressed on the future. Different techniques such as immobilisation of terpene synthases on a solid phase within a flow reactor, or batch incubations combined with supercritical CO_2 extractions of the volatile products, can be tested to further improve the methodology.

CHAPTER 6

DETAILED EXPERIMENTAL PROCEDURES

6. Synthetic procedures

6.1 General synthetic procedures

All chemicals were purchased from Sigma-Aldrich, Acros Chemicals or Alpha Aesar unless otherwise stated. Anhydrous tetrahydrofuran, diethyl ether, toluene, acetonitrile, hexane, dimethylformamide and dichloromethane were dried using molecular sieves (3 Å), previously activated at 200 ℃ for at least 24 h[299] or were obtained from a MBraun SPS800 solvent purification system. Reactions were performed under N_2 atmosphere unless otherwise stated. Glassware was cleaned and dried at 200 ℃ for at least for 16 h before use.

^{1}H NMR and [13]H NMR spectrum were measured on a Bruker Avance 500 NMR spectrometer, Bruker Avance DPX400 spectrometer, Bruker Fourier 300 and a Bruker DPX 250 spectrometer. ^{1}H NMR spectrum are reported as chemical shifts in parts per million downfield from tetramethylsilane, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, bs = broad singlet, bd = broad doublet), coupling constant, integration and assignment, respectively. All the coupling constants are reported in Hz. [13]C spectrum are reported as chemical shift downfield from tetramethylsilane. Assignments are made to the limitations of COSY, DEPT 90/135, gradient HSQC and gradient HMBC spectrum. ^{19}F and ^{31}P NMR spectrum were recorded on a Jeol Eclipse +300 NMR spectrometer or Bruker Avance 500 NMR spectrometer and are reported in chemical shift downfield from $CFCl_3$ and 85% H_3PO_4 respectively followed by multiplicity and coupling constant in Hz if appropriate. IR spectrum were recorded on a Perkin-Elmer 1600 series FTIR spectrometer and samples were prepared as thin films of neat liquid on potassium bromide discs (st = stretching) EI[+] mass spectrum were measured on a Micromass LCT premiere XE mass spectrometer. ES[-] mass spectrum were measured on a Micromass LCT premiere XE spectrometer fitted with a Waters 1525 Micro binary HPLC pump. Reverse phase HPLC was performed on a system comprising of a Dionex P680 pump and a Dionex UVD170U detector unit. GCMS was performed on a Hewlett Packard 6890 GC fitted with a J&W scientific DB-5MS column (30 m x 0.25 mm internal diameter) and a Micromass GCT Premiere detecting in the range *m/z* 50-800 in EI+ mode with scanning once a second with a scan time of 0.9 s. Injections were performed in split mode (split ratio 5:1) at 50 °C. Chromatograms were begun with an oven temperature of 50 °C rising at 4 °C min-1 for 25 min (up to 150 °C) and then at 20 °C min-1 for 5 min (250 °C final temperature).

Flash column chromatography refers to the procedure of Still[300] and vacuum chromatography was performed according to the method of Green.[301] All reactions and separations were monitored by thin layer chromatography using pre-coated aluminium plates of silica G/UV254 (Fluka) TLC. TLC plates were visualised with UV light (254 nm) and TLC stain solution: CAM (40g of ammonium pentamolybdate + 1.6 g of cerium(IV) sulfate + 800ml of diluted sulfuric acid (1:9, with water, v/v)) or phosphomolybdic acid (10% solution in EtOH).

Ion exchange resin DOWEX 40-W was received from Aldrich in H^+ form. The resin was converted into ammonium form by continuous washing with concentrated NH_4OH over 24 h and equilibrated with ion-exchange buffer (25 mM NH_4HCO_3 containing 2% i-PrOH). After use, the resin was regenerated by washing with aqueous HCl solution (5 M), water, concentrated NH_4OH and equilibrated with ion exchange buffer. Amberlyst (A26, H^+ form) was regenerated after use by thorough washing with NaOH solution (0.1 M), water and aqueous HCl solution (1 M) respectively.

Diphosphates were prepared from the corresponding alcohols using a modification of the procedure described by Davisson.[302]

6.2   Synthesis of 14Me-FDP (137)

6.2.1   Preparation of ethyl 7-methyl-3-oxo-oct-6-enoate (133)
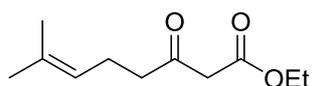
To a suspension of NaH (1.6 g 60% in mineral oil, 40.0 mmol) in anhydrous THF (33 mL) at 0 ℃ ethyl acetoacetate (3.12 g, 24.0 mmol) and then n-Buli (16.2 mL, 2.2 M in hexanes, 35.6 mmol) were added. This solution was stirred for a further 30 min then a cooled solution of 3,3-dimethylallyl bromide (3.0 g, 20.0 mmol) in anhydrous THF (10 mL) was added *via* syringe. The reaction was quenched by the addition of saturated aqueous NH_4Cl solution (20 mL). Organic material was extracted with hexane (3 x 15 mL) and the combined organic extracts were washed with water (3 x 20 mL), brine (3 x 20 mL), dried over $MgSO_4$ and concentrated under reduced pressure. Purification was performed by flash chromatography on

silica gel using a mixture of hexane and ethyl acetate (gradient of solvents from 20:1 to 10:1) as eluent. The compound was isolated as yellow oil (3.2 g, 80%).

HRMS EI[+] [M[+]] found 198.1256, $C_{11}H_{18}O_3$ requires 198.1256. **IR** KBr (cm^{-1}) 2979 (C-H st), 2916 (C-H st), 2360, 2342, 1746 (C=O st), 1717 (C=O st), 1646 (C=C st), 1446 (C-O st), 1410, 1367, 1313, 1236, 1178, 1096, 1039. **^{1}H NMR** (400 MHz, CDCl$_3$) δ 5.03 (s, 1 H, C=CH), 4.16 (q, J = 7.0, 2 H, OCH$_2$CH$_3$), 3.40 (s, 2 H, O=CCH$_2$C=O), 2.53 (t, J = 7.0, 2 H, CH$_2$CH$_2$C=O), 2.25 (dd, J = 14.0, 7.0, 2 H, C=CHCH$_2$), 1.64 (s, 3 H, CHCH$_3$), 1.58 (s, 3 H, CHCH$_3$), 1.25 (t, J = 7.0, 3 H, OCH$_2$CH$_3$).**^{13}C NMR** (101 MHz, CDCl$_3$) δ 202.7 (C=O), 167.3 (C=OOEt), 133.2 (C=CH), 122.3 (C=CH), 61.4 (C=OCCH$_2$), 49.5 (C=OCH$_2$COOEt), 43.1 (CH$_2$), 25.7 (CH$_2$), 22.3 (OCH$_2$CH$_3$), 17.7 (CH$_3$C=CH), 14.2 (CH$_3$C=CH).

6.2.2 Preparation of (*3Z*)-1-ethoxycarbonyl)-6-methylhepta-1,5-dien-2-yl trifluoromethanesulfonate (159)
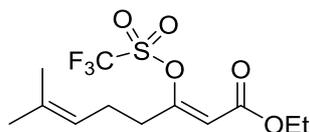
To a stirred solution of **133** (2.9 g, 14.7 mmol) in anhydrous DCM (440 mL) at 0 ºC, lithium trifluoromethane sulfonate (2.8 g, 17.7 mmol) and triethylamine (2.6 mL, 18.7 mmol) were added. After 1 h, trifluoromethane sulfonic anhydride (5.0 g, 17.7 mmol) was added to the solution and the resulting orange solution was stirred at this temperature for 1 h. The reaction was quenched by the addition of saturated aqueous NH$_4$Cl solution (70 mL). Organic material was extracted with DCM (3 x 40 mL) and the combined organic extracts were washed with saturated aqueous NaHCO$_3$ solution (3 x 100 mL) and brine (3 x 100 mL), dried over MgSO$_4$ and concentrated under reduced pressure. The crude product was purified by a flash chromatography column on silica with a mixture of hexane and ethyl acetate (30:1) as eluent. The compound was isolated as yellow oil (4.0 g, 83%).

IR: KBr (cm^{-1}) 2917 (C-H st), 2360, 2342, 1733 (C=O st), 1683 (C=C st), 1428 (C-F st), 1370 (C-O st), 1206 (S=O st), 1142, 1027, 923 (C=C-H). **^{1}H NMR** (250 MHz, CDCl$_3$) δ 5.74

(s, 1H, C=OTfCH̲C=O), 5.04 (t, J = 6.0 , 1 H, (CH$_3$)$_2$C=CH̲), 4.24 (q, J = 7.0 , 2H, OCH̲$_2$CH$_3$), 2.45-2.33 (m, 2H, CHCH$_2$CH̲$_2$), 2.26 (dd, J = 14.0, 7.0 , 2H, CHCH̲$_2$CH$_2$), 1.70 (s, 3H, HC=C(CH̲$_3$)$_2$), 1.61 (s, 3H, HC=C(CH̲$_3$)$_2$), 1.30 (t, J = 7.0 , 3H, OCH$_2$CH̲$_3$).^{13}**C NMR** (101 MHz, CDCl$_3$) δ 162.6 (C̲=O), 158.6 (C̲O), 134.6 (C̲=CH), 120.8 (C=C̲H), 112.1 (COTfC̲HCOOEt), 61.3 (OC̲H$_2$CH$_3$), 34.6 (C̲H$_2$), 25.7 (C̲H$_2$), 24.6 (OCH$_2$C̲H$_3$), 17.7 (C̲H$_3$C=CH), 14.1 (C̲H$_3$C=CH).^{19}**F NMR** (300 MHz, CDCl$_3$) δ -74.7.

6.2.3 Preparation of (2*E*)-ethyl 3-ethyl-7-methylocta-2,6-dienoate (134)
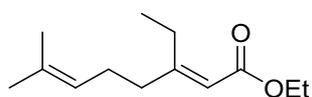
To a stirred suspension of CuI (9.2 g, 48.8 mmol) in anhydrous THF (140 mL) at 0 ºC, under argon atmosphere, ethyl magnesium bromide (16 mL, 3M, 48.8 mmol) was added. The resulting black solution was stirred for 30 min. Then the solution was cooled to -78 ºC and the triflate **159** (4.0 g, 12.2 mmol) was added. After 1 h the reaction was completed and was quenched by the addition of saturated aqueous NH$_4$Cl solution (50 mL). The copper salts formed during the quench were dissolved by adding NH$_4$OH (35%, 50 mL) to the biphasic mixture and gentle stirring for 1 h. The blue coloured aqueous layer was extracted with diethyl ether (3 x 50 mL). Combined organic extracts were washed with 10% aqueous NH$_4$OH solution (3 x 100 mL), water (2 x 100 mL) and brine (3 x 100 mL). The organic material was dried over MgSO$_4$ and concentrated under reduced pressure. The crude product was purified by flash chromatography with a mixture of hexane and ethyl acetate (gradient from 30:1 to 20:1). The title compound was isolated as yellow oil (3.3 g, 88%).

HRMS EI$^+$ [M] found 210.1615, C$_{13}$H$_{22}$O$_2$ requires 210.1620. **IR**: KBr (cm^{-1}) 2972 (C=C-H st), 2931 (C-H st), 2360, 2342, 1716 (C=O st), 1644 (C=C st), 1456, 1378 (C-O st), 1309, 1274, 1205, 1144, 1039. ^{1}**H NMR** (250 MHz, CDCl$_3$) δ 5.54 (s, 1 H, (Et)C=C(CH̲C=OOEt), 5.02 (s, 1 H,(CH$_3$)$_2$C=CH̲), 4.07 (q, J = 7.0, 2 H, OCH̲$_2$CH$_3$), 2.55 (q, J = 7.5, CH̲$_2$CH$_3$ 2 H), 2.09 (bs, 4 H, 2 x CH$_2$), 1.62 (s, 3 H, HC=CCH̲$_3$), 1.54 (s, 3 H, HC=CCH̲$_3$), 1.21 (t, J = 7.0, 3 H, OCH$_2$CH̲$_3$), 1.00 (t, J = 7.5, 3 H, CH$_2$CH̲$_3$). ^{13}**C NMR** (63 MHz, CDCl$_3$) δ 166.6 (C̲=OOEt), 165.7 (EtC̲C=CH), 132.6 (C̲(CH$_3$)$_2$), 123.3 (C̲HC=OOEt), 114.9 (C=C̲H), 59.6

(OCH$_2$), 38.1 (CH$_2$), 26.4 (CH$_2$), 25.8 (<u>C</u>H$_2$CH$_3$), 25.5 (OCH$_2$<u>C</u>H$_3$), 17.8 (<u>C</u>H$_3$C), 14.4 (<u>C</u>H$_3$C), 13.1 (CH$_2$<u>C</u>H$_3$).

6.2.4   Preparation of (*E*)-3-ethyl-7-methylocta-2,6-dien-1-ol (160)
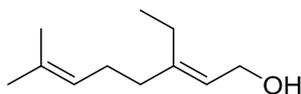
To a stirred solution of the ester **134** (2.3 g, 10.1 mmol) in anhydrous toluene (50 mL), diisobutylaluminiumhydride (1.5 M in toluene, 20.2 mL, 30.3 mmol) was added. The solution was kept at this temperature and stirred for 1 h. The reaction was diluted with DCM (30 m) and quenched by the addition of HCl (2M, 40 mL). This mixture was warmed up to room temperature and stirred vigorously for 16 h. The clear aqueous layer was extracted with DCM (3 x 50 mL), the combined organic extracts were washed with water (3 x 50 mL) and brine (3 x 50 mL). The organic extracts were dried over MgSO$_4$ and concentrated under reduced pressure. The crude product was purified by flash chromatography column with a mixture of hexane and ethyl acetate (10:4) as eluent. The title compound was isolated as yellow oil (1.5 g, 85%).

HRMS EI$^+$ [M$^+$ + Na] found 191.1412, C$_{11}$H$_{20}$ONa requires 191.1410. **^{1}H NMR** (250 MHz, CDCl$_3$) δ 5.36 (t, *J* = 7.0, 1 H, C(Et)=C<u>H</u>COH), 5.10 (m, 1 H, (CH$_3$)$_2$C=C<u>H</u>), 4.15 (d, *J* = 7.0, 2 H, C<u>H</u>$_2$OH), 2.06 (m, 6 H, 2 x CH$_2$, C<u>H</u>$_2$CH$_3$), 1.68 (s, 3 H, CHC<u>H</u>$_3$), 1.60 (s, 3 H, CHC<u>H</u>$_3$), 0.98 (t, *J* = 7.5, 3 H, CH$_2$C<u>H</u>$_3$). **^{13}C NMR** (63 MHz, CDCl$_3$) δ 145.8 (C(Et)), 131.8 ((CH$_3$)$_2$<u>C</u>=C), 124.2 (<u>C</u>HCH$_2$OH) , 122.9 (CH), 59.2 (CH$_2$OH), 36.5 (CH$_2$), 26.7 (CH$_2$), 25.8 (<u>C</u>H$_2$CH$_3$), 23.7 (CH$_2$<u>C</u>H$_3$), 17.8 (<u>C</u>H$_3$CH), 13.8 (<u>C</u>H$_3$CH).

6.2.5   Preparation of (*E*)-1-bromo-3-ethyl-7-methylocta-2,6-diene (161)
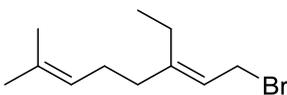
To a stirred solution of the alcohol **160** (1.5 g, 9.1 mmol) in anhydrous THF (46 mL) at -45 ºC was added a freshly distilled Et$_3$N (2.5 mL, 18.3 mmol) and methanosulfonyl chloride (1.3 g, 11.8 mmol). After 45 min LiBr (3.2 g, 36.7 mmol) was added, then the mixture was warmed to 0 ºC and left at this temperature for 1 h. The mixture was diluted with hexane (20 mL) and quenched by the addition of saturated NH$_4$Cl solution (20 mL). Organic material was extracted with hexane (3 x 20 mL), combine organic extracts were washed with saturated aqueous NaHCO$_3$ solution (3 x 20 mL), water (3 x 20 mL) and brine (3 x 20 mL), dried over MgSO$_4$ and concentrated under reduced pressure. The crude reaction was analysed by ^{1}H NMR spectroscopy and used immediately in the next step without further purification.

^{1}H NMR (400 MHz, CDCl$_3$) δ 5.49 (t, *J* = 8.5, 1 H, C**H**CH$_2$CBr), 5.09 (s, 1 H, (CH$_3$)$_2$C=C**H**), 4.04 (d, *J* = 8.5, 2 H, CH$_2$Br), 2.26 -1.93 (m, 6 H, 2 x CH$_2$, C**H**$_2$CH$_3$), 1.69 (s, 3 H, CHC**H**$_3$), 1.60 (s, 3 H, CHC**H**$_3$), 1.04 (t, *J* = 7.5, 3 H,CH$_2$C**H**$_3$). Data in agreement with previously published data by Rawat *et al.*[199]

6.2.6 Preparation of (*E*)-ethyl 7-ethyl-11-methyl-3-oxododeca-6,10-dienoate (162)
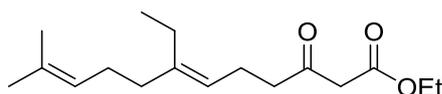
To a suspension of NaH (0.7 g 60% in mineral oil, 31.4 mmol) in anhydrous THF (24 mL) at 0 ºC, ethyl acetoacetate (3.7 g, 28.6 mmol) was added and the solution was stirred for 20 min. Then n-Buli (2.2 M in hexanes, 13.4 mL, 29.5 mmol) was added and this solution was further stirred for 30 min. A cooled solution of the crude bromide **161** (2.2 g) in anhydrous THF (5 mL) was added to the orange-red solution. After 1 h the reaction was quenched by the addition of saturated aqueous NH$_4$Cl solution (15 mL). Organic material was extracted with hexane (3 x 15 mL) and the combined organic extracts were washed with water (3 x 15 mL) and brine (3 x 15 mL). The organic material was dried over MgSO$_4$ and concentrated under reduced pressure. Purification was performed by flash chromatography column with a

mixture of hexane and ethyl acetate (gradient of solvents from 15:1 to 10:1) as eluent. The product was isolated as yellow oil (2.0 g, 78% yield).

HRMS ES$^+$ [M-CO$_2$Et] found 208.1836, C$_{14}$H$_{24}$O requires 208.1827. ^{1}**H NMR** (400 MHz, CDCl$_3$) δ 5.02-4.96 (m, 2 H, 2 x C=CH), 4.14 (q, 2 H, J = 7.0, C=OOC$\underline{H}_2$CH$_3$), 3.36 (s, 2 H, C=OC$\underline{H}_2$C=O), 2.51 (t, J = 8.5, 2 H CH$_2$C$\underline{H}_2$C), 2.25 (q, 2 H, J = 7.0, C$\underline{H}_2$CH$_3$), 1.98-1.94 (m, 6 H, 3 x CH$_2$), 1.6 (s, 3 H, CC$\underline{H}_3$), 1.53 (s, 3 H, CC$\underline{H}_3$), 1.21 (t, J = 7.0 , 3 H, COOCH$_2$C$\underline{H}_3$), 0.89 (t, J = 7.5 , 3 H, CH$_2$C$\underline{H}_3$).^{13}**C NMR** δ (126 MHz, CDCl$_3$) δ 202.5 ($\underline{C}$=OOEt), 167.2 (C=O), 142.7 ($\underline{C}$=CH), 131.5 ($\underline{C}$=CH), 124.4 (C=$\underline{C}$H), 121.8 (C=$\underline{C}$H), 61.4 (C=OO$\underline{C}$H$_2$CH$_3$), 49.5 (C=O$\underline{C}$H$_2$CO$_2$Et), 43.5 ($\underline{C}$H$_2$CH$_3$), 36.6 (CH$_2$), 27.0 (CH$_2$), 25.7 (CH$_2$), 23.3 (CH$_2$), 22.0 (COOCH$_2$$\underline{C}H_3$) , 17.8 (C$\underline{C}H_3$), 14.2 (C$\underline{C}H_3$), 13.2 (CH$_2$$\underline{C}H_3$).

6.2.7 Preparation of (*1Z,5E*)-1-(ethoxycarbonyl)-6-ethyl-10-methylundeca-1,5,9-trien-2-yl diethyl phosphate (163)
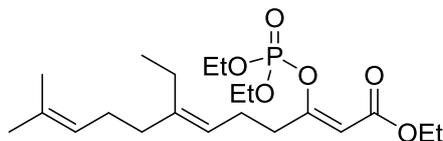
To a suspension of NaH (100 mg 60% mineral oil, 2.5 mmol) at 0 ºC in anhydrous diethyl ether (12 mL), **162** (0.5 g, 1.8 mmol) was added and the resulting solution was stirred for 20 min. Diethyl chlorophosphate (0.5 g, 2.8 mmol) was added to the colourless solution. After 1 h the reaction was quenched by the addition of saturated aqueous NH$_4$Cl solution (5 mL). Organic material was extracted with diethyl ether (3 x 5 mL), combine organic extracts were washed with saturated aqueous NaHCO$_3$ solution (3 x 10 mL) and brine (3 x 10 mL). The organic material was dried over MgSO$_4$ and concentrated under reduce pressure. The crude was analysed by ^{1}H NMR, judged sufficiently pure and used immediately without further purification.

^{1}**H NMR** (500 MHz, CDCl$_3$) δ 5.34 (s, 1 H, C=C$\underline{H}$C=OOEt). 5.10-5.4 (m, 2 H, 2 x C=CH), 4.31 – 4.19 (m, 4 H, (CH$_3$C$\underline{H}_2$)$_2$P=O), 4.14 (q, J = 7.0, 2 H, OC$\underline{H}_2$CH$_3$), 2.45 (t, J = 7.5, 2 H, CH$_2$), 2.28 (dd, J = 15.0, 7.0, 2 H, CH$_2$), 2.10-1.98 (m, 6 H, 3 x CH$_2$), 1.67 (s, 3H, CH$_3$), 1.59

(s, 3H, CH$_3$), 1.35 (t, J = 7.0, 6 H, (C$\underline{H}$$_3CH_2$)$_2$P=OO), 1.26 (t, J = 7.0, 3 H, OCH$_2$C$\underline{H}$$_3$), 0.95 (t, J = 7.5, 3 H, CH$_2$C$\underline{H}$$_3$).

6.2.8 Preparation of (2E,6E)-ethyl 7-ethyl-3,11-dimethyldodeca-2,6,10-trienoate (164)

This methylation was performed according to the method described by Coates[303]. To a stirred suspension of CuI (1.4 g, 7.0 mmol) in anhydrous ether (7 mL) at 0 ºC under Ar atmosphere, MeLi (1.6 M in hexane, 9 mL, 14.4 mmol) was added. After 20 min the reaction mixture was cooled to -78 ºC and a solution of the compound **163** (1.1 g of crude) in anhydrous ether (7 mL) was added *via* syringe over 5 min. After 2 h the reaction was warmed to -45 ºC and MeI (0.5 mL, 1.2 mmol) was added. The solution was stirred for another 45 min at this temperature. Reaction was quenched by the addition of saturated aqueous NH$_4$Cl solution (8 mL). The copper salts formed during quench were dissolved by diluting the reaction with ether and addition of NH$_4$OH (35%, 50 mL), then the biphasic mixture was gentle stirred for 1 h. Organic material was extracted from the blue colour aqueous layer with ether (3 x 20 mL) and the combined organic extracts were washed with 10% aqueous NH$_4$OH solution (3 x 25 mL), water (3 x 25 mL), brine (3 x 25 mL), dried over MgSO$_4$ and concentrated under reduced pressure. The crude product was purified by flash column chromatography with a mixture of hexane and ethyl acetate (10:1) as eluent. The product was isolated as yellow oil (0.3 g, 65%).

HRMS ES$^+$ [M] found 278.2243, C$_{18}$H$_{20}$O$_2$ requires 278.2246. **^{1}H NMR** (250 MHz, CDCl$_3$) δ 5.72-5.60 (s, 1 H, C$\underline{H}$C=OOEt), 5.16-4.96 (m, 2 H, 2 x C=C$\underline{H}$), 4.10 (q, J = 7.0, 2 H, C=OOC$\underline{H}$$_2CH_3$), 2.23-2.10 (bs, 6 H, 3 x CH$_2$), 2.09-1.91 (m, 5 H, C$\underline{H}$$_3$C=CH, CH$_2$), 1.73-1.63 (s, 3 H, (C$\underline{H}$$_3$)$_2$C=CH), 1.63-1.54 (s, 3 H (C$\underline{H}$$_3$)$_2$C=CH), 1.24 (t, J = 7.0, 3 H, C=OOCH$_2$C$\underline{H}$$_3$), 1.03-0.83 (t, J = 7.5, 3 H, CH$_2$C$\underline{H}$$_3$). **^{13}C NMR** (63 MHz, CDCl$_3$) δ 167.0 ($\underline{C}$=OOEt), 159.8 ($\underline{C}$=CH), 142.1 ($\underline{C}$=CH), 131.5 ($\underline{C}$=CH), 124.5 (C=$\underline{C}$H), 122.5 (C=$\underline{C}$H),

115.7 (C=$\underline{C}$H), 59.6 (O$\underline{C}$H$_2$CH$_3$), 41.4 (CH$_2$), 36.6 (CH$_2$), 27.0 (CH$_2$), 25.8(CH$_2$), 23.3 (CH$_3$$\underline{C}$=CH), 19.0 ($\underline{C}H_3$)$_2$C=CH), 17.8 ($\underline{C}H_3$)$_2$C=CH), 14.5 C=OOCH$_2$$\underline{C}H_3$, 13.3 CH$_2$$\underline{C}H_3$).

6.2.9 Preparation of (*2E, 6E*)-7-ethyl-3,11-dimethyldodeca-2,6,10-trien-1-ol (165)
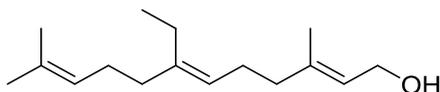
To a stirred solution of the ester **164** (300 mg, 1.0 mmol) in anhydrous toluene (6 mL) at -78 ºC, diisobutylaluminium hydride (1.5 M, 2.3 mL, 3.4 mmol) was added. The mixture was stirred for 1 h. The mixture was diluted with DCM (20 mL) and quenched with HCl (2 M, 20 mL). The biphasic mixture was stirred for 16 h at room temperature. Organic material was extracted with DCM (3 x 20 mL) and the pooled organic extracts were washed with brine (3 x 40 mL), dried over MgSO$_4$ and concentrated under reduce pressure. The crude product was purified by flash chromatography column using a mixture of hexane and ethyl acetate (2:1) as eluent. The title compound was isolated as yellow oil (150 mg, 60% yield).

HRMS ES$^+$ [M] found 236.2118, C$_{16}$H$_{28}$O requires 236.2140. **^{1}H NMR** (250 MHz, CDCl$_3$) δ 5.48-5.35 (t, *J* = 7.5, 1 H, C=CH), 5.17-5.00 (m, 2 H C=CH, C=C$\underline{H}$CH$_2$OH), 4.22-4.09 (d, 2 H, C$\underline{H}_2$OH), 2.22-1.93 (m, 10 H, 4 x CH$_2$, C$\underline{H}_2$CH$_3$), 1.72-1.65 (bs, 6 H, (CH$_3$)$_2$C=C, CH$_3$C=C), 1.63-1.57 (s, 3 H, (CH$_3$)$_2$C=C), 1.02-0.89 (t, *J* = 7.5, 3 H, C$\underline{H}_3$CH$_2$). **^{13}C NMR** (63 MHz, CDCl$_3$) δ 141.4 ($\underline{C}$=CH), 139.9 ($\underline{C}$=CH), 131.4 ($\underline{C}$=CH), 124.6 (C=$\underline{C}$H), 123.5 (C=$\underline{C}$H), 123.4 (C=$\underline{C}$H), 59.5 (CH$_2$OH), 40.0 ($\underline{C}$H$_2$CH$_3$), 36.6 (CH$_2$), 27.1 (CH$_2$), 26.1 (CH$_2$), 25.8 (CH$_2$), 23.3 ($\underline{C}$H$_3$C=C), 17.8 (($\underline{C}$H$_3$)$_2$=C), 16.4 (($\underline{C}$H$_3$)$_2$=C), 13.4 ($\underline{C}$H$_3$CH$_2$).

6.2.10 Preparation of (*2E,6E*)-7-ethyl-3,11-dimethyldodeca-2,6,10-trien-1-yl acetate (352)
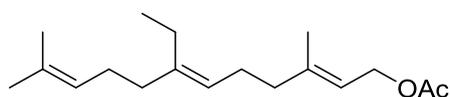
To a stirred solution of alcohol **165** (15 mg, 0.06 mmol) in DCM (1 mL) at room temperature in an open flask, pyridine (20 μL, 0.25 mmol) and acetic anhydride (18 μL, 0.2 mmol) were added. The mixture was stirred overnight. The reaction was diluted with DCM (12 mL) and quenched by the addition of 10% HCl aqueous solution (8 mL). Organic material was extracted with DCM (2 x 15 mL), combined organic extracts were washed with saturated $NaHCO_3$ aqueous solution (3x 10 mL), brine (3 x 20 mL), dried over Na_2SO_4 and solvent was removed under reduce pressure. The crude product was purified by clash chromatography column with mixture of hexane and ethyl acetate (40:1) as eluent to afford the product as pale yellow oil (16 mg, 90%).

HRMS EI$^+$ [M] found 278.2250, $C_{18}H_{30}O_2$ requires 278.2246. **^{1}H NMR** (400 MHz, CDCl$_3$) δ 5.35 (t, J = 8.0, 1H, C=CH), 5.08 (m, 2 H, 2 x C=CH), 4.59 (d, J = 7.0, 2 H, CH$_2$OAc), 2.22 – 1.93 (m, 13 H, 5 x CH$_2$, OC=OCH$_3$), 1.70 (s, 3 H, CH$_3$), 1.68 (s, 3 H, CH$_3$), 1.60 (s, 3 H, CH$_3$), 0.95 (t, J = 7.5, 3 H, CH$_2$CH$_3$). **^{13}C NMR** (63 MHz, CDCl$_3$) δ 171.3 (OC=OCH$_3$), 141.5 (C=CH), 139.7 (C=CH), 131.4 (C=CH), 124.6 (C=CH), 123.3 (C=CH), 118.4 (C=CH), 61.5 (CH$_2$OAc) , 40.7 (OC=OCH$_3$), 40.0 (CH$_2$), 36.6 (CH$_2$), 27.1 (CH$_2$), 25.8 (CH$_2$), 23.3 (CH$_2$), 21.2 (CH$_3$), 17.8 (CH$_3$), 16.6 (CH$_3$), 13.4 (CH$_3$).

6.2.11 Preparation of a mixture of (*E*)-β -, (*Z*)-α - and (*E*)- α- 14Me-farnesenes

353   **354**   **355**
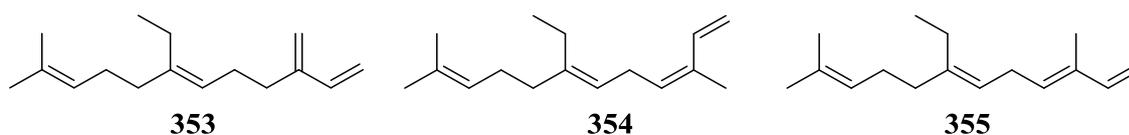
The following procedure is similar to the one reported by Keinan.[304] To a stirred solution of acetate **352** (10 mg, 0.04 mmol) in anhydrous THF (1 mL) at room temperature, Tetrakis(triphenylphosphine)palladium (5 mg, 0.005 mmol) was added and the solution was stirred for 4 h. Reaction mixture was diluted in hexane (3 mL). The resulting solution was passed through short pad of silica. Farnesene mixture was eluted with hexane (5 mL) and solvent was removed by continuous evaporation with stream of N_2 to afford the title mixture of compounds (3 mg, 38%). The oil was dissolved in pentane and analysed by GC-MS.

6.2.12 Preparation of (*2E,6E*)-1-tris-(ammonium)-diphosphate -7-ethyl-3,11-dimethyldodeca-2,6,10-triene (137)
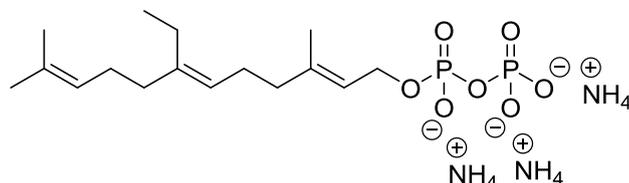
To a stirred solution of the alcohol **165** (150 mg, 0.6 mmol) in anhydrous DMF (8 mL) at 0 °C, S-collidine (0.5 mL, 3.8 mmol) and methanesulphonyl chloride (0.07 mL, 0.9 mmol) were added sequentially. After 20 min of stirring at this temperature, lithium chloride (0.4 g, 9.6 mmol) was added to the solution. After 2 h the milky solution was quenched by the addition of cold water (6 mL). The biphasic mixture was diluted with pentane. Organic material was extracted with pentane (3 x 5 mL), organic extracts together were washed with saturated aqueous CuSO$_4$ solution (3 x 15 mL), water (3 x 15 mL), saturated aqueous NaHCO$_3$ solution (3 x 15 mL), dried over MgSO$_4$ and concentrated under reduced pressure. The crude was used in the next step without further purification.

Tris-(tetrabutylammonium) hydrogendiphosphate (1.2 g, 1.3 mmol) was placed in a round bottom flask and then a solution of crude allylchloride (170 mg) in acetonitrile (2 mL) was added. The reaction was stirred at room temperature for 16 h. Then the acetonitrile was concentrated under reduce pressure. The remaining yellow oil was dissolved in buffer (4 mL, 25 mM NH$_4$HCO$_3$, 2% isopropanol) and pass thought an ion exchange column DOWEX 40 W (NH$_4$$^+$ form). The ion exchange column was monitored by TLC using a mixture of isopropanol / buffer / NH$_4$OH (6:2:2) as eluent. The fractions containing product were collected and freeze dried. The yellow solid was triturated with dry methanol and concentrated. The yellow solid was washed with CHCl$_3$ and the remaining white solid was dried under reduce pressure. The white solid was crystallised in methanol affording white crystals (85 mg, 39% yield over two steps).

HRMS ES$^-$ [M + 2H] found 395.1400, C$_{16}$H$_{29}$O$_7$P$_2$ requires 395.1389. **¹H NMR** (500 MHz, D$_2$O) δ 5.46-5.34 (m, 1 H, C=CH), 5.20-5.06 (m, 2 H, C=CH, C=CHCH$_2$OH), 4.49-4.36 (m,

2 H, CH$_2$OPP), 2.19-1.88 (m, 10 H, C$\underline{H}_2$CH$_3$, 4 x CH$_2$), 1.70-1.54 (m, 9 H, 2 x (CH$_3$)$_2$=C, CH$_3$C=C), 0.94-0.87 (m, 3 H C$\underline{H}_3$CH$_2$). ^{31}**P NMR** (202 MHz, MeOD) δ -9.00 – -9.32 (d, J = 19.0), -9.59 – -9.91 (d, J = 19.0). Data in agreement with previously published data by Rawat *et al.*[199]

6.3   Synthesis of 15Me-FDP (138)

6.3.1   Preparation of (*E*)-ethyl 7,11-dimethyl-3-oxododeca-6,10-dienoate (177)

To a suspension of NaH (60% dispersion in mineral oil, 1.7 g, 46.2 mmol) in anhydrous THF (30 mL) at 0 ºC, a solution of ethyl acetoacetate (5.0 g, 4.9 mL, 39 mmol) in anhydrous THF (20 mL) was added, followed, after 30 min, by the addition of *n*-BuLi (2.5 M solution in hexanes, 18.3 mL, 46.2 mmol). The resulting mixture was stirred for 30 min and then geranyl bromide (3.0 g, 14 mmol) was added drop-wise *via* syringe. The resulting mixture was allowed to stir for 1h and then the reaction was quenched by addition of saturated aqueous NH$_4$Cl solution (100 mL, 0.1 M). Organic material was extracted with hexane (2 x 50 mL), combine organic extracts were washed with water (2 x 50 mL), brine (50 mL) and dried over MgSO$_4$. Solvent was removed under reduced pressure and the yellow residue was purified by flash chromatography column with a mixture of hexane and ethyl acetate (20:1) as eluent to afford the title compound as colourless oil (14.8 g, 81%).

HRMS ES$^+$ [M] found 266.1888. C$_{16}$H$_{26}$O$_3$ requires 266.1882. ^{1}**H NMR** δ (500 MHz, CDCl$_3$) 5.1 (m, 2 H 2 x C=CH), 4.23 (q, 2 H, J = 7.0, OC$\underline{H}_2$CH$_3$), 3.4 (s, 2 H O=CC$\underline{H}_2$C=O), 2.6 (t, 2 H, J = 7.0, CH$_2$), 2.3 (m, 2 H, CH$_2$), 2.1 (m, 2 H CH$_2$), 2.0 (t, 2 H, J = 7.0, C$\underline{H}_2$), 1.7 (s, 1 H, CC$\underline{H}_3$), 1.65 (s, 1 H, CC$\underline{H}_3$), 1.63 (s, 1 H CC$\underline{H}_3$), 1.32 (t, 3 H, J = 7.0, OCH$_2$C$\underline{H}_3$). ^{13}**C NMR** δ (125 MHz, CDCl$_3$) 209.1 ($\underline{C}$=OOEt), 168.1 (C=O), 136.8 ($\underline{C}$=CH), 131.5 ($\underline{C}$=CH), 124.1 (C=$\underline{C}$H), 122.1 (C=$\underline{C}$H), 61.3 (C=O$\underline{C}$H$_2$C=OOEt), 49.4 (CH$_2$), 43.0 (CH$_2$), 39.6 (CH$_2$), 26.6 (CH$_2$), 25.7 (CH$_3$), 22.2 (CH$_2$), 17.8 (CH$_3$), 15.6 (CH$_3$), 14.1 (CH$_3$).

6.3.2 Preparation of (*2E,6E*)-ethyl 7,11-dimethyl-3-ethyldodeca-2,6,10-trienoate (179)

To a solution of β-keto ester **5.15** (290 mg, 1.1 mmol) and lithium trifluoromethanesulfonate (340 mg, 2.2 mmol) in anhydrous DCM (25 mL) at 0 °C, Triethylamine (0.3 mL, 2.4 mmol) and trifluoromethanesulfonic anhydride (0.4 mL, 2.4 mmol) were added and the mixture was stirred at 0 °C for 2 h. Saturated aqueous NH₄Cl solution was added (20 mL) and then the organic material was extracted with DCM (3 x 10 mL), combine organic extracts were washed with water (30 mL) and brine (30 mL) before drying over MgSO₄ solvent evaporation under reduced pressure. The vinylic triflate (**178**) was isolated as dark oil which was used in the next step without further purification.

To a stirred suspension of CuI (230 mg, 1.2 mmol) in anhydrous THF (20 mL) at 0 °C under argon atmosphere, ethyl magnesium bromide (2.0 M in diethyl ether, 0.6 mL, 1.2 mmol) was added and the mixture was stirred for 30 min. The stirred opaque black solution was then cooled to -78 °C. A solution of the enol triflate (400 mg, 1.0 mmol) in anhydrous THF (10 mL) was added *via* a cannula and the reaction was stirred at this temperature for 2.5 h. The reaction was quenched by the addition of saturated aqueous NH₄Cl solution (20 mL). Resulting emulsions were dissolved by diluting with diethyl ether (40 mL), addition of concentrated NH₄OH (30 mL) and stirring over night. Organic material was extracted with ethyl acetate (3 x 10 mL) and the pooled organic extracts were washed with water (2 x 30 mL), brine (30 mL), dried over MgSO₄ and solvent was removed under reduce pressure. Crude product was purified by flash chromatography column with a mixture of hexane and ethyl acetate (20:1) as eluent. The title compound was isolated as colourless oil (120 mg, 41% over two steps).

HRMS ES⁺ [M + H] found 279.2336. $C_{18}H_{31}O_2$ requires 279.2324. **¹H NMR** δ (500 MHz, CDCl₃) 5.49 (s, 1 H, C=CH), 4.98 (m, 2 H, C=CH), 4.03 (q, 2 H, J = 7.0, OC$\underline{H}_2$CH₃), 2.53 (q, 2 H, J = 7.5, CH₃C$\underline{H}_2$), 1.85 - 2.05 (m, 6 H, 3 x CH₂), 1.95 (t, 2 H, J = 7.0 CH₂), 1.65 (s, 3 H, CCH₃), 1.48 (s, 3 H, CCH₃), 1.46 (s, 3 H, CCH₃), 1.16 (t, 3 H, J = 7.0, OCH₂C$\underline{H}_3$), 0.9 (t, 3 H, J = 7.5, CH₂C$\underline{H}_3$). **¹³C NMR** δ (125 MHz, CDCl₃) 166.5 (C=O), 165.5 ($\underline{C}$=CH), 136.1 ($\underline{C}$=CH), 131.2 ($\underline{C}$=CH), 124.2 (C=$\underline{C}$H), 123.0 (C=$\underline{C}$H), 114.9 (C=$\underline{C}$H), 59.4 O$\underline{C}$H₂CH₃),

39.7 (CH$_2$), 38.0 (CH$_2$), 26.7 (CH$_2$), 26.1 (CH$_2$), 25.7 (CH$_3$), 25.3 (CH$_2$), 17.7 (CH$_3$), 16.0 (CH$_3$), 14.3 (CH$_3$), 13.0 (CH$_3$).

6.3.3 Preparation of (*2E,6E*) 7,11-dimethyl-3-ethyldodeca-2,6,10-trien-1-ol (180)

To a stirred suspension of ethyl ester **179** (145 mg, 0.5 mmol) in anhydrous THF (10 mL) at - 78 °C was added DIBAL-H (1.5 M in toluene, 870 µL, 1.3 mmol), the solution was stirred at this temperature for 2 h. The reaction was quenched by addition of HCl (2M, 10 mL). Organic material was extracted with diethyl ether (2 x 10 mL) and the combine organic extracts were washed with water (20 mL), brine (20 mL) and dried over MgSO$_4$. Solvent was removed under reduced pressure. Crude product was purified by flash chromatography column with hexane and ethyl acetate (4:1) as eluent to give the title compound as colourless oil (86 mg, 69% yield).

HRMS ES$^+$ [M] found 236.2130, C$_{16}$H$_{28}$O requires 236.2140. **^{1}H NMR** (400 MHz, CDCl$_3$) δ 5.38 (t, J = 7.0 , 1 H, C=CH), 5.20 – 5.01 (m, 2 H, 2 x C=CH), 4.16 (d, J = 7.0, 2 H, C$\underline{H}_2$OH), 2.22 – 1.93 (m, 10 H, 5 x CH$_2$), 1.68 (s, 3 H, CCH$_3$), 1.60 (s, 6 H, 2 x CCH$_3$), 0.99 (t, J = 7.5, 3 H, C$\underline{H}_2$CH$_3$). **^{13}C NMR** δ$_C$ (62.5 MHz, CDCl$_3$) 141.3 ($\underline{C}$=CH), 139.8 ($\underline{C}$=CH), 131.3 ($\underline{C}$=CH), 124.4 (C=$\underline{C}$H), 123.3 (C=$\underline{C}$H), 123.3(C=$\underline{C}$H), 59.5 (CH$_2$OH), 39.9 (CH$_3$$\underline{C}H_2$), 36.5 (CH$_2$), 26.9 (CH$_2$), 26.0 (CH$_2$), 25.7 (CH$_2$), 23.2 ($\underline{C}H_3$C=C), 17.7 ($\underline{C}H_3$)$_2$C=C, 16.4 ($\underline{C}H_3$)$_2$C=C, 13.4 ($\underline{C}H_3CH_2$).
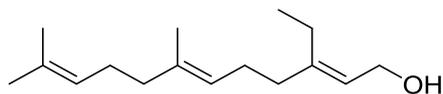
6.3.4 Preparation of trisammonium (*2E,6E*)-3-ethyl-7,11-dimethyldodeca-2,6,10-trienyl diphosphate (138)
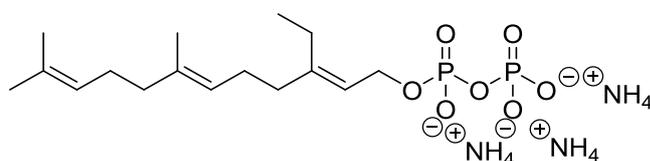
To a stirred solution of the alcohol **180** (110 mg, 0.5 mmol) in anhydrous DMF (6.2 mL) at 0 ℃, LiCl (0.3 g, 7.3 mmol), S-collidine (350 μL, 0.28 mmol) and mesylchloride (54 μL, 0.69 mmol) were added. The milky solution formed after 15 min was stirred for an additional 2.5 h. The mixture was diluted in cold pentane (4 mL) and cold water (25 mL). Organic material was extracted with pentane (3 x 10 mL), combine organic extracts were washed with saturated solution of $CuSO_4$ (3 x 10 mL), saturated aqueous $NaHSO_4$ solution (2 x 10 mL), brine (2 x 10 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure and the crude product was used in the next step without further purification.

To a solution of the crude allylic chloride in anhydrous CH_3CN (1.3 mL), tris-(tetrabutylammonium) hydrogendiphosphate (1 g, 1.1 mmol) was added and the mixture was stirred at room temperature for 15 h. Solvent was removed under reduced pressure and the residue was dissolved in ion-exchange buffer (25 mM NH_4HCO_3 containing 2% i-PrOH, 1 mL). This solution was slowly passed through an ion exchange column DOWEX 50W-X8 (100-200 mesh) (NH_4^+ form) that had been pre-equilibrated with ion exchange buffer. Once ion exchange was complete, fractions containing product (as judged by TLC run using as eluent a mixture of i-PrOH:c.NH_3:H_2O 6:3:1) were lyophilised to dryness. The white solid was extracted with MeOH (3 x 10 mL) and the MeOH concentrated affording yellow solid which was cleaned with diethyl ether (3 x 3 mL) to afford pure white solid (34 mg, 18%).The pellet was further purified by reverse-phase HPLC (150 × 21.2 mm Phenomenex Luna column, eluting with 10% B for 20 min, then a linear gradient to 60% B over 25 min and finally a linear gradient to 100% B over 5 min.; solvent A: 25 mM NH_4HCO_3 in water, solvent B: CH_3CN, flow rate 5.0 mL/min, detecting at 220 nm). Once purification was complete the solution was again lyophilised to dryness giving the title compound as a fluffy white solid (68 mg, 36% yield).

HRMS ES⁻ [M + 2D] found 395.1403, $C_{16}H_{29}O_7P_2$ requires 395.1389. **¹H NMR** (500MHz, D_2O) δ 5.32 (m, 1 H, C=C*H*), 5.10 (m, 1 H, C=C*H*), 5.06 (m, 1 H C=C*H*), 4.36 (m, 1 H C*H*₂OH), 1.88 – 2.09 (m, 10 H, 5 x C*H*₂), 1.55 (s, 3 H, C*H*₃), 1.49 (s, 6 H, 2 x C*H*₃), 0.86 (t, 3 H, J = 7.5, C*H*₃CH₂). **³¹P NMR** δ (202.5 MHz, D_2O) -10.41 (d, J_{PP} = 22.5), -8.30 (d, J_{PP} = 22.5). Data in agreement with previously published by Gibbs *et al.*[305]

6.4 Synthesis of 14,15-diMeFDP (139)

6.4.1 Preparation of (*2E,6E*)-ethyl 3,7-diethyl-11-methyldodeca-2,6,10-trienoate (189)
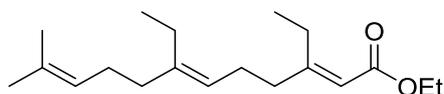
To a stirred solution of β-keto ester **162** (0.35 g, 1.5 mmol) and lithium trifluoromethanesulfonate (780 mg, 5.0 mmol) in anhydrous DCM (38 mL) under argon at 0 °C, Triethylamine (0.7 mL, 5 mmol) and trifluoromethanesulfonic anhydride (0.32 mL, 1.9 mmol) were added. The mixture was stirred at 0 °C for 2 h. The reaction was quenched by the addition of saturated aqueous NH_4Cl solution (20 mL) and then diluted with DCM (20 mL). Organic material was extracted with DCM (2 x 10 mL), combined organic material was washed with water (30 mL) and brine (30 mL), dried over $MgSO_4$ and solvent was removed under reduced pressure. The crude vinylic triflate was recovered as dark oil which was judged to be sufficiently pure by thin layer chromatography to carry on the next step without further purification.

To a stirred suspension of CuI (950 mg, 5 mmol) in anhydrous THF (12 mL) under argon atmosphere at 0°C, ethylmagnesium bromide (3.0 M in diethyl ether, 3.3 mL, 10 mmol) was added drop-wise. The solution was stirred for 30 minutes, whereupon an opaque black colour was formed. The stirred reaction mixture was then cooled to -78 °C and a solution of the enol triflate (580 mg, 1.3 mmol) in anhydrous THF (4 mL) was added *via* cannula. The reaction was stirred at this temperature for 2.5 h. The reaction was quenched by the addition of saturated aqueous NH_4Cl solution (20 mL). Resulting emulsions were dissolved by addition of concentrated aqueous NH_4OH solution and stirring over night. The aqueous layer was extracted with ethyl acetate (3 x 10 mL) and the combined organic extracts were washed with water (2 x 30 mL) and brine (30 mL) and dried over $MgSO_4$. Solvent was removed under reduced pressure. The residual oil was purified by flash chromatography column with a mixture of hexane and ethyl acetate (19:1) as eluent. The title compound was isolated as colourless oil (185 mg, 52%).

HRMS EI$^+$ found 292.2407, $C_{19}H_{32}O_2$ requires 292.2402. **^{1}H NMR** (400 MHz, CDCl$_3$) δ 5.55 (s, 1 H, C=CH), 5.11 – 4.87 (m, 2 H, 2 x C=CH), 4.07 (q, *J* = 7.0, 2 H, OCH$_2$CH$_3$), 2.55

(q, J = 7.5, 2H, C$\underline{H}_2$CH$_3$), 2.22 – 2.05 (m, 4 H, 2 x CH$_2$), 2.00 – 1.89 (m, 6 H, 3 x CH$_2$), 1.61 (s, 3 H, CH$_3$), 1.53 (s, 3 H, CH$_3$), 1.21 (t, J = 7.0, 3 H, CH$_2$C$\underline{H}_3$), 1.01 (t, J = 7.5, 3 H, CH$_2$C$\underline{H}_3$), 0.89 (t, J = 7.5, 3 H, CH$_2$C$\underline{H}_3$).**13C NMR** (62.5 MHz, CDCl$_3$) δ 166.4 ($\underline{C}$=OOEt), 165.5 ($\underline{C}$=CH), 141.9 ($\underline{C}$=CH), 131.3 ($\underline{C}$=CH), 124.3 (C=$\underline{C}$H), 122.5 (C=$\underline{C}$H), 114.8 (C=$\underline{C}$H), 59.1 (O$\underline{C}$H$_2$CH$_3$), 38,3 (CH$_2$), 36.4 (CH$_2$), 26.8 (CH$_2$), 25.8 (CH$_2$), 25.7 (CH$_2$), 25.3 (CH$_2$), 23.1 (CH$_3$), 17.7 (CH$_3$), 14.3 (CH$_3$), 13.2 (CH$_3$), 13.0 (CH$_3$).

6.4.2 Preparation of (*2E,6E*)-3,7-diethyl-11-methyldodeca-2,6,10-trien-1-ol (187)
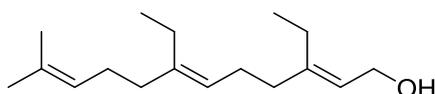
To a stirred suspension of the ethyl ester **189** (180 mg, 0.6 mmol) in anhydrous toluene (3.1 mL) at -78 °C, DIBAL-H (1.5 M in toluene, 1.3 mL, 1.8 mmol) was added. The resulting solution was stirred at this temperature for 2 h. The reaction was quenched by addition of HCl 2M (10 mL), diluted with DCM (10 mL) and stirred for 1 h at room temperature. The aqueous layer was extracted with DCM (2 x 10 mL) and the pooled organic material was washed with saturated aqueous NaHCO$_3$ solution (3 x 10 mL), brine (2 x 15 mL), dried over MgSO$_4$ and concentrate under reduced pressure. The crude product was purified by flash chromatography column with hexane and ethyl acetate (3:1) to afford the title compound as colourless oil (160 mg, 73% yield).

HRMS APCI [M-OH] found 233.2275,C$_{17}$H$_{29}$ requires 233.2269. **1H NMR** (400MHz, CDCl$_3$) δ 5.38 (t, 1 H, J = 7.0, C=CH), 5.09 (m, 2 H, 2 x C=CH), 4.16 (d, 2 H, J = 7.0, CHC$\underline{H}_2$), 1.97-2.13 (m, 12 H, 6 x CH$_2$), 1,68 (s, 3 H, CCH$_3$), 1.60 (s, 3 H, CCH$_3$), 0.94-1.01 (m, 6 H, 2 x CH$_2$C$\underline{H}_3$). **13C NMR** δ$_C$ (62.5 MHz, CDCl$_3$) 145.69 ($\underline{C}$=CH), 141.25 ($\underline{C}$=CH), 131.26 ($\underline{C}$=CH), 124.46 (C=$\underline{C}$H), 123.43 (C=$\underline{C}$H), 122.89 (C=$\underline{C}$H), 59.09 ($\underline{C}$H$_2$OH), 36.75 (CH$_2$), 36.51 (CH$_2$), 26.96 (CH$_2$), 26.22 (CH$_2$), 25.65 (CH$_2$), 23.54 (CH$_2$), 23.21 (CH$_3$), 17.68 (CH$_3$), 13.65 (CH$_3$), 13.20 (CH$_3$).

6.4.3 Preparation of trisammonium (2*E*,6*E*)-3,7-diethyl-11-methyldodeca-2,6,10-trienyl diphosphate (139)
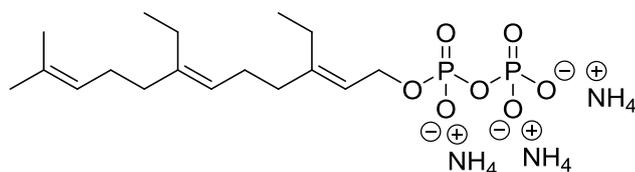
To a stirred suspension of LiCl (270 mg, 6.4 mmol) in anhydrous DMF (5.3 mL) at 0 °C, S-collidine (0.3 mL, 2.4 mmol) and menthansulfonyl chloride (50 μL, 0.64 mmol) were added and the solution was stirred for 15 min during which time a white cloudy precipitate was formed. A solution of the alcohol **187** (100 mg, 0.40 mmol) in dry DMF (1 mL) was added drop-wise and the reaction mixture was stirred at 0 °C for 3 h. The mixture was diluted in cold pentane (10 mL) and cold water (15 mL) was added. The organic material was extracted with pentane (3 x 10 mL). The combined organic extracts were washed with saturated aqueous $CuSO_4$ solution (3 x 10 mL), saturated aqueous $NaHSO_4$ solution (2 x 10 mL), brine (2 x 10 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure and the crude allylic chloride was used in the next step without further purification.

To a solution of the crude allylic chloride in anhydrous CH_3CN (1 mL), tris-(tetrabutylammonium) hydrogendiphosphate (0.7 g, 1.8 mmol) was added. The mixture was stirred at room temperature for 15 h. The solvent was removed under reduced pressure and the residue was dissolved in ion-exchange buffer (25 mM NH_4HCO_3 containing 2% *i*-PrOH, 1 mL). This solution was slowly passed through a column containing DOWEX 50W-X8 (100-200 mesh) cation exchange resin (NH_4^+ form) that had been pre-equilibrated with two column volumes of ion-exchange buffer. The column was slowly eluted with ion-exchange buffer. Once ion exchange was complete, fractions containing product (as judged by TLC in 6:3:1 *i*-PrOH:c.NH_3:H_2O) was lyophilised to dryness. The resulting white solid was triturated with MeOH (3 x 10 mL) and the combined organic extracts were concentrated to dryness under reduced pressure affording yellow solid which was cleaned with diethyl ether (3 x 3 mL) to give the title compound as a white solid (64 mg, 36%). The solid residue from the triturating step was further purified by reverse-phase HPLC (150 × 21.2 mm Phenomenex Luna column, eluting with 10% B for 20 min, then a linear gradient to 60% B over 25 min and finally a linear gradient to 100% B over 5 min.; solvent A: 25 mM NH_4HCO_3 in water,

solvent B: CH₃CN, flow rate 5.0 mL/min, detecting at 220 nm). Once purification was complete the solution was again lyophilised to dryness giving the title compound as a fluffy white solid (34 mg, 19% yield).

HRMS ES⁻ [M+2H] found 409.2539, $C_{17}H_{29}O_7$ requires 409.2545. **¹H NMR** (400MHz, D₂O) δ 5.32 (t, *J* = 6.5, 1 H, C=CH), 5.07 (t, *J* = 6.0, 2 H, 2 x C=CH), 4.37 (m, 2 H C<u>H</u>₂OH), 1.92-2.03 (m, 12 H, 6 x CH₂), 1,56 (s, 3 H, CH₃), 1.50 (s, 3H, CH₃), 0.8-0.91 (m, 6 H, 2 x CH₂C<u>H</u>₃). **³¹P NMR** (202.5 MHz, D₂O) δ -10.41 (d, *J*$_{PP}$ = 22.5), -8.30 (d, *J*$_{PP}$ = 22.5).

6.5 Synthesis of 12Me-FDP (136)

6.5.1 Preparation of 2-((*2E,6E*)-3,7,11-trimethyldodeca-2,6,10-trienyloxy)-tetrahydro-2H-pyran (207)
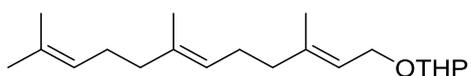
To a stirred solution of 3,4-Dihydropyran (2.3 g, 27.0 mmol) and farnesol (5 g, 22.5 mmol) in DCM (50 mL) at room temperature, *para*-toluene sulfonic acid (0.18 g, 1.3 mmol) was added. The mixture was stirred for 15 h. reaction was quenched by the addition of aqueous saturated NaHCO₃ solution (20 mL). Organic material was extracted with DCM (3 x 10 mL). The pooled organic extracts were washed with brine (3 x 20 mL), dried over MgSO₄ and the solvents removed under reduce pressure. The crude product was purified by flash chromatography column with a mixture of hexane and ethyl acetate (60:1) as eluent to afford the title compound as colourless oil (5.9 g, 86%).

HRMS ES⁺ [M+H] $C_{20}H_{35}O_2$ calculated: 307.2637; found: 307.2630. **¹H NMR** (300 MHz, CDCl₃) δ 5.37 (t, *J* = 6.5 , 1 H, C=C<u>H</u>CH₂), 5.17 – 5.06 (m, 2 H, 2 x C=C<u>H</u>CH₂), 4.64 (t, *J* = 3.5 , 1 H, OCHO), 4.25 (dd, *J* = 12.0, 6.5 , 1 H, CHC<u>H</u>₂O), 4.04 (dd, *J* = 12.0, 7.5 , 1 H, CHC<u>H</u>₂O), 3.91 (dt, *J* = 11.0, 7.0, 3.5, 1 H, OC<u>H</u>₂CH₂), 3.52 (dt, *J* = 8.5, 5.0 , 1 H, OC<u>H</u>₂CH₂), 2.19 – 1.93 (m, 8 H, 4 x CH₂), 1.90 – 1.72 (m, 2 H, CH₂), 1.69 (s, 6 H, CH₃), 1.61 (s, 6 H, CH₃), 1.58 – 1.47 (m, 4 H, CH₂).**¹³C NMR** (75 MHz, CDCl₃) δ 140.3 (<u>C</u>=CH), 135.3 (<u>C</u>=CH), 131.3 (<u>C</u>=CH), 124.3 (C=<u>C</u>H), 123.9 (C=<u>C</u>H), 120.5 (C=<u>C</u>H), 97.8 (OCHO),

63.7 (C=CH$\underline{C}$H$_2$O), 62.3 (O$\underline{C}$H$_2$CH$_2$) , 39.7 (CH$_2$), 39.6 (CH$_2$), 30.7 (CH$_2$), 26.7 (CH$_2$), 26.2 (CH$_2$), 25.7 (CH$_2$), 25.5 (CH$_2$) , 19.6 (CH$_3$), 17.7 (CH$_3$), 16.4 (CH$_3$), 16.0 (CH$_3$).

6.5.2 Preparation of (*2E,6E,10E*)-2,6,10-trimethyl-12-(tetrahydro-2H-pyran-2-yloxy)dodeca-2,6,10-trien-1-ol (204)
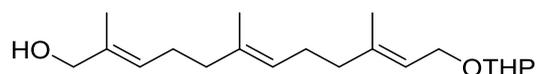
To a stirred suspension of selenium oxide (210 mg, 1.9 mmol) in DCM (40 mL) at 0 ºC, 70% aqueous solution of *tert*-butyl hydroperoxide (8 mL, 58.5 mmol) was added. After 10 min a solution of **207** (5.8 g, 19 mmol) in DCM (10 mL) was added *via* a syringe. The solution was warmed to room temperature and allowed to stir for another 12 h. The reaction was diluted with water (30 mL). Organic material was extracted with DCM (3 x 20 mL), the combined organic extracts were washed with brine (3 x 40 mL), dried over MgSO$_4$ and solvent was removed under reduce pressure. The crude product was purified by flash chromatography column with a mixture of hexane and ethyl acetate (gradient from 60:1 to 40:1) to afford the title compound as yellow oil (1.3 g, 21%).

HRMS ES$^+$ [M+Na] C$_{20}$H$_{34}$O$_3$Na calculated: 345.2406; found: 345.2402. **^{1}H NMR** (300 MHz, CDCl$_3$) δ **^{1}H NMR** (300 MHz, CDCl$_3$) δ 5.36 (m, 2 H, C=C$\underline{H}$CH$_2$), 5.10 (t, *J* = 7.0, 1 H, C=C$\underline{H}$CH$_2$), 4.62 (t, *J* = 3.5, 1 H, OCHO), 4.31 – 4.16 (m, 1 H, CHC$\underline{H}_2$O), 4.02 (m, 3 H, CHC$\underline{H}_2$O, HOC$\underline{H}_2$), 3.88 (m, 1 H, OC$\underline{H}_2$CH$_2$), 3.51 (m, 1 H, OC$\underline{H}_2$CH$_2$), 2.23 – 1.96 (m, 8 H, 4 x CH$_2$), 1.87 – 1.76 (m, 2H, CH$_2$), 1.66 (d, *J* = 5.5, 6 H, 2 x CH$_3$), 1.59 (s, 3 H, CH$_3$), 1.55 – 1.46 (m, 4 H, 2 x CH$_2$). **^{13}C NMR** (75 MHz, CDCl$_3$) δ 140.2 ($\underline{C}$=CH), 134.8 ($\underline{C}$=CH), 134.7 ($\underline{C}$=CH), 125.8 (C=$\underline{C}$H), 124.2 (C=$\underline{C}$H), 120.6 (C=$\underline{C}$H), 97.7 (OCHO), 68.9 (C=CH$\underline{C}$H$_2$O), 63.6 (C=CH$\underline{C}$H$_2$O), 62.3 (O$\underline{C}$H$_2$CH$_2$), 39.6 (CH$_2$), 39.3 (CH$_2$), 30.7 (CH$_2$), 26.2 (CH$_2$), 26.1 (CH$_2$), 25.5 (CH$_2$), 19.6 (CH$_2$), 16.4 (CH$_3$), 16.0 (CH$_3$), 13.7 (CH$_3$).

6.5.3 Preparation of 2-((*2E,6E,10E*)-3,7,11-trimethyltrideca-2,6,10-trienyloxy)-tetrahydro-2H-pyran (205)
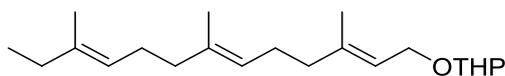
To a stirred solution of the alcohol **204** (320 mg, 1.0 mmol) in anhydrous THF (5 mL) at −45 ºC, freshly distilled Et$_3$N (264 μl, 2.0 mmol) and methanesulfonyl chloride (167 μL, 1.4 mmol) were added. After 45 min, LiBr (403 mg, 5.9 mmol) was added to the milky solution. The mixture was warmed up to room temperature and left at this temperature for 1.5 h. The mixture was diluted with hexane (10 mL) and quenched by the addition of saturated aqueous NH$_4$Cl solution (10 mL). Organic material was extracted with hexane (3 x 10 mL). The combined organic extracts were washed with saturated aqueous NaHCO$_3$ solution (3 x 20 mL), water (3 x 20 mL) and brine (3 x 20 mL), dried over MgSO$_4$ and solvent was removed under reduce pressure. The crude colourless oil was immediately used in the next step without further purification.

To a stirred suspension of CuI (375 mg, 1.9 mmol) in anhydrous Et$_2$O (4 mL) under Ar atmosphere at 0 ºC, MeLi (1.6 M in hexane, 3.1 mL, 4.9 mmol) was added. After 20 min the reaction mixture was cooled to −78 ºC and a solution of the crude allylic bromide **203** (400 mg), in of anhydrous diethyl ether (7 mL), was added *via* a syringe over 5 min. After 30 min the reaction was completed as judged by TLC. The reaction was quenched by the addition of saturated aqueous NH$_4$Cl solution (8 mL). Copper salts were dissolved by the addition of concentrated NH$_4$OH (10 mL) and stirring the biphasic mixture for 12 h. The organic material was extracted with ether (3 x 15 mL) and the combined organic extracts were washed with 10% aqueous NH$_4$OH solution (3 x 25 mL), water (3 x 25 mL), brine (3 x 25 mL), dried over MgSO$_4$ and solvent was removed under reduce pressure. The crude product was purified by flash chromatography column with a mixture of hexane and ethyl acetate (gradient from hexane to 70:1) as eluent, to afford the title compound as yellow oil (160 mg, 53% yield over two steps).

HRMS ES$^+$ [M+Na] C$_{21}$H$_{36}$O$_2$Na calculated: 343.2613; found: 343.2615. **^{1}H NMR** (300 MHz, CDCl$_3$) δ 5.36 (t, *J* = 6.5, 1 H, C=CHCH$_2$), 5.10 (m, 2 H, 2 x C=CHCH$_2$), 4.63 (t, *J* = 3.5, 1 H, OCHO), 4.24 (dd, *J* = 12.0, 6.5, 1 H, CHCH$_2$O), 4.02 (dd, *J* = 12.0, 7.5, 1 H,

CHC$\underline{\text{H}}_2$O), 3.89 (dt, J = 7.0, 4.0 , 1 H, OC$\underline{\text{H}}_2$CH$_2$), 3.50 (dt, J = 7.0, 4.0 , 1 H, OC$\underline{\text{H}}_2$CH$_2$), 2.18 – 1.92 (m, 10 H, 5 x CH$_2$), 1.83(m, 2 H, CH$_2$), 1.68 (s, 3 H, CH$_3$), 1.59 – 1.44 (m, 10 H, 2 x CH$_2$, 2 x CH$_3$), 0.97 (t, J = 7.5 , 3 H, C$\underline{\text{H}}_3$CH$_2$). ^{13}C NMR (126 MHz, CDCl$_3$) δ 140.5 (HC=$\underline{\text{C}}$), 136.9 (HC=$\underline{\text{C}}$), 135.4 (HC=$\underline{\text{C}}$), 124.1 (H$\underline{\text{C}}$=C), 122.9 (H$\underline{\text{C}}$=C), 120.7 (H$\underline{\text{C}}$=C), 97.9 (OCHO), 63.8 (CH$\underline{\text{C}}$H$_2$O), 62.4(O$\underline{\text{C}}$H$_2$CH$_2$), 39.9 (CH$_2$), 39.8 (CH$_2$), 32.5 (CH$_2$), 30.9 (CH$_2$), 26.7 (CH$_2$), 26.4 (CH$_2$), 25.7 (CH$_2$), 19.8 (CH$_2$), 16.6 (2 x CH$_3$), 16.2 (CH$_3$), 13.0 (CH$_3$).

6.5.4 Preparation of (*2E,6E,10E*)-3,7,11-trimethyltrideca-2,6,10-trien-1-ol (209)
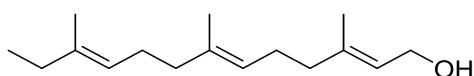
To a stirred solution of **205** (160 mg, 0.5 mmol) in MeOH (5 mL) pyridine *para*-toluenesulfonic acid (400 mg, 1.6 mmol) was added. The solution was stirred for 24 h, diluted with diethyl ether (20 mL) and quenched by the addition of saturated aqueous NaHCO$_3$ solution (20 mL). Organic material was extracted with diethyl ether (5 x 10 mL), the combined organic extracts were washed with brine (3 x 20 mL), dried over MgSO$_4$ and solvent was removed under reduce pressure. The crude product was purified by flash chromatography column with hexane and ethyl acetate (gradient from 70:1 to 50:1) to afford the title compound as colourless oil (100 mg, 81%).

HRMS ES$^+$ [M+H] C$_{16}$H$_{29}$O calculated: 237.2218; found: 237.2211. ^{1}H NMR (250 MHz, CDCl$_3$) δ 5.35 (t, J = 7.0, 1 H, C=C$\underline{\text{H}}$CH$_2$), 5.03 (m, 2 H, 2 x C=C$\underline{\text{H}}$CH$_2$), 4.08 (d, J = 7.0, 2 H, C$\underline{\text{H}}_2$OH), 2.22 – 1.80 (m, 10 H 5 x CH$_2$), 1.61 (s, 3 H, CH$_3$), 1.53 (s, 6 H, 2 x CH$_3$), 0.91 (t, J = 7.5 , 3 H, C$\underline{\text{H}}_2$CH$_3$). ^{13}C NMR (126 MHz, CDCl$_3$) δ 139.9 (HC=$\underline{\text{C}}$), 136.9 (HC=$\underline{\text{C}}$), 135.4 (HC=$\underline{\text{C}}$), 123.8 (H$\underline{\text{C}}$=C), 123.3 (H$\underline{\text{C}}$=C), 122.7 (H$\underline{\text{C}}$=C), 59.4 (CH$_2$OH), 39.7 (CH$_2$), 39.6 (CH$_2$), 32.3 (CH$_2$), 26.6 (CH$_2$), 26.3 (CH$_2$), 16.3 (CH$_3$), 16.0 (CH$_3$), 15.9 (CH$_3$), 12.8 (CH$_3$).

6.5.5 Preparation of trisammonium (2*E*,6*E*,11*E*)-11-ethyl-11-methyldodeca-2,6,10-trienyl diphosphate (136)
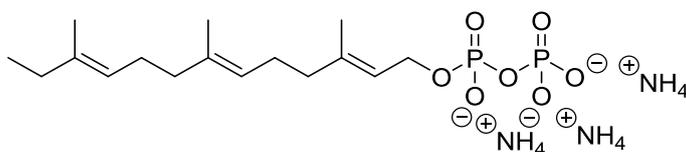
To a stirred solution of the alcohol **209** (70 mg, 0.3 mmol) in anhydrous DMF (4 mL) at 0 ºC, s-collidine (221 µL, 1.8 mmol) and methanesulfonyl chloride (34 µl, 0.4 mmol) were added. After 20 min stirring at this temperature, lithium chloride (201 mg, 4.7 mmol) was added to the solution. After 3 h the reaction was quenched by the addition of cold water (3 mL). The biphasic mixture was diluted with pentane (5 mL) and organic material was extracted with pentane (3 x 3 mL), the combined organic extracts were washed with saturated aqueous $CuSO_4$ solution (3 x 10 mL), water (3 x 10 mL), saturated aqueous $NaHCO_3$ solution (3 x 10 mL), dried over $MgSO_4$ and solvent were removed under reduce pressure. The crude product was used in the next step without further purification.

To a stirred solution of crude allylic chloride (72 mg) in acetonitrile (2 mL), tris-(tetrabutylammonium)hydrogen diphosphate (800 mg, 0.8 mmol) was added. The reaction was stirred at room temperature for 16 h. Acetonitrile was removed under reduced pressure, the remaining yellow oil was dissolved in buffer (4 mL, 25 mM of NH_4HCO_3, 2% isopropanol) and passed thought an ion exchange column DOWEX 40 W (NH_4^+ form). The eluent from the ion exchange column was monitored by TLC (isopropanol / buffer / NH_4OH 6:2:2). Fractions containing product were collected and freeze dried. The yellow solid was triturated with anhydrous methanol (4 x 8 mL), the combined methanol extracts were concentrated under reduce pressure. The yellow solid was washed with diethyl ether (3 x 1 mL), the remaining white solid was dried under vacuum to afford the title compound as a white solid (48 mg, 36%). The solid was dissolved in H_2O (11 mL) to afford 10 mM stock solution.

HRMS ES⁻ [M+2H] $C_{16}H_{29}O_7P_2$ calculated: 395.1389; found: 395.1385. **¹H NMR** (400 MHz, D_2O) δ 5.37 (t, *J* = 6.0, 1 H, C=C<u>H</u>CH$_2$), 5.07 (m, 2 H, 2 x C=C<u>H</u>CH$_2$), 4.41 (t, *J* = 6.0, 2 H, C<u>H</u>$_2$OP=OO$_2$), 2.16 – 1.81 (m, 10 H, 5 x CH$_2$), 1.65 (s, 3 H, CH$_3$), 1.53 (bd, *J* = 8.0 , 6

H, 2 x CH$_3$), 0.90 (t, J = 7.5, 3 H, CH$_2$C$\underline{H}_3$) ppm; **^{31}P NMR** (202.5 MHz, D$_2$O) δ −9.9 (bd, J_{PP} = 56.5).

6.6 Synthesis of 8nor-FDP (140)

6.6.1 Preparation of (*E*)-2-((3,7-dimethylocta-2,6-dien-1-yl)oxy)tetrahydro-2H-pyran (223)
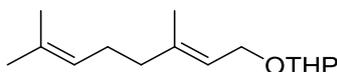
To a stirred solution of geraniol (10.0 g, 65.0 mmol) in DCM (200 mL), DHP (10.9 g, 13 mmol) and PPTS (160 mg, 0.6 mmol) were added. The colourless solution was stirred for 16 h and then quenched with saturated NaHCO$_3$ solution (60 mL). The organic material was extracted with DCM (2 x 30 mL). The combine organic extracts were washed with brine (3 x 100 mL), dried over MgSO$_4$ and concentrated under reduced pressure. Purification of the crude product by vacuum chromatography with hexane and ethyl acetate as eluent (40:1 to 30:1) gave the title compound as a colourless oil (13.8 g, 89%).

HRMS ES$^+$ [M+H] found 239.2022, C$_{15}$H$_{27}$O$_2$ requires 239.2011. **^{1}H NMR** (300 MHz, CDCl$_3$) δ 5.35 (t, J = 7.0, 1 H, C=CH), 5.08 (t, J = 6.0, 1 H, (CH$_3$)$_2$=C$\underline{H}$), 4.66 − 4.57 (bs, 1 H, OCHO), 4.23 (dd, J = 12.0, 6.0, 1H, C=CHC$\underline{H}_2$O), 4.02 (dd, J = 12.0, 7.5 , 1 H, C=CHC$\underline{H}_2$O), 3.95-3.85 (m, 1 H, OC$\underline{H}_2$CH$_2$), 3.58 − 3.40 (m, 1 H, OC$\underline{H}_2$CH$_2$), 2.16 − 1.97 (m, 4 H 2 x CH$_2$), 1.90 − 1.73 (m, 2 H, CHC$\underline{H}_2$), 1.67 (s, 6 H, 2 x CH$_3$C), 1.62 − 1.44 (m, 7 H CH$_3$, 2 x CH$_2$ from OTHP). **^{13}C NMR** (75 MHz, CDCl$_3$) δ 140.4 ($\underline{C}$=CH), 131.7 ($\underline{C}$=CH), 124.1 (C=$\underline{C}$H), 120.6 (C=$\underline{C}$H), 97.9 (OCHO), 63.7 (C=CH$\underline{C}$H$_2$O), 62.4 (O$\underline{C}$H$_2$CH$_2$), 39.7 (CH$_2$), 30.8 (CH$_2$), 26.5 (CH$_2$), 25.8 (CH$_2$), 25.6 (CH$_2$), 19.7 (CH$_3$), 17.8 (CH$_3$), 16.5 (CH$_3$).

6.6.2 Preparation of (*2E,6E*)-2,6-dimethyl-8-((tetrahydro-2H-pyran-2-yl)oxy)octa-2,6-dien-1-ol (222)

HO$\diagup$$\diagdown$$\diagup$$\diagdown$$\diagup$OTHP
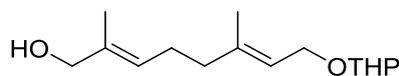
To a stirred solution of THP protected geraniol **223** (2 g, 8.4 mmol) in DCM (20 mL) at 0ºC, SeO$_2$ (93 mg, 0.84 mmol), *t*-BuOOH (3.7 mL, 26.8 mmol) and salicylic acid (110 mg, 0.89 mmol) were added. Reaction mixture was kept at this temperature for 5 h and then water (10 mL) was added. The organic material was extracted with DCM (3 x 15 mL), combine organic extracts were washed with saturated aqueous NaHCO$_3$ solution (2 x 15 mL), brine (3 x 15 mL), dried over MgSO$_4$ and concentrate under reduce pressure. Purification of the crude product by flash chromatography column with hexane and Ethyl acetate (3:1) as eluent gave the title compound as pale yellow oil (1.3 g, 60%).

HRMS EI$^+$ [M + Na] found 277.1768, C$_{15}$H$_{26}$O$_3$Na requires 277.1780. **^{1}H NMR** (400 MHz, CDCl$_3$) δ 5.38 – 5.28 (m, 2 H, 2 x C=CH), 4.60 (bs, 1 H, OCHO), 4.21 (dd, *J* = 11.5, 6.0, 1 H, C=CHC$\underline{H}_2$O), 4.05 – 3.97 (m, 1 H, C=CHC$\underline{H}_2$O), 3.95 (s, 2 H, C$\underline{H}_2$OH), 3.86 (t, *J* = 8.0 , 1 H, OC$\underline{H}_2$CH$_2$), 3.53 – 3.45 (m, 1 H, OC$\underline{H}_2$CH$_2$), 2.18 – 2.11 (m, 2 H, CH$_2$), 2.09 –2.02 (m, 2 H, CH$_2$), 1.98 – 1.75 (m, 4 H, CH$_2$), 1.65 (s, 3 H, CH$_3$), 1.63 (s, 3 H, CH$_3$), 1.57 – 1.49 (m, 2 H, CH$_2$).**^{13}C NMR** (75 MHz, CDCl$_3$) δ 139.8 (C=CH), 135.2 (C=CH), 125.4 (C=CH), 120.9 (C=CH), 97.8 (OCHO), 68.8 (CH$_2$OH), 63.7 (C=CHCH$_2$O), 62.3 (OCH$_2$CH$_2$), 39.2 (CH$_2$), 30.7 (CH$_2$), 25.8 (CH$_2$), 25.5 (CH$_2$), 19.5 (CH$_2$), 16.4 (CH$_3$), 13.7 (CH$_3$).

6.6.3 Preparation of 2-(((*2E,6E*)-3,7,10-trimethylundeca-2,6,9-trien-1-yl)oxy)tetrahydro-2H-pyran (217)

$\diagup$$\diagdown$$\diagup$$\diagdown$$\diagup$$\diagdown$$\diagup$OTHP
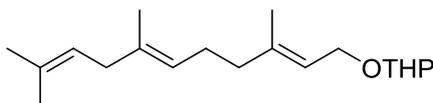
To a stirred solution of alcohol **222** (200 mg, 0.8 mmol) in THF at -45 ºC (4 mL), Et$_3$N (200 µL, 1.57 mmol) and mesyl chloride (84 µL, 1.1 mmol) were added, reaction was kept at this temperature for 1h. Then LiBr (406 mg, 4.7 µL) was added and the milky solution was warm to 0 ºC and stirred for 4 h. Water was added to the reaction and organic material was extracted with diethyl ether (3 x 8 mL). Combined organic extracts were washed with aqueous saturated NaHCO$_3$ solution (3 x 10 mL), brine (3 x 10 mL), dried over MgSO$_4$ and concentrate under reduce pressure. The crude bromide was used without further purification in the next step.

To a suspension of CuI (295 mg, 1.6 mmol) in THF (3 mL) at 0 ºC, (2-methylprop-1-en-1-yl) magnesium bromide (0.5 M in THF, 7.9 mL, 3.9 mmol) was added and the reaction was stirred for 30 min. The black solution was cooled to − 78 ºC and a solution of crude bromide (260 mg) in THF (4 mL) was added *via* cannula. The reaction was stirred at this temperature for 1 h and then quenched by the addition a saturated aqueous NH$_4$Cl solution (5 mL). The white precipitate formed was dissolved by the addition of concentrate NH$_4$OH (5 mL), dilution with diethyl ether (10 mL) and stirred the biphasic mixture overnight. The organic material was extracted with diethyl ether (6 x 10 mL) and the combined organic extracts were washed with 10% aqueous NH$_4$OH solution (2 x 15 mL), brine (3 x 20 mL), dried over MgSO$_4$ and concentrated under reduce pressure. Purification of the crude product by flash chromatography column with hexane and ethyl acetate (gradient from 40:1 to 30:1) as eluent gave the title compound as colourless oil (200 mg, 87%).

HRMS EI$^+$ [M+Na] found 315.2314, C$_{19}$H$_{32}$O$_2$Na requires 315.2300. **^{1}H NMR** (400 MHz, CDCl$_3$) δ 5.35 (t, J = 7.0, 1 H, C=CH), 5.14 – 5.07 (m, 2 H, 2 x C=CH), 4.64 – 4.61 (m, 1 H, OCHO), 4.23 (dd, J = 11.5, 6.5, 1 H, CH$_2$OCH), 4.07 – 3.98 (m, 1 H, CH$_2$OCH), 3.89 (ddd, J = 11.0, 8.0, 3.5, 1 H, OCH$_2$CH$_2$), 3.55 – 3.44 (m, 1 H, OCH$_2$CH$_2$), 2.63 (d, J = 7.0, 2 H, C=CHCH$_2$C), 2.16 – 2.00 (m, 4 H, 2 x CH$_2$), 1.84 (m, 2 H, CH$_2$), 1.71 (s, 3 H, CH$_3$), 1.67 (s, 3 H, CH$_3$), 1.61 (s, 3 H, CH$_3$), 1.57 (s, 3 H, CH$_3$), 1.55 – 1.46 (m, 4 H, 2 x CH$_2$). **^{13}C NMR** (101 MHz, CDCl$_3$) δ 140.4(HC=C), 135.0 (HC=C),, 132.6 (HC=C), 123.8 (HC=C), 122.7 (HC=C), 120.7 (HC=C), 97.9 (OCHO), 63.8 (CH$_2$OCH), 62.4 (OCH$_2$CH$_2$), 39.8 (CH$_2$), 38.3 (CH$_2$), 30.8 (CH$_2$), 26.4 (CH$_2$), 25.9 (CH$_2$), 25.6 (CH$_2$), 19.7 (CH$_3$), 17.8 (CH$_3$), 16.6 (CH$_3$), 16.3 (CH$_3$).

6.6.4 Preparation of (*2E,6E*)-3,7,10-trimethylundeca-2,6,9-trien-1-ol (216)
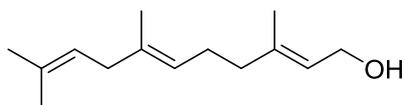
To a stirred solution of THP ether **217** (150 mg, 0.5 mmol) in methanol (4 mL) PPTS (202 mg, 0.8 mmol) was added in a open air flask. The resulting clear solution was stirred for 16 h. After this time the methanol was concentrated under reduce pressure and the resulting oil was dissolve in diethyl ether (10 mL). The ethereal extract was washed with saturated aqueous NaHCO$_3$ solution (3 x 6 mL), brine (2 x 6 mL), dried over MgSO$_4$ and concentrated under reduce pressure. The crude product was purified by flash chromatography column with hexane and ethyl acetate (2:1) as eluent to afford the title compound as colourless oil (75 mg, 68%).

HRMS EI$^+$ [M-OH] found 190.1725, C$_{14}$H$_{22}$ requires 190.1722. **^{1}H NMR** (400 MHz, CDCl$_3$) δ 5.41 (t, *J* = 7.0 , 1 H, C=C<u>H</u>), 5.11 (m, 2 H, 2 x C=C<u>H</u>), 4.14 (d, *J* = 7.0, 2 H, CHC<u>H</u>$_2$OH), 2.64 (d, *J* = 7.5, 2 H, CHC<u>H</u>$_2$C), 2.18 – 1.99 (m, 4 H, 2 x CH$_2$), 1.71 (s, 3 H, CH$_3$), 1.67 (s, 3 H, CH$_3$), 1.61 (s, 3 H, CH$_3$), 1.57 (s, 3 H, CH$_3$). **^{13}C NMR** (63 MHz, CDCl$_3$) δ 140.0 (<u>C</u>=CH), 135.2 (<u>C</u>=CH), 132.7 (<u>C</u>=CH), 123.7 (C=<u>C</u>H), 123.5 (C=<u>C</u>H), 122.7 (C=<u>C</u>H), 59.5 (CHC<u>H</u>$_2$OH), 39.7 (CHC<u>H</u>$_2$C), 38.3 (CH$_2$), 26.4 (CH$_2$), 25.9 (CH$_3$), 17.8 (CH$_3$), 16.4 (CH$_3$), 16.3 (CH$_3$).

6.6.5 Preparation of (*2E,6E*)-3,7,10-trimethylundeca-2,6,9-trien-1-yl acetate (356)
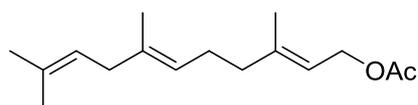
To a stirred solution of alcohol **216** (17 mg, 0.08 mmol) in DCM (1 mL) at room temperature, pyridine (66 μL, 0.8 mmol) and acetic anhydride (40 μL, 0.4 mmol) were added, in a open air flask. The mixture was stirred overnight. The reaction was diluted with DCM (10 mL) and quenched by the addition of 10% HCl aqueous solution (10 mL). Organic material was

extracted with DCM (3 x 10 mL), combined organic extracts were washed with saturated NaHCO$_3$ aqueous solution (3 x 15 mL), brine (3 x 15 mL), dried over Na$_2$SO$_4$ and solvent was removed under reduce pressure. The crude product was purified by flash chromatography column with mixture of hexane and ethyl acetate (60:1) as eluent to afford the product as pale yellow oil (17 mg, 61%).

HRMS EI$^+$ [M] found 250.1928, C$_{14}$H$_{22}$ requires 250.1933. **^{1}H NMR** (250 MHz, CDCl$_3$) δ 5.33 (t, J = 7.0, 1 H, C=CH), 5.19 − 5.02 (m, 2 H, 2 x C=CH), 4.58 (d, J = 7.0, 2 H, CHCH$_2$OAc), 2.64 (d, J = 7.5, 2 H, CHCH$_2$C), 2.20 − 1.97 (m, 7 H, 2 x CH$_2$, C=OCH$_3$), 1.70 (m, 6 H, 2 x CH$_3$), 1.61 (s, 3 H, CH$_3$), 1.57 (s, 3 H, CH$_3$).**^{13}C NMR** (63 MHz, CDCl$_3$) δ 171.3 (C=O), 142.4 (C=CH), 135.2 (C=CH), 132.7 (C=CH), 123.6 (C=CH), 122.7 (C=CH), 118.4 (C=CH), 61.6 (CH$_2$OAc), 39.7 (CH$_2$), 38.3 (CH$_2$), 26.4 (CH$_2$), 25.9 (CH$_3$), 21.2 (CH$_3$), 17.8 (CH$_3$), 16.6 (CH$_3$), 16.3 (CH$_3$).

6.6.6 Preparation of a mixture of (*E*)-β -, (*Z*)-α - and (*E*)- α-8nor-farnesenes
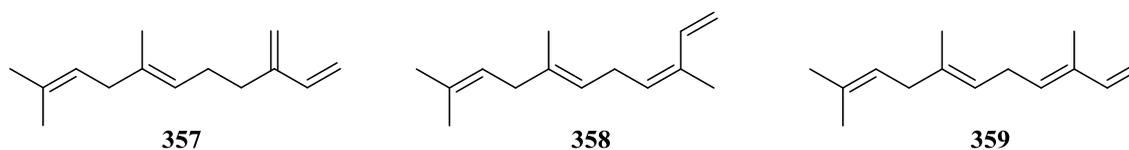
The following procedure is similar to the one reported by Heathcock.[306] The mixture of alcohol (12 mg, 0.06 mmol), pyridinium *p*-toluenesulfonate (7 mg, 0.03 mmol) in dichloroethane (1.5 mL) was stirred in a seal tube at 150 ºC for 10 min. The reaction mixture was cooled to room temperature, diluted with dichloroethane (2 mL). Organic material was washed with water (2 x 2 mL), brine (2 x 2 mL) and dried over Mg$_2$SO$_4$. The organic material was passed through short pad of silica and de compound was eluted with pentane (3 mL). The sample was analysed directly by GC-MS

6.6.7 Preparation of trisammonium (2*E*,6*E*)-10-methylundeca-2,6,9-trienyl diphosphate (140)
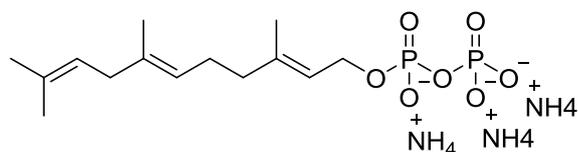
To a stirred solution of the alcohol **216** (53 mg, 0.3 mmol) in anhydrous DMF (5 mL) at 0 ºC, s-collidine (2.35 mL, 19 mmol) and methanesulfonyl chloride (364 μL, 4.7 mmol) were added. After 20 min stirring at this temperature, lithium chloride (2.1 g, 50.0 mmol) was added to the solution. After 3 h at this temperature, the reaction was quenched by the addition of cold water (30 mL). The biphasic mixture was diluted with pentane and organic material was extracted with pentane (3 x 20 mL), the combined organic extracts were washed with saturated aqueous $CuSO_4$ solution (3 x 30 mL), water (3 x 30 mL), saturated aqueous $NaHCO_3$ solution (3 x 20 mL), dried over $MgSO_4$ and solvent were removed under reduce pressure. The crude chloride was used in the next step without further purification.

To a stirred solution of crude allylic chloride (750 mg) in acetonitrile (13 mL) tris-(tetrabutylammonium) hydrogen diphosphate (7 g, 7.8 mmol) was added. The reaction was stirred at room temperature for 16 h. Acetonitrile was removed under reduced pressure, the remaining yellow oil was dissolved in buffer (15 mL, 25 mM of NH_4HCO_3, 2% isopropanol) and passed thought an ion exchange column DOWEX 40 W (NH_4^+ form). The eluent from the ion exchange column was monitored by TLC (isopropanol / buffer / NH_4OH 6:2:2). Fractions containing product were collected and freeze dried. The yellow solid was diluted in buffer (15 mL). The crude was purified by reverse-phase HPLC (150 × 21.2 mm Phenomenex Luna column, eluting with 10% B for 20 min, then a linear gradient to 60% B over 25 min and finally a linear gradient to 100% B over 5 min.; solvent A: 25 mM NH_4HCO_3 in water, solvent B: CH_3CN, flow rate 5.0 mL/min, detecting at 220 nm) in injections of 1.5 mL. The flow through of the HPLC was freeze dried and title compound was recovered as a white solid (60 mg, 57% over two steps).

HRMS ES⁻ [M + 2H] found 352.0857, $C_{16}H_{29}O_7P_2$ requires 352.0852. **¹H NMR** (400 MHz, D_2O) δ 5.42 (t, *J* = 7.0, 1 H, C=CH), 5.20 (dd, *J* = 12.5, 7.5, 2 H, 2 x C=CH), 4.43 (t, *J* = 6.5,

2 H, CH$_2$OH), 2.65 (d, J = 7.5, 2 H, CHCH$_2$C(CH$_3$)=CH), 2.12 (dd, J = 15.0, 7.0, 2 H, CH$_2$), 2.08 – 2.02 (m, 2 H, CH$_2$), 1.67 (s, 6 H, 2 x CH$_3$), 1.59 (s, 3 H, CH$_3$), 1.57 (s, 3 H, CH$_3$). **^{31}P NMR** (202.5 MHz, D$_2$O) δ -12.06 (d, J_{PP} = 18.0), -10.69 (d, J_{PP} = 18.0). Data in agreement with previously publish data by Gibbs *et al.*[218]

6.7 Synthesis of 14OH-FDP (142)

6.7.1 Preparation of 1,1-diethoxy-2-methylpropan-2-ol (360)
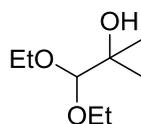
To a stirred solution of ethyl diethoxyacetate (3.0 g, 17.0 mmol) in anhydrous THF (40 mL) at -78 ºC methyl magnesium bromide (5.7 mL, 3M, 17.0 mmol) was added drop-wise. The resulting solution was stirred at this temperature for 2.5 h. The reaction mixture was then diluted with diethyl ether (20 mL) and quench by the addition of 20% solution of NH$_4$Cl (30 mL). The organic material was extracted with hexane (3 x 30 mL), combined organic extracts were washed with brine (3 x 40 mL), dried over MgSO$_4$ and solvent was removed under reduce pressure. The crude product was purified by vacuum chromatography with hexane and ethyl acetate (7:1) as eluent, to afford the title compound as colourless oil (1.01 g, 41% yield).

HRMS EI$^+$ [M + Na] found 185.1148, C$_8$H$_{18}$O$_3$Na requires 185.1154. **^{1}H NMR** (300 MHz, CDCl$_3$) δ 4.08 (s, 1 H, CH), 3.80 (dq, J = 9.0, 7.0, 2 H, OCH$_2$CH$_3$), 3.53 (dq, J = 9.0, 7.0, 2 H, OCH$_2$CH$_3$), 1.17 (t, J = 7.0, 6 H, 2 x OCH$_2$CH$_3$), 1.12 (s, 6 H, 2 x CH$_3$). **^{13}C NMR** (75 MHz, CDCl$_3$) δ 108.8 (HOC(CH$_3$)$_2$), 72.7 (HC(OEt)$_2$), 66.1 (2 x OCH$_2$CH$_3$), 24.0 (2 x OCH$_2$CH$_3$), 15.6 (2 x CH$_3$).

6.7.2 Preparation of 1,1-diethoxypropan-2-one (267)
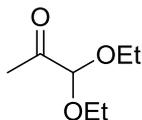
To a stirred suspension of CuI (4.1 g, 21.0 mmol) in anhydrous THF (80 mL) at 0°C, methylmagnesium bromide (1.6 M in diethyl ether, 30 mL, 48.0 mmol) was added drop-wise. The solution was stirred for 30 minutes at this temperature, whereupon yellow precipitate was dissolved. The mixture was cooled to − 78 ºC and then a solution of ethyl diethoxyacetate (2.0 g, 9.7 mmol) in anhydrous THF (10 mL) was added. The reaction mixture was stirred for 2 h and quenched by the addition of saturated aqueous NH$_4$Cl solution. The mixture was diluted with diethyl ether (30 mL) and concentrate NH$_4$OH (20 mL) was added, the biphasic mixture was stirred for 30 min. Organic material was extract with diethyl ether (3 x 30 mL), the combined organic extracts were washed with brine (2 x 50 mL), dried over MgSO$_4$ and concentrate under reduce pressure. The crude product was purified by vacuum chromatography with hexane and ethyl acetate (10:1) as eluent to afford the title compound as a colour less oil (0.9 g, 62%).

HRMS EI$^+$ [M] found 146.0940, C$_7$H$_{14}$O$_3$ requires 146.0943. **^{1}H NMR** (400 MHz, CDCl$_3$) δ 4.52 (s, 1 H, C$\underline{H}$(OEt)$_2$), 3.69 (dq, *J* = 9.5, 7.0, 2 H OC$\underline{H}_2$CH$_3$), 3.55 (dq, *J* = 9.5, 7.0 , 2 H, OC$\underline{H}_2$CH$_3$), 2.19 (s, 3 H, CH$_3$), 1.24 (t, *J* = 7.0, 6 H, OCH$_2$C$\underline{H}_3$).**^{13}C NMR** (101 MHz, CDCl$_3$) δ 204.5 (C=O), 102.9 (CH), 63.4 (OC$\underline{H}_2$CH$_3$), 24.6 (CH$_3$), 15.3 (OCH$_2$C$\underline{H}_3$).

6.7.3 Preparation of (*E*)-N-cyclohexyl-1,1-diethoxypropan-2-imine (268)
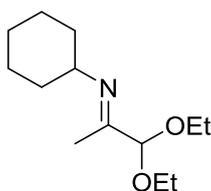
To a flame round bottom flask containing activated molecular sieves 4Å, solution of α ,α-diethoxyacetone **267** (100 mg, 0.7 mmol) in anhydrous DCM (2 mL) and previously distilled cyclohexylamine (101 µL, 0.9 mmol) were added. The reaction was stirred for 5 h. The mixture was filtrated through a short pad of celite® and washed with DCM. The solvent was removed under reduced pressure to afford the pure title compound as pale yellow oil (162 mg, 98%).

HRMS EI$^+$ [M + H] found 228.1958, $C_{13}H_{26}NO_2$ requires 228.1964. **^{1}H NMR** (300 MHz, CDCl$_3$) δ 4.54 (s, 1 H, N=CC$\underline{H}$(OEt)$_2$), 3.67 (dt, J = 14.0, 7.0, 2 H, OC$\underline{H}_2$CH$_3$), 3.46 (dt, J = 14.0, 7.0, 2 H, OC$\underline{H}_2$CH$_3$), 3.36 − 3.23 (m, 1 H, CH$_2$C$\underline{H}$N), 1.83 (s, 3 H, CH$_3$), 1.76 (bd, J = 12.0, 2 H, CH$_2$), 1.60 (bd, J = 14.0, 2 H, CH$_2$), 1.49 − 1.12 (m, 12 H, 3 x CH$_2$, 2 x (OCH$_2$C$\underline{H}_3$)).**^{13}C NMR** (75 MHz, CDCl$_3$) δ 164.9 (C=N), 106.9 (s, 1H, N=$\underline{C}$CH(OEt)$_2$), 63.1 (CH$_2\underline{C}$HN) , 58.9 (O$\underline{C}$H$_2$CH$_3$), 33.3 (CH$_2$), 25.6 (CH$_2$), 24.9 (OCH$_2\underline{C}$H$_3$), 15.2 (CH$_2$), 11.2 (CH$_3$).

6.7.4   Preparation of trimethyl((6-methylhepta-1,5-dien-2-yl)oxy)silane (257)
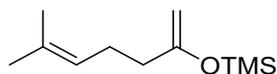
To a stirred solution of DIPA (1.4 mL, 9.9 mmol) in anhydrous THF (4 mL) at 0 ℃, n-butyl lithium (3.9 mL, 2.5 M, 9.9 mmol) was added. The reaction mixture was stirred at this temperature for 30 min, whereupon pale yellow colour was observed. To the resulting LDA solution was cooled to -78 ℃, a solution of 6-methylhept-5-en-2-one (0.5 g, 3.9 mmol) in anhydrous THF (6 mL) was added. The mixture was stirred at this temperature for 1 h and then freshly redistilled chlorotrimethylsilane (1.1 g, 9.9 mmol) was added. The reaction was warmed to 0 ℃ and stirred for 45 min. The reaction was quenched by the addition of a saturated NH$_4$Cl aqueous solution (5 mL). Organic material was extracted with diethyl ether (3 x 5 mL), combined organic extracts were washed with brine (3 x 10 mL), dried over MgSO$_4$ and solvent was removed under reduced pressure to afford yellow oil (0.7 g, 90%). The sample was analysed by NMR spectroscopy using deactivated CDCl$_3$ as solvent.

HRMS EI$^+$ [M + H] found 199.1519, C$_{11}$H$_{23}$OSi requires 199.1518. **^{1}H NMR** (300 MHz, CDCl$_3$) δ 4.91 (t, J = 7.0 , 1 H, ((CH$_3$)$_2$CC<u>H</u>), 3.84 (s, 2 H, C=CH$_2$), 2.02 − 1.76 (m, 4 H, 2 x (CH$_2$)), 1.48 (s, 3 H, (CH$_3$)), 1.41 (s, 3 H, (CH$_3$)), 0.01 (s, 9 H, Si(CH$_3$)$_3$). **^{13}C NMR** δ (75 MHz, CDCl$_3$) δ 131.86(CH$_2$=<u>C</u>OSi), 131.81 ((CH3)$_2$<u>C</u>CH), 123.92((CH3)$_2$C<u>C</u>H), 90.13(<u>C</u>H$_2$=COSi), 36.75 (CH$_2$), 25.83(CH$_3$), 25.65 (CH$_3$), 17.84 (CH$_2$), 4.22 (SiCH$_3$), 3.08 (SiCH$_3$), 0.27 (SiCH$_3$).

6.7.5   Preparation of 1-hydroxy-6-methylhept-5-en-2-one (258)
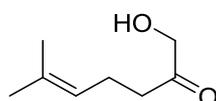
To a stirred solution of silyl enolate **257** (0.8, 3.9 mmol) in DCM (20 mL) at -78 ℃, a solution of mCPBA (870 mg 70%, 3.5 mmol) in DCM (130 mL) was added drop-wise. The mixture was stirred for 1 h. Another portion of mCPBA (0.3 g, 70%, 1.2 mmol) was added to the heterogeneous mixture and stirred for an additional 2 h. A 10% aqueous HCl solution (20 mL) was added and the biphasic mixture was warmed to 0 ℃ and stirred for 1 h. The excess of HCl was neutralised with saturated solution of K$_2$CO$_3$ (50 mL) and the mixture stirred overnight. The precipitate was filtrated, the organic material was extracted with DCM (3 x 50 mL), the combined organic extracts were washed with brine, dried over MgSO$_4$ and solvent was removed under reduce pressure. The crude product was purified by vacuum chromatography with mixture of hexane and ethyl acetate (4:1) as eluent to afford the title compound as pale yellow oil (20 mg, 4%).

HRMS EI$^+$ [M + Na] found 165.0891, C$_8$H$_{14}$O$_2$Na requires 277.1780. **^{1}H NMR** (400 MHz, CDCl$_3$) δ 5.04 (t, J = 7.0, 1 H, C=CH), 4.22 (s, 2 H, C<u>H</u>$_2$OH), 3.12 (bs, 1 H, CH$_2$O<u>H</u>), 2.43 (t, J = 7.5, 2 H, CH$_2$C<u>H</u>$_2$C=O), 2.31 (q, J = 7.0, 2 H, C<u>H</u>$_2$CH$_2$C=O), 1.67 (s, 3 H, CH$_3$), 1.61 (s, 3 H, CH$_3$).**^{13}C NMR** (101 MHz, CDCl$_3$) δ 209.7 (C=O), 133.7 (<u>C</u>=CH), 122.0 (C=<u>C</u>H), 68.4 (CH$_2$OH), 38.6 (CH$_2$), 25.8 (CH$_2$), 22.5 (CH$_3$), 17.8 (CH$_3$).

6.7.6 Preparation of ethyl 4,4-diethoxy-3-oxobutanoate (272)
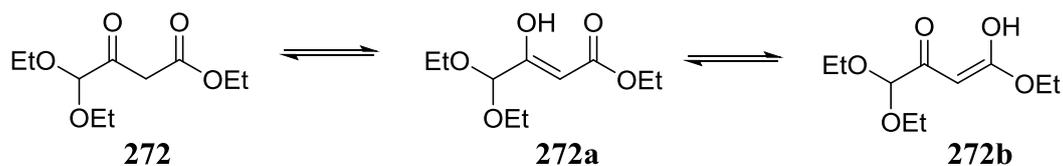
To a stirring suspension of sodium hydride (20.0 g, 97,8 mmol) in anhydrous THF (170 mL) a mixture of anhydrous ethyl acetate (13 mL, 127 mmol) and ethyl diethoxyacetate (20.0 g, 98.0 mmol) was added over 20 min. Then, the reaction was refluxed for 3 h before allow cooling to room temperature and additional stirring for 16 h. The reaction was quenched by the rapid addition of aqueous acetic acid (100 mL, 15% v/v) at 0 °C and the organic material was extracted with diethyl ether (3 x 30 mL). The combined organic layers were washed sequentially with water (2 x 40 mL), brine (3 x 40 mL), dried over $MgSO_4$ and then concentrated in vacuum to afford the title compound (17.3 g, 81%) as a mixture of tautomers **272, 272a, 272b** (0.3:1:1).

HRMS EI$^+$ [M + Na] found 241.1043, $C_{10}H_{18}O_5Na$ requires 241.1052. **^{1}H NMR** (300 MHz, CDCl$_3$) δ 4.87 (d, J = 1.0, 1 H, C=CH **5.40b**), 4.65 (d, J = 1.0, 1 H, C=CH, **5.40a**), 4.19 (m, J = 20.0, 7.0, 1.0, 2 H, C=OOCH$_2$), 3.82 − 3.43 (m, 5 H, 2 x OCH$_2$, HC(CH$_2$)$_2$), 2.32 (s, 2 H,C=OCH$_2$C=O, **5.40**), 1.34 − 1.14 (m, 9 H, 3 x CH$_3$). **^{13}C NMR** (75 MHz, CDCl$_3$) δ 198.9 (C=OOCH$_2$), 167.3 (C=OCH), 102.1(CH), 63.6 (C=OOCH$_2$), 61.4 (OCH$_2$), 44.3 (C=OCH$_2$C=O), 15.2 (CH$_3$), 14.2 (2 x CH$_3$).

6.7.7 Preparation of 1,1-diethoxy-6-methylhept-5-en-2-one (269)
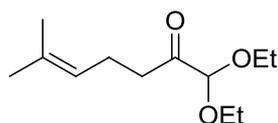
To a suspension of NaH (0.76 g, 19.1 mmol, 60% in mineral oil) previously washed with anhydrous hexane (3 x 5 mL) in anhydrous THF (80 mL) at 0 °C a solution of **272** (4.5 g, 19

mmol) in THF (15 mL) was added drop-wise, the mixture was further stirred for 1 h. Then a solution of the allylic bromide (2.8 g, 19.1 mmol) in anhydrous THF (5 mL) was added dropwise. Reaction was warm to room temperature and stirred for 16 h. The reaction was cooled to 0 °C, quenched by the addition of saturated solution of NH$_4$Cl (25 mL) and the organic material was extracted with diethyl ether (3 x 20 mL). Combined organic extracts were washed with brine (3 x 50 mL), dried over MgSO$_4$ and concentrated under reduce pressure to afford 5 g of crude α-keto ester. The crude product was dissolved in absolute EtOH (233 mL) and water (400 mL) was added to the solution. Then a solution 5% NaOH (240 mL) was added, the resulting cloudy solution was refluxed for 4 h. The reaction was neutralised with aqueous solution of HCl (6M) and concentrated under reduce pressure. The organic material was extracted with diethyl ether (4 x 100 mL), the combine organic layers were washed with brine (3 x 70 mL), concentrated under reduce pressure to afford 1.2 g (30% over two steps) of pure compound.

HRMS ES$^+$ [M] found 214.1569, C$_{12}$H$_{22}$O$_3$ requires 214.1571. **^{1}H NMR** (250 MHz, CDCl$_3$) δ 5.05 (t, J = 7.0, 1 H, C=CH), 4.54 (s, 1 H, HC(OEt)$_2$), 3.76 – 3.43 (m, 4 H, 2 x OCH$_2$CH$_3$), 2.58 (t, J = 7.5, 2 H, CH$_2$C=O), 2.23 (dd, J = 14.5, 7.0, 2 H, HCCH$_2$), 1.75 – 1.51 (m, 6 H, 2 x OCH$_2$CH$_3$), 1.22 (t, J = 7.0 , 6 H, 2 x CH$_3$C=). **^{13}C NMR** (63 MHz, CDCl$_3$) δ 206.2 (C=O), 123.0 (CH$_3$C=C), 102.7 (HC=C), 63.3 (CH), 37.2(OCH$_2$CH$_3$), 25.7 (CH$_2$), 21.9 (2 x OCH$_2$CH$_3$), 17.7 (CH$_2$), 15.2 (2 x CH$_3$).

6.7.8 Preparation of (*E*)-2-((5-(3,3-dimethyloxiran-2-yl)-3-methylpent-2-en-1yl)oxy)tetrahydro-2H-pyran (252)
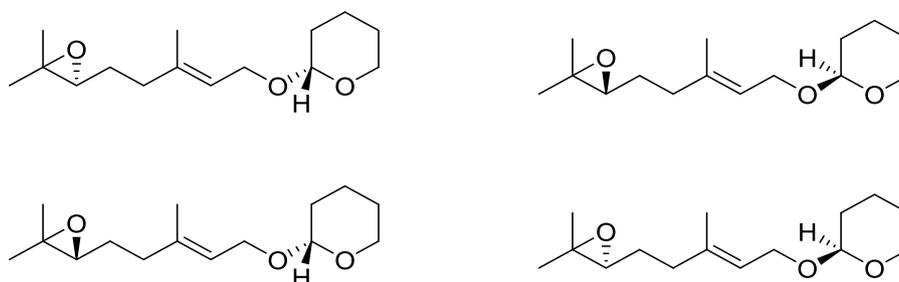
To a stirred solution of **223** (13.3 g, 56.0 mmol) in DCM (250 mL) at 0 °C, a solution of *m*-chloroperbenzoic acid (14.4 g, 70-75%, 61.0 mmol) in DCM (150 mL) was added by

cannula. The resulting slurry was stirred for 5 h at 0 ℃ and then Ca(OH)$_2$ (15 g, 200.0 mmol) was added, the suspension was stirred for further 40 min at room temperature. After filtration the residue was washed with DCM (50 mL), combine organic filtrate were washed with saturated solution of NaHCO$_3$ (2 x 70 mL), brine (3 x 70 mL) and concentrated under reduce pressure. Purification of the crude product by vacuum chromatography, with hexane and ethyl acetate (30:1) as eluent, gave the title compound as pale yellow oil (9.7 g, 69%).

HRMS EI$^+$ [M + Na] found 277.1781, C$_{15}$H$_{26}$O$_3$Na requires 277.1780. **^{1}H NMR** (300 MHz, CDCl$_3$) δ 5.39 (t, *J* = 7.0 , 1 H, C=CH), 4.61 (t, *J* = 3.5 , 1 H OCHCH$_2$), 4.24 (dd, *J* = 12.0, 6.5, 1 H, CH$_2$OCH), 4.01 (dd, *J* = 12.0, 7.5 , 1 H, CH$_2$OCH), 3.95 − 3.80 (m, 1 H, OCH$_2$CH$_2$), 3.59 − 3.43 (m, 1 H, OCH$_2$CH$_2$), 2.70 (t, *J* = 12.5, 1 H, CH from epoxide), 2.28 − 2.05 (m, 2 H, CH$_2$), 1.85 − 1.48 (m, 11 H, 4 x CH$_2$, CH$_3$), 1.29 (s, 3 H, CH$_3$), 1.25 (s, 3 H, CH$_3$). **^{13}C NMR** (75 MHz, CDCl$_3$) δ 99.3, 99.2, 98.5, 66.4, 66.3, 65.8, 63.9, 63.8, 63.7, 62.4, 62.3, 61.9, 61.6, 61.2, 61.1, 60.7, 60.0, 59.9, 59.8, 59.7, 58.5, 58.4, 35.3, 35.3, 34.9, 30.6, 30.5, 25.4, 24.9, 24.6, 24.4, 19.5, 19.4, 19.1, 18.7, 17.0, 16.9, 16.8, 16.7.

6.7.9 Preparation of (*E*)-4-methyl-6-((tetrahydro-2H-pyran-2-yl)oxy)hex-4-enal (253)
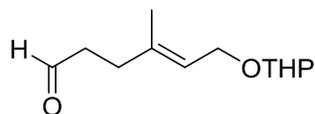
To a stirred solution of epoxide **252** (9.7 g, 38.0 mmol) in THF (75 mL) and water (45 mL) at 0 ℃, periodic acid (9.6 g, 42.0 mmol) and sodium periodate (4.0 g, 19.0 mmol) were added. The solution was stirred for 30 min and then brine (50 mL) was added. The organic material was extracted with diethyl ether (4 x 60 mL), combine ethereal extracts were washed with (2 x 80 mL), dried over magnesium sulphate and concentrated under reduced pressure. Purification of crude product by vacuum chromatography, with hexane and ethyl acetate (30:1) as eluent, gave the title compound as yellow oil.

HRMS EI$^+$ [M + Na] found 235.1306, C$_{12}$H$_{20}$O$_3$Na requires 235.1310. **^{1}H NMR** (300 MHz, CDCl$_3$) δ 9.75 (bs, 1 H, HCO), 5.36 (t, *J* = 7.0, 1 H, C=CHCH$_2$), 4.58 (t, *J* = 3.5, 1 H, OCHO), 4.21 (dd, *J* = 12.5, 6.5, 1 H, CH$_2$OCH), 3.99 (dd, *J* = 12.0, 7.0, 1 H, CH$_2$OCH), 3.85

(m, 1 H, OCH$_2$CH$_2$), 3.54 – 3.37 (m, 1 H, OCH$_2$CH$_2$), 2.55 (t, J = 8.0, 2 H, CH$_2$), 2.34 (t, J = 7.5, 2 H, CH$_2$), 1.91 – 1.71 (m, 2 H, CH$_2$), 1.67 (s, 3 H, CH$_3$), 1.53 (m, J = 14.0, 5.5, 4 H, 2 x CH$_2$). **^{13}C NMR** (75 MHz, CDCl$_3$) δ 202.2 (C=O), 138.0 (HC=C), 121.7 (HC=C), 98.1 (OCHO), 63.6 (CH$_2$OCH), 62.4 (OCH$_2$CH$_2$), 41.9 (CH$_2$), 31.6 (CH$_2$), 30.7 (CH$_2$), 25.5 (CH$_2$), 19.7 (CH$_2$), 16.6 (CH$_3$).

6.7.10 Preparation of (*E*)-4-methyl-6-((tetrahydro-2H-pyran-2-yl)oxy)hex-4-en-1-ol (254)
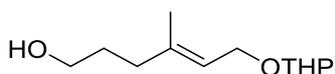
To a stirred solution of the aldehyde **253** (2.3 g, 11.0 mmol) in ethanol (100 mL) at 0 ºC sodium borohydride (480 mg, 13.0 mmol) was added. The reaction mixture was stirred for 4 h. Then a mixture acetone / water (70 mL, 1:10) was added, and the resulting mixture was concentrated under reduce pressure. The aqueous residue was extracted with diethyl ether (5 x 30 mL), the combined ethereal extracts were washed with HCl solution 1 M (2 x 50 mL), dried over MgSO$_4$ and concentrated under reduced pressure. The crude product was purified by vacuum chromatography, with hexane ethyl acetate (3:1) as eluent, to afford the title compound as colourless oil (1.9 g, 83%).

HRMS EI$^+$ [M + Na] found 237.1471, C$_{12}$H$_{22}$O$_3$Na requires 237.1467. **^{1}H NMR** (300 MHz, CDCl$_3$) δ 5.37 (t, J = 7.0, 1 H, C=CHCH$_2$), 4.59 (t, J = 3.0, 1 H, OCHO), 4.21 (dd, J = 12.0, 6.5, 1 H, CH$_2$OCH), 3.99 (dd, J = 12.0, 7.0, 1 H, CH$_2$OCH), 3.91 – 3.78 (m, 1 H, OCH$_2$CH$_2$), 3.60 (t, J = 6.0, 2 H, CH$_2$OH), 3.54 – 3.42 (m, 1 H, OCH$_2$CH$_2$), 2.08 (t, J = 7.5, 2 H, CH$_2$CH$_2$C(CH$_3$)=C), 1.92 – 1.72 (m, 2 H, CH$_2$), 1.66 (s, 5 H, CH$_3$, CH$_2$), 1.59 – 1.44 (m, 4 H, 2 x CH$_2$). **^{13}C NMR** (75 MHz, CDCl$_3$) δ 140.0 (HC=C), 120.9 (HC=C), 98.0 (OCHO), 63.7 (CH$_2$OCH), 62.6 (OCH$_2$CH$_2$), 62.4 (CH$_2$OH), 36.0 (CH$_2$), 30.7 (CH$_2$), 30.6 (CH$_2$), 25.5 (CH$_2$), 19.6 (CH$_2$), 16.4 (CH$_3$).

6.7.11 Preparation of (*E*)-2-((6-iodo-3-methylhex-2-en-1-yl)oxy)tetrahydro-2H-pyran (255)
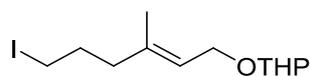
To a stirred solution of alcohol **254** (0.7 g, 3.5 mmol) in anhydrous DCM (5 mL), toluene-*p*-sulfonyl chloride (890 mg, 4.6 mmol) and pyridine (370 μL, 4.6 mmol) were added. The reaction mixture was stirred for 16 hours at room temperature in the dark. Then, the mixture was cooled to 0 °C, diluted with DCM (7 mL) and quenched by the addition of water (7 mL). Organic material was extracted with DCM (3 x 5 mL). The combine organic extracts were washed with solution 1 M HCl (3 x 10 mL), saturated solution of $NaHCO_3$ (2 x 10 mL), brine (3 x 10 mL), dried over Na_2SO_4 and solvent was removed under reduce pressure. The resulting yellow oil was used in the next step without further purification. Crude product was dissolved in anhydrous acetone (10 mL) and then anhydrous sodium iodide (2.9 g, 17.8 mmol) was added. The mixture was stirred at room temperature for 24 h. The acetone was concentrated under reduce pressure and $Na_2S_2O_3$ (5 mL) was added to the residue. Organic material was extracted with hexane (4 x 10 mL), the combine organic extracts were washed with brine (3 x 20 mL) and dried over $MgSO_4$. Solvent was removed under reduce pressure. Purification of the crude product by vacuum chromatography with hexane and ethyl acetate (40:1) as eluent gave the title compound as a yellow oil (630 mg, 57%).

HRMS EI$^+$ [M] found 324.0588, $C_{12}H_{21}O_2I$ requires 324.0586. **^{1}H NMR** (300 MHz, CDCl$_3$) δ 5.40 (t, *J* = 7.0, 1 H, C=C$\underline{H}$CH$_2$), 4.61 (t, *J* = 3.5, 1 H, OCHO), 4.23 (dd, *J* = 12.0, 6.5, 1 H, C$\underline{H}_2$OCH), 4.07 – 3.96 (m, 1 H, C$\underline{H}_2$OCH), 3.94 – 3.82 (m, 1 H, OC$\underline{H}_2$CH$_2$), 3.56 – 3.42 (m, 1 H, OC$\underline{H}_2$CH$_2$), 3.16 (t, *J* = 7.0, 2 H, IC$\underline{H}_2$CH$_2$), 2.20 – 2.07 (m, 2 H, CH$_2$C$\underline{H}_2$C=CH), 2.01 – 1.88 (m, 2 H, CH$_2$), 1.87 – 1.70 (m, 2 H, CH$_2$), 1.66 (s, 3 H, CH$_3$), 1.63 – 1.45 (m, 4 H, CH$_2$).

6.7.12 Preparation of (*E*)-(4-methyl-6-((tetrahydro-2H-pyran-2-yl)oxy)hex-4-en-1-yl)triphenylphosphonium iodide (251)
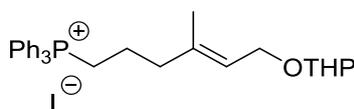
To a stirred solution of iodide **255** (63 mg, 1.9 mmol) in anhydrous diethyl ether (20 mL), previously purified triphenyl phosphine (0.6 g, 2.2 mmol) was added. The solution was refluxed for 24 h. The reaction mixture was warmed to room temperature. The white precipitate was filtrated, washed with anhydrous cold diethyl ether and dried under high vacuum to give the title compound as a white solid (734 mg, 67%).

HRMS EI[+] found 459.2463, $C_{30}H_{36}O_2P$ requires 459.2453.[1]**H NMR** (400 MHz, CDCl₃) δ 7.91 − 7.61 (m, 15 H, 3 x Ph), 5.32 (t, *J* = 6.5, 1 H, C=CHCH₂), 4.54 (s, 1 H, OCHO), 4.19 (dd, *J* = 12.0, 6.5, 1 H, CH₂OCH), 3.96 (dd, *J* = 12.0, 7.0, 1 H, CH₂OCH), 3.87 − 3.77 (m, 1 H, OCH₂CH₂), 3.74 − 3.61 (m, 2 H, Ph₃PCH₂), 3.46 (dd, *J* = 10.0, 5.5 , 1 H, OCH₂CH₂), 2.38 (t, *J* = 7.0 , 2 H, CH₂CH₂C=CH), 1.87 − 1.61 (m, 4 H, 2 x CH₂), 1.59 − 1.42 (m, 7 H, CH₃, 2 x CH₂). **¹³C NMR** (75 MHz, CDCl₃) δ 137.8 (HC=C), 135.2 (Ph), 133.8 (Ph), 133.6 (Ph), 130.7 (Ph), 130.5 (Ph), 122.6 (HC=C), 118.6 (Ph), 117.5 (PCH₂), 98.5 (OCHO), 63.7 (CH₂OCH), 62.7 (OCH₂CH₂), 30.8 (CH₂), 25.4 (CH₂), 22.0 (CH₂), 20.3 (CH₂), 19.8 (CH₂), 16.5 (CH₃). **³¹P NMR** δ (202.5 MHz, CDCl₃) 25.2 (s)

6.7.13 Preparation of 2-(((2E,6Z)-7-(diethoxymethyl)-3,11-dimethyldodeca-2,6,10-trien-1-yl)oxy)tetrahydro-2H-pyran (276)
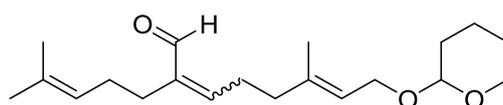
Isomer: a and b

To a stirred suspension of Phosphonium salt **251** (200 mg, 0.34 mmol) in anhydrous THF (3 mL) at − 78 ºC n-BuLi (2.5 M solution in hexanes, 136 µL, 0.3 mmol) was added. The

resulting orange solution was stirred for 30 min and then ketone **269** (56 mg, 0.3 mmol) was added. The reaction mixture was kept at -78 ℃ for 3 h and then warmed to -45 ℃ for another additional 6 h. The reaction was quenched by the addition of saturated solution of NH_4Cl (3 mL) warmed to room temperature and diluted with diethyl ether (3 mL). The organic material was extracted diethyl ether Et_2O (3 x 5 mL), organic extracts were washed with $Na_2S_2O_3$ (3 x 10 mL), brine (2 x 10 mL), dried over $MgSO_4$ and concentrated under reduce pressure. The excess of triphenyl phosphorous oxide was removed by dissolving the oil in cold hexane and filtration of the white precipitate followed by evaporation under reduce pressure. The crude product (80mg) was dissolved in a mixture THF / acetic acid (5 mL, 3:2) and stirred at room temperature for 10 min. The reaction mixture was diluted with 5 mL of water. The organic material was extracted with diethyl ether (4 x 10 mL), the combine organic extracts were washed with saturated aqueous solution of $NaHCO_3$ (3 x 15 mL), brine (2 x 20 mL) and concentrate under reduce pressure. Purification of the crude product by flash chromatography column with hexane and ethyl acetate (20:1) as eluent gave the title compound as a mixture of isomers 6:4 (25 mg, 30%).

HRMS EI$^+$ [M] found 320.2348, $C_{20}H_{32}O_3$ requires 320.2352. **^{1}H NMR** (400 MHz, CDCl$_3$) δ 10.09 (s, 1 H, HC=O, *Z* isomer), 9.35 (s, 1 H, HC=O, *E* isomer), 6.42 (t, *J* = 8.0 , 1 H, HC=OC=C<u>H</u>), 5.40 (dd, *J* = 13.0, 5.5, 1 H, C=CH), 5.07 (dt, *J* = 14.0, 7.0 , 1 H, C=CH), 4.61 (dd, *J* = 4.0, 2.5, 1 H, OCHO), 4.25 (dt, *J* = 12.0, 6.0, 1 H, C<u>H</u>$_2$OCH), 4.10 – 3.95 (m, 1 H, C<u>H</u>$_2$OCH), 3.91 – 3.79 (m, 1 H, OC<u>H</u>$_2$CH$_2$), 3.50 (dd, *J* = 10.5, 4.5, 1H, OC<u>H</u>$_2$CH$_2$), 2.69 (dd, *J* = 15.5, 8.0, 1 H, HC=OC=CHC<u>H</u>$_2$), 2.51 – 2.42 (dd, *J* = 15.5, 8.0, 1 H, HC=OC=CHC<u>H</u>$_2$), 2.22 (ddd, *J* = 21.0, 150, 7.5, 4 H, 2 x CH$_2$), 2.05 (dd, *J* = 14.0, 7.0 , 2 H, CH$_2$), 1.90 – 1.72 (m, 2 H, CH$_2$), 1.72 – 1.64 (m, 8 H, 2 x CH$_3$, CH$_2$), 1.63 – 1.48 (m, 5 H, CH$_3$, CH$_2$). **^{13}C NMR** (101 MHz, CDCl$_3$) δ 195.3 (HC=O, isomer b), 191.0 (HC=O, isomer a), 154.6 (HC=O<u>C</u>=CH, b), 148.8 (HC=O<u>C</u>=CH, a), 143.5 (HC=OC=<u>C</u>H, b), 140.0 (HC=OC=<u>C</u>H, a) , 138.5 (<u>C</u>=CHCH2OTHP, b), 138.3 (<u>C</u>=CHCH2OTHP, a), 132.6 (C=<u>C</u>HCH2OTHP, b), 132.4 (C=<u>C</u>HCH2OTHP, a), 123.7 ((CH$_3$)$_2$<u>C</u>=CH, b), 123.6 ((CH$_3$)$_2$<u>C</u>=CH, a), 122.3 ((CH$_3$)$_2$C=<u>C</u>H, b), 122.1 ((CH$_3$)$_2$C=<u>C</u>H, a), 98.1 (OCHO), 63.7 (<u>C</u>H$_2$OCH, b), 63.6 (<u>C</u>H$_2$OCH, a) , 62.5 (O<u>C</u>H$_2$CH$_2$, b), 62.4 (O<u>C</u>H$_2$CH$_2$, a) , 39.4 (CH$_2$, b), 38.3(CH$_2$, a), 30.8(CH$_2$, b), 30.5 (CH$_2$, a), 30.2 (CH$_2$, b), 29.8 (CH$_2$, a), 27.4 (CH$_2$), 27.2 (CH$_2$, b), 27.1 (CH$_2$, a), 25.9 (CH$_2$, b), 25.8 (CH$_2$, a), 25.6 (CH$_2$), 25.0 (CH$_2$, b), 24.4 (CH$_2$, a), 19.7 (CH$_3$, b), 19.7 (CH$_3$, a), 17.9 (CH$_3$, b), 17.8 (CH$_3$, a), 16.5 (CH$_3$, b), 16.5 (CH$_3$, a).

6.7.14 Preparation of (*2Z,6E*)-6-methyl-2-(4-methylpent-3-en-1-yl)-8-((tetrahydro-2H-pyran-2-yl)oxy)octa-2,6-dien-1-ol (277)
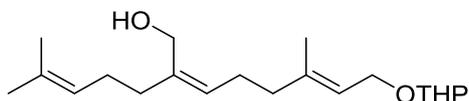
To a stirred solution of aldehyde **276** (25 mg, 0.1 mmol) in EtOH (1.5 mL) at 0 ºC, Sodium borohydride (6 mg, 0.1 mmol) was added. The mixture was stirred for 2 h. The reaction was quenched by the addition of acetone (3 mL) and water (5 mL). Organic solvent was removed under reduce pressure. The organic material was extracted from the aqueous residue with hexane (5 x 5 mL), combined organic extracts were washed with saturated aqueous $NaHCO_3$ solution (2 x 10 mL) and brine (3 x 10 mL). Solvent was removed under reduce pressure. The crude product was purified, and the two isomers separate, by vacuum chromatography with mixture of hexane and ethyl acetate (gradient 50:1 to 30:1) as eluent to afford the title compound as colourless oil (9 mg, 36%)

HRMS ES$^+$ [M + Na] found 245.2390, $C_{20}H_{34}O_3Na$ requires 345.2406. **^{1}H NMR** (300 MHz, CDCl$_3$) δ 5.37 (m, 2 H, 2 x C=CH), 5.11 (bs, 1 H, C=CH), 4.63 (t, *J* = 3.5, 1 H, OCHO), 4.24 (dd, *J* = 11.5, 6.0, 1 H, CH$_2$OCH), 4.02 (bs, 2 H, CH$_2$OH), 3.93 – 3.82 (m, 1 H, CH$_2$OCH), 3.74 (t, *J* = 6.5, 1 H, OCH$_2$CH$_2$), 3.61 – 3.44 (m, 1 H, OCH$_2$CH$_2$), 2.25 – 2.00 (m, 8 H, 4 x CH$_2$), 1.89 – 1.77 (m, 2 H, CH$_2$), 1.68 (s, 6 H, CH$_3$), 1.64 – 1.48 (m, 7 H, CH$_3$, 2 x CH$_2$).**^{13}C NMR** (126 MHz, CDCl$_3$) δ 139.8 (C=CH), 139.0 (C=CH), 131.7 (C=CH), 127.8 (C=CH), 124.2 (C=CH), 121.3 (C=CH), 97.9 (OCHO), 63.6 (CH$_2$OCH), 62.2 (OCH$_2$CH$_2$), 60.2 (CH$_2$OH), 39.5 (CH$_2$), 35.0 (CH$_2$), 30.6 (CH$_2$), 27.1 (CH$_2$), 25.8 (CH$_2$), 25.7 (CH$_2$), 25.5 (CH$_2$), 19.5 (CH$_3$), 17.7 (CH3), 16.5 (CH$_3$).

6.7.15 Preparation of (*2Z,6E*)-6-methyl-2-(4-methylpent-3-en-1-yl)-8-((tetrahydro-2H-pyran-2-yl)oxy)octa-2,6-dien-1-yl acetate (278)
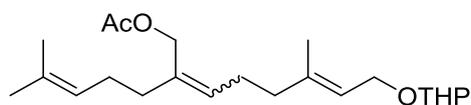
To a stirred solution of alcohol **277** (21 mg, 0.1 mmol) in DCM (2 mL) at room temperature, pyridine (50 μL, 0.6 mmol) and acetic anhydride (50 μL, 0.5 mmol) were added. The mixture was stirred at this temperature overnight. The reaction was diluted with DCM (10 mL) and 10% aqueous HCl solution (5 mL) was added, the mixture was stirred for 15 min at room temperature. Organic material was extracted with DCM (3 x 5 mL), combined organic extracts were washed with aqueous saturated NaHCO$_3$ solution (3 x 10 mL), brine (2 x 15 mL) and dried over MgSO$_4$. Solvent was removed under reduced pressure. The crude product was purified by flash chromatography column with mixture of hexane and ethyl acetate (gradient from 40:1 to 30:1) as eluent to afford the title mixture of compounds as a colourless oil (17 mg, 71%).

HRMS ES$^+$ [M] found 364.2609, C$_{22}$H$_{36}$O$_4$ requires 364.2614. **^{1}H NMR** (300 MHz, CDCl$_3$) δ 5.46 (t, *J* = 7.0, 1 H, C=CH), 5.37 (t, *J* = 8.0, 1 H, C=CH), 5.10 (s, 1 H, C=CH), 4.62 (t, *J* = 3.5, 1 H, OCHO), 4.58 (s, 1 H, C$\underline{H}_2$OAc isomer), 4.48 (s, 2 H, C$\underline{H}_2$OAc isomer), 4.24 (dd, *J* = 12.0, 6.5 , 1 H, C$\underline{H}_2$OCH), 4.07 − 3.95 (m, 1 H, C$\underline{H}_2$OCH), 3.94 − 3.83 (m, 1 H, OC$\underline{H}_2$CH$_2$), 3.58 − 3.45 (m, 1 H, OC$\underline{H}_2$CH$_2$), 2.19 (dd, *J* = 15.5, 7.5, 2 H, CH$_2$), 2.13 − 2.02 (m, 9 H, CO=OC$\underline{H}_3$, 3 x CH$_2$), 1.83 (ddd, *J* = 22.0, 13.0, 7.5, 2 H, CH$_2$), 1.68 (s, 6 H, 2 x CH$_3$), 1.64 − 1.46 (m, 7 H, CH$_3$, 2 x CH$_2$).**^{13}C NMR** (75 MHz, CDCl$_3$) δ 171.1 ($\underline{C}$=OOCH$_3$), 139.6 ($\underline{C}$=CH), 134.2 ($\underline{C}$=CH), 132.1 ($\underline{C}$=CH), 130.1 (C=$\underline{C}$H), 123.8 (C=$\underline{C}$H), 121.0 (C=$\underline{C}$H), 97.9 (OCHO), 68.7 ($\underline{C}$H$_2$C=OOCH$_3$), 63.6 ($\underline{C}$H$_2$OCH), 62.3 (O$\underline{C}$H$_2$CH$_2$), 35.2 (C=OO$\underline{C}$H$_3$), 30.7 (CH$_2$), 28.4 (CH$_2$), 26.9 (CH$_2$), 26.0 (CH$_2$), 25.8 (CH$_2$), 25.5 (CH$_2$), 21.1 (CH$_2$), 19.6 (CH$_3$), 17.7 (CH$_3$), 16.5 (CH$_3$).

6.7.16 Preparation of (*2Z,6E*)-8-hydroxy-6-methyl-2-(4-methylpent-3-en-1-yl)octa-2,6-dien-1-yl acetate (249)
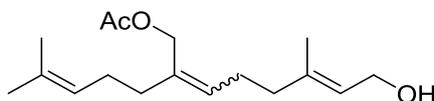
To a stirred solution of protected alcohol **278** (17 mg, 0.05 mmol) in methanol (2 mL) at room temperature, pyridinium p-toluenesulfonate (41 mg, 0.2 mmol) was added. The mixture was stirred overnight. The reaction was diluted with diethyl ether (10 mL) and saturated aqueous $NaHCO_3$ solution (10 mL) was added. The organic material was extracted with Et_2O (5 x 10 mL), combined organic extracts were washed with brine (2 x 30 mL), dried over $MgSO_4$ and concentrated under reduced pressure. Crude product was purified by flash chromatography column with mixture of hexane and ethyl acetate (4:1) as eluent to afford the title mixture of isomers as colourless oil (9 mg, 68%).

HRMS EI[+] [M + Na] found 303.1930, $C_{17}H_{28}O_3Na$ requires 303.1936. **^{1}H NMR** (300 MHz, $CDCl_3$) δ 5.43 − 5.28 (m, 2 H, 2 x C=CH), 5.04 (bs, 1 H, C=CH), 4.52 (s, 1 H, CH₂OAc isomer), 4.42 (s, 1 H, CH₂OAc isomer), 4.08 (d, *J* = 7.0 , 2 H, CH₂OH), 2.13 (dd, *J* = 15.5, 7.7 , 2 H, CH₂), 2.02 (s, 9 H, C=OOCH₃, 3 x CH₂), 1.61 (bs, *J* = 2.0, 6 H, 2 x CH₃), 1.54 (s, 3 H, CH₃). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 171.1 (C=OOCH₃), 139.1 (C=CH), 134.5 (C=CH), 132.2 (C=CH), 130.0 (C=CH), 124.1 (C=CH), 123.9 (C=CH), 68.7 (CH₂OAc), 59.5 (CH₂OH), 39.3 (CH₂), 28.6 (C=OOCH₃), 27.0 (CH₂), 26.0 (CH₂), 25.8 (CH₂), 21.2 (CH₃), 17.8 (CH₃), 16.3 (CH₃).

6.8 Synthesis of 8OH- FDP (141)

6.8.1 Preparation of (*2E,6E*)-8-hydroxy-3,7-dimethylocta-2,6-dien-1-yl acetate (237)
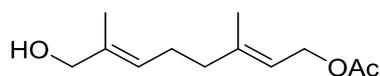
To a stirred solution of THP geranyl acetate (1.0 g, 5.1 mmol) in DCM (20 mL), SeO$_2$ (47 mg, 0.42 mmol), t-BuOOH (1.8 mL, 13.4 mmol) and salicylic acid (60 mg, 0.4 mmol) were added at 0 ºC. Reaction mixture was kept at this temperature for 5 h and then water (10 mL) was added. The organic material was extracted with DCM (3 x 15 mL), combine organic extracts were washed with saturated aqueous NaHCO$_3$ solution (2 x 15 mL), brine (3 x 15 mL), dried over MgSO$_4$ and concentrate under reduce pressure. Purification of the crude product by vacuum chromatography with hexane and Ethyl acetate (gradient from 5:1 to 3:1) as eluent gave the title compound as pale yellow oil (480 mg, 44%).

HRMS EI$^+$ [M + Na] found 235.1308, C$_{12}$H$_{20}$O$_3$Na requires 235.1310. **^{1}H NMR** (300 MHz, CDCl$_3$) δ 5.38 – 5.26 (m, 2 H, 2 x C=CH), 4.55 (d, J = 7.0, 2 H, CHCH$_2$C=OAc), 3.95 (s, 2 H, CH$_2$OH), 2.12 (m, 4 H, 2 x CH$_2$), 2.03 (s, 3 H, OC=OCH$_3$), 1.68 (s, 3 H, CH$_3$), 1.63 (s, 3 H, CH$_3$).**^{13}C NMR** (75 MHz, CDCl$_3$) δ 171.4 (CHCH$_2$C=OOMe), 141.9 (C=CH), 135.3 (C=CH), 125.2 (C=CH), 118.6 (C=CH), 68.8 (CHCH$_2$C=OOMe), 61.5 (CH$_2$OH), 39.1 (CH$_2$), 25.7 (CH$_2$), 21.1 (OC=OCH$_3$), 16.5 (CH$_3$) , 13.7 (CH$_3$).

6.8.2 Preparation of (*2E,6E*)-2,6-dimethyl-8-((tetrahydro-2H-pyran-2-yl)oxy)octa-2,6-dienal (235)
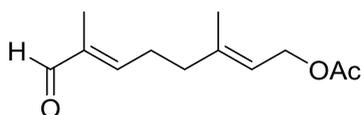
To a stirred solution of alcohol **237** (0.4 g, 1.9 mmol) in DCM (5 mL) containing molecular sieves 3Å at 0 ºC, PDC (920 mg, 2.5 mmol) was added and the solution was stirred for 4 h at this temperature. The reaction mixture was filtrate through celite® and washed with diethyl ether (50 mL). The organic filtrates were concentrate under reduce pressure. The crude product was purified by vacuum chromatography with a mixture of hexane and ethyl acetate (10:1) as eluent to afford the title compound as pale yellow oil (270 mg, 67%)

HRMS EI$^+$ [M + H] found 211.1329, C$_{12}$H$_{19}$O$_3$ requires 211.1334. **^{1}H NMR** (300 MHz, CDCl$_3$) δ 9.43 – 9.29 (m, 1 H, C=OCH), 6.43 (t, J = 7.0, 1 H, C=CH), 5.35 (t, J = 7.0, 1 H, C=CH), 4.56 (d, J = 6.0, 2 H, C=CHCH$_2$OC=O), 2.47 (q, J = 7.0 , 2 H, CHCH$_2$), 2.21 (t, J =

7.5, 2 H, CH$_2$), 2.05 – 1.99 (m, 3 H, HC=OCC$\underline{H}_3$), 1.71 (s, 6 H CH$_3$, OC=OCH$_3$).^{13}C **NMR** (75 MHz, CDCl$_3$) δ 195.3 (O=CH), 171.1 ($\underline{C}$=OCH$_3$), 153.6 ($\underline{C}$=CH) , 140.5($\underline{C}$=CH), 139.7 (C=$\underline{C}$H), 119.6 (C=$\underline{C}$H), 61.2 (CHC$\underline{H}_2$OC=O), 37.8 (CH$_2$), 27.0 (CH$_2$), 21.1(OCH$_3$), 16.5 (CH$_3$), 9.3 (CH$_3$).

6.8.3 Preparation of (*5E,9E*)-3,3,5,9-tetramethyl-11-((tetrahydro-2H-pyran-2-yl)oxy)undeca-1,5,9-trien-4-ol (240)
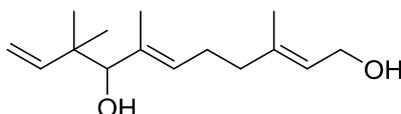
To freshly distilled diethyl ether (2 mL) containing Mg (240 mg, 10.0 mmol) tunings, previously activated at 180 ºC, small crystal of I$_2$ and 1,2-dibromoethane (2.8 μL, 0.03 mmol) were add. To the pale purple mixture at 0 ºC, a solution of 3, 3-dimethylallyl bromide (0.5 g 3.3 mmol) and the aldehyde **235** (130 mg, 0.7 mmol) in Et$_2$O (7 mL) were added. The mixture was slowly taken to reflux whereupon the purple colour disappeared and a cloudy solution was formed. The mixture was refluxed for 2.5 h until clear solution was formed with a yellow grey precipitate on the bottom. The reactions was cooled to 0 ºC and quenched by the addition of water (15 mL). The organic material was extracted with diethyl ether (3 x 15 mL), the combined organic extracts were washed with brine (3 x 20 mL), dried over Na$_2$SO$_4$ and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography column with a mixture of hexane and ethyl acetate (1:1) as eluent to afford the title compound as colourless oil (110 mg, 71%).

HRMS EI$^+$ [M-H$_2$O] found 221.1903, C$_{15}$H$_{24}$O requires 221.1905, ^{1}**H NMR** (250 MHz, CDCl$_3$) δ 5.84 (dd, *J* = 17.5, 11.0, 1 H, H$_2$C=$\underline{H}$C), 5.32 (t, *J* = 7.0, 1 H, C=C$\underline{H}$), 5.24 (t, *J* = 6.5, 1 H, C=C$\underline{H}$), 5.05 – 4.91 (m, 2 H, $\underline{H}_2$C=HC), 4.06 (d, *J* = 7.0 , 2 H, C$\underline{H}_2$OH), 3.68 (s, 1 H, C$\underline{H}$OH), 2.08 (dt, *J* = 20.5, 7.5, 4 H, 2 x CH$_2$), 1.60 (s, 3 H, CH$_3$), 1.54 (s, 3 H, CH$_3$), 0.94 (s, 3 H, CH$_3$), 0.91 (s, 3 H, CH$_3$).^{13}**C NMR** (75 MHz, CDCl$_3$) δ 145.7 ($\underline{C}$=CH), 139.0 ($\underline{C}$=CH), 135.3 (H$_2$C=H$\underline{C}$), 128.6 (C=$\underline{C}$H), 123.9 (C=$\underline{C}$H), 113.0 (H$_2\underline{C}$=HC), 83.7 (C), 59.3 (C$\underline{H}_2$OH), 42.0 ($\underline{C}$HOH), 39.1 (CH$_2$), 25.8 (CH$_2$), 25.1 (CH$_3$), 22.5 (CH$_3$), 16.2 (CH$_3$), 14.3 (CH$_3$).

6.8.4 Preparation of (*2E,6E*)-8-hydroxy-3,7,11-trimethyldodeca-2,6,10-trien-1-yl acetate (241)
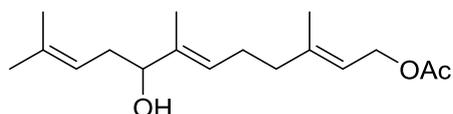
To a stirred solution of farnesyl acetate (5.0 g, 19.0 mmol) in DCM (40 mL) at room temperature, selenium dioxide (210 mg, 1.9 mmol) and t-butyl peroxide 70% solution (8.4 mL, 16.4 mmol) were added. The solution was stirred for 4 h. Then, Water (10 mL) was added and the mixture was extracted with DCM (3 x 15 mL). The combined organic extracts were washed with 10% NaOH aqueous solution (2 x 30 mL) and brine (3x 40 mL). After drying over MgSO$_4$ and concentration under reduce pressure, the crude product was purified by flash chromatography column with hexane and ethyl acetate (gradient from 10:1 to 4:1) as eluent to afford two separate acetoxyfarnesyl alcohols, (0.4 g, 8%) and (0.7 g, 13%).

HRMS EI$^+$ [M + Na] found 303.1924, C$_{17}$H$_{28}$O$_3$Na requires 303.1936, **^{1}H NMR** (500 MHz, CDCl$_3$) δ 5.41 – 5.29 (m, 2 H, 2 x C=CH), 5.08 (t, *J* = 7.0, 1 H, C=CH), 4.58 (d, *J* = 7.0, 2 H, CH$_2$OH), 3.97 (dd, *J* = 7.5, 6.0 , 1 H, CHOH), 2.33 − 2.05 (m, 6 H, CH$_2$), 2.05 (s, 3 H, C=OCH$_3$), 1.71 (s, 3 H, CH$_3$), 1.70 (s, 3 H, CH$_3$), 1.63 (s, 3 H, CH$_3$), 1.61 (s, 3 H, CH$_3$).**^{13}C NMR** (126 MHz, CDCl$_3$) δ 171.2 (C=OCH$_3$), 141.9 (C=CH), 137.5 (C=CH), 134.7 (C=CH), 125.5 (C=CH), 120.3 (C=CH), 118.8 (C=CH), 77.3 (CH$_2$OH), 61.5 (CHOH), 39.2 (CH$_2$), 34.4 (CH$_2$), 26.0 (CH$_2$), 25.8 (C=OCH$_3$), 21.1 (CH$_3$), 18.1 (CH$_3$), 16.6 (CH$_3$), 11.8 (CH$_3$).

6.8.5 Preparation of (*2E,6E*)-3,7,11-trimethyldodeca-2,6,10-triene-1,8-diol (238)
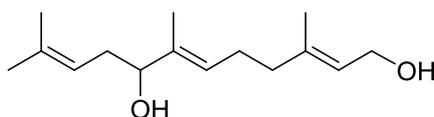
To a suspension of lithium aluminium hydride (30 mg, 0.8 mmol) in anhydrous diethyl ether (1 mL) at 0 ºC, a solution of the acetoxyfarnesyl alcohol **241** (100 mg, 0.35 mmol) in anhydrous diethyl ether (2 mL), was added drop-wise *via* syringe. The grey solution was stirred for 20 min. The reaction was diluted with diethyl ether (10 mL), quenched by the addition of 10% aqueous HCl solution (4 mL) and stirred for an additional 1 h at room temperature. Organic material was extracted with diethyl ether (3 x 10 mL). The combined organic extracts were washed with saturated $NaHCO_3$ aqueous solution (3 x 15 mL), brine (3 x 20 mL), dried over $MgSO_4$ and concentrated under reduce pressure. The crude product was purified by flash chromatography column with hexane and ethyl acetate (2:1) as eluent to afford the title compound as pale yellow oil (74 mg, 87%).

HRMS EI[+] [M-H_2O] found 220.1827, $C_{15}H_{24}O$ requires 220.1827, **^{1}H NMR** (500 MHz, CDCl_3) δ 5.35 (dt, J = 14.0, 7.0 , 2 H, 2 x C=CH), 5.06 (t, J = 7.0 , 1 H, C=CH), 4.11 (d, J = 6.5 , 2 H, CH_2OH), 3.95 (t, J = 6.5 , 1 H, CHOH), 2.38 – 1.94 (m, 6 H, 3 x CH_2), 1.69 (s, 3 H, CH_3), 1.65 (s, 3 H, CH_3), 1.61 (s, 3 H, CH_3), 1.59 (s, 1 H, CH_3).**^{13}C NMR** (126 MHz, CDCl_3) δ 139.0 (C=CH), 137.3 (C=CH), 134.6 (C=CH), 125.7 (C=CH), 124.0 (C=CH), 120.3 (C=CH), 77.3 (CH_2OH), 59.3 (CHOH), 39.2 (CH_2), 34.3 (CH_2), 25.9 (CH_2), 25.8 (CH_3), 18.1 (CH_3), 16.3 (CH_3), 11.8 (CH_3).

6.9 Synthesis of 10F-FDP (290)
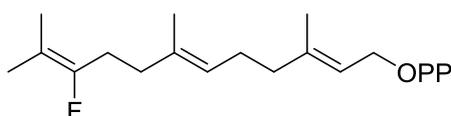
To a stirred suspension of LiCl (110 g, 2.7 mmol) in anhydrous DMF (3 mL) at 0 °C, S-collidine (0.2 mL, 1.0 mmol) and methanesulfonyl chloride (20 μL, 250 μmol) were added. The resulting solution was stirred for 15 min during which time a white cloudy precipitate formed. 10F farnesol (40 mg, 0.2 mmol) was added drop-wise as a solution in anhydrous DMF (1.5 mL), the reaction mixture was stirred to 0 °C for 4 h. The mixture was diluted in cold pentane (7 mL) and cold water (15 mL). Organic material was extracted with pentane (3 x 10 mL). Combined organic extracts were washed with saturated $CuSO_4$ aqueous solution

(3 x 15 mL), saturated NaHSO$_4$ aqueous solution(2 x 15 mL) and brine (2 x 20 mL) before drying (MgSO$_4$) and filtration. The solvent was concentrated under reduced pressure and the crude allylic chloride (43 mg) was used in the next step without further purification.

To a solution of the crude allylic chloride in anhydrous CH$_3$CN (1 mL) was added tris-(tetrabutylammonium) hydrogendiphosphate (270 mg, 0.3 mmol) and the mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure and the residue was dissolved in ion-exchange buffer (25 mM NH$_4$HCO$_3$ containing 2% i-PrOH, 4 mL). This solution was slowly passed through a column containing DOWEX 50W-X8 (100-200 mesh) cation exchange resin (NH$_4^+$ form) that had been pre-equilibrated. Once ion exchange was complete, fractions containing product were lyophilised to dryness. The resulting white solid was triturated with MeOH (3 x 10 mL), the first fraction was discard and rest of organic extracts were concentrated to dryness under reduced pressure affording yellow solid which was cleaned with CDCl$_3$ (3 x 3 mL) to give the partially pure title compound as a white solid (29 mg, 38%). The solid residue from the trituration step was further purified by reverse-phase HPLC (150 × 21.2 mm Phenomenex Luna column, eluting with 10% B for 20 min, then a linear gradient to 60% B over 25 min and finally a linear gradient to 100% B over 5 min.; solvent A: 25 mM NH$_4$HCO$_3$ in water, solvent B: CH$_3$CN, flow rate 5.0 mL/min, detecting at 220 nm). Once purification was complete the solution was again lyophilised to dryness giving the title compound as a fluffy white solid (21 mg, 28% yield).

HRMS ES$^-$ [M-H] found 399.1126, C$_{15}$H$_{26}$O$_7$FP$_2$ requires 399.1138. **^{1}H NMR** (400 MHz, D$_2$O) δ 5.43 (t, J = 7.0, 1 H, C=CH), 5.19 (t, J = 6.5, 1 H, C=CH), 4.43 (t, J = 6.5, 2 H, CH$_2$OPP), 2.35 (dt, J = 25.0, 7.0 , 2 H, CFCH$_2$), 2.12 (dd, J = 13.5, 6.0, 4 H, 2 x CH$_2$), 2.07 – 2.00 (m, 2 H, CH$_2$), 1.68 (s, 3 H, CH$_3$), 1.60 (s, 3 H, CH$_3$), 1.56 (d, J = 2.9, 3 H, 1 x (C$\underline{H}_3$)$_2$C=CF), 1.53 (d, J = 2.5 , 3 H, 1 x (C$\underline{H}_3$)$_2$C=CF). **^{31}P NMR** δ (202.5 MHz, ^{2}D$_2$O) -9.5 (d, J_{PP} = 20.8), -5.7 (d, J_{PP} = 20.8).**^{19}F NMR** (282.8 MHz, CDCl$_3$) δ -113.9 (t, J = 27.5, (CH3)$_2$C=CFCH$_2$). Data in agreement with previously publish data by Cane et al.[307]

6.10 Synthesis of (*3E,7E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene

6.10.1 Preparation of (*2E,6E*)-3,7,11-trimethyldodeca-2,6,10-trienal (361)
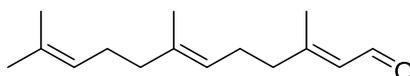
To a stirred solution of farnesol (60 mg, 27 μmol) in hexane (7 mL) at 0 ºC, MnO$_2$ (490 mg 5.6 mmol) was added and the mixture was stirred at this temperature for 4 h. The reaction mixture was filtrated over neutral celite and the filtrate was concentrate under reduce pressure. The crude product was purified by flash chromatography column with hexane and ethyl acetate (10:1) as eluent, to afford colourless oil (48 mg, 80%).

HRMS ES$^+$ [M] found 220.1823, C$_{15}$H$_{24}$O requires 220.1827. **^{1}H NMR** (300 MHz, CDCl$_3$) δ 9.99 (d, *J* = 8.0, 1 H, HC=O), 5.88 (d, *J* = 8.0, 1 H, C=CHC=OH), 5.17 − 4.98 (m, 2 H, 2 x C=CH), 2.28 − 2.18 (m, 4 H, CH$_2$), 2.17 (bd, *J* = 1.0, 3 H, CH$_3$C=CHC=OH), 2.11 − 1.93 (m, 4 H, 2 x CH$_2$), 1.67 (s, 4 H, 2 x CH$_2$), 1.59 (d, *J* = 2.0, 6 H, 2 x CH$_3$). **^{13}C NMR** (75 MHz, CDCl$_3$) δ 191.4 C=O, 164.1 (HC=C), 136.6 (HC=C), 131.6 (HC=C), 127.4 (HC=C), 124.1 (HC=C), 122.5 (HC=C), 40.6 (CH$_2$), 39.7 (CH$_2$), 26.6 (CH$_2$), 25.8 (CH$_2$), 25.7 (CH$_3$C=CHC=OH), 17.7 (CH$_3$), 17.7 (CH$_3$), 16.1 (CH$_3$).

6.10.2 Preparation of (*3E,7E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (362)
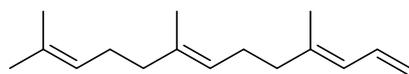
Methyltriphenylphosphonium bromide (115 mg, 0.3 mmol) and potassium *t*-butoxide (36 mg, 0.3 mmol) were dissolved in THF (0.7 mL) at room temperature. The resulting solution was stirred at this temperature for 10 min and then the yellow mixture was cooled to -78 ºC. A solution of the aldehyde **361** (48 mg, 0.2 mmol) in THF (0.5 mL) was added. After stirring the reaction for 1 h at this temperature water (0.4 mL) was added, the white precipitate was filtrated and the yellow filtrate was evaporate using a stream of N$_2$. The crude product was

purified by flash chromatography column with hexane and diethyl ether (10:1) as eluent to afford the title compound as yellow oil (29 mg, 62%).

HRMS EI[+] [M] found 218.2038, $C_{16}H_{26}$ requires 218.2035, **^{1}H NMR** (300 MHz, CDCl$_3$) δ 6.59 (dt, J = 17.0, 10.5, 1 H, C=CHC<u>H</u>=CH$_2$), 5.86 (d, J = 11.0, 1 H, C=C<u>H</u>CH=CH$_2$), 5.12 (bs, 2 H, 2 x C=CH), 5.08 (dd, J = 8.0, 1.5, 1 H, C=CHCH=C<u>H$_2$</u>), 4.98 (dd, J = 10.0, 2.0, 1 H, C=CHCH=C<u>H$_2$</u>), 2.25 − 1.93 (m, 8 H, 4 x CH$_2$), 1.77 (s, 3 H, CH$_3$), 1.68 (s, 3 H, CH$_3$), 1.60 (s, 6 H CH$_3$).**^{13}C NMR** (75 MHz, CDCl$_3$) δ 139.6 (<u>C</u>=CH), 135.4 (<u>C</u>=CH), 133.4 (<u>C</u>=CH), 131.4 (C=<u>C</u>H), 125.5 (C=<u>C</u>H), 124.3 (C=<u>C</u>H), 123.9 (C=<u>C</u>H), 114.6 (CH=<u>C</u>H$_2$), 39.9 (CH$_2$), 39.7 (CH$_2$), 26.7 (CH$_2$), 26.4 (CH$_2$), 25.8 (CH$_3$), 17.7 (CH$_3$), 16.7 (CH$_3$), 16.0 (CH$_3$).

6.11 Synthesis of FDP (17)

6.11.1 Preparation of tris-(tetrabutylammonium)hydrogen diphosphate (171)
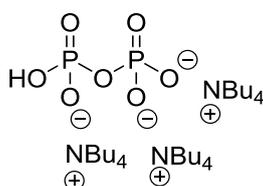
A solution of 3.5 g (16.0 mmol) of disodium dihydrogen diphosphate in aqueous ammonium hydroxide (10%, 17 mL) was passed through a column of Amberliyst (Acidic form). The free acid was eluted with water (120 mL). The resulting solution (pH 1.2) was immediately titrated to pH 7.3 with 40% (w/w) aqueous tetra-n-butylammonium hydroxide solution. The solution was dried by lyophilisation to yield 15.2 g of a white solid. The white solid was partially dried by treating with of acetonitrile (3 x 90 mL) and azeotropic removal of solvent by rotary evaporation to yield a sticky solid. This material was warmed to 40 ℃ in ethyl acetate (250 mL) before gravity filtration. The clear, colourless filtrate was concentrated by rotary evaporation until White solid stars crashing out and then cooled to -20 ℃. The short, white powder was filtrated under N$_2$ and dried under high vacuum. The title compound was recovered as a hygroscopic white solid (9.2 g, 65%).

HRMS EI⁻ found 176.9356, $H_3O_7P_2$ requires 176.9354. **¹H NMR** (400 MHz, CDCl₃) δ 3.38 – 3.27 (m, 2 H, NCH₂), 1.61 (dt, *J* = 16.0, 8.0, 2 H, CH₂), 1.49 – 1.33 (m, 2 H, CH₂), 0.93 (t, *J* = 7.5 , 3 H, CH₃). **¹³C NMR (101 MHz, CDCl₃)** δ 58.7 (NCH₂), 24.2 (CH₂), 19.7 (CH₂), 13.8 (CH₃). **³¹P NMR** (202 MHz, CDCl₃) δ -5.67

6.11.2 Preparation of trisammonium (2*E*,6*E*)-11-methyldodeca-2,6,10-trienyl diphosphate (17)
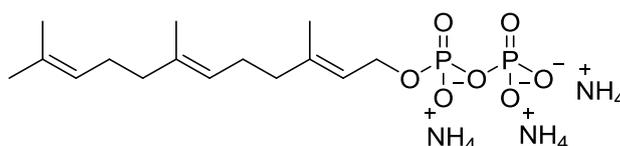
To a stirred solution of farnesol (700 mg, 3.1 mmol) in anhydrous DMF (42 mL) at 0 °C, s-collidine (2.35 mL, 19 mmol) and methanesulfonyl chloride (364 μl, 4.7 mmol) were added. After 20 min stirring at this temperature, lithium chloride (2.1 g, 50.0 mmol) was added to the solution. After 3 h at this temperature, the reaction was quenched by the addition of cold water (30 mL). The biphasic mixture was diluted with pentane and organic material was extracted with pentane (3 x 20 mL), the combined organic extracts were washed with saturated aqueous $CuSO_4$ solution (3 x 30 mL), water (3 x 30 mL), saturated aqueous $NaHCO_3$ solution (3 x 20 mL), dried over $MgSO_4$ and solvent were removed under reduce pressure. The crude chloride was used in the next step without further purification.

To a stirred solution of crude allylic chloride (750 mg) in acetonitrile (13 mL) tris-(tetrabutylammonium)hydrogen diphosphate (7 g, 7.8 mmol) was added. The reaction was stirred at room temperature for 16 h. Acetonitrile was removed under reduced pressure, the remaining yellow oil was dissolved in buffer (15 mL, 25 mM of NH_4HCO_3, 2% isopropanol) and passed thought an ion exchange column DOWEX 40 W (NH_4^+ form). The eluent from the ion exchange column was monitored by TLC (isopropanol / buffer / NH_4OH 6:2:2, staining with phosphomolibdic acid 10% in EtOH stain). Fractions containing product were collected and freeze dried. The yellow solid was diluted in buffer (15 mL). The crude was purified by reverse-phase HPLC (150 × 21.2 mm Phenomenex Luna column, eluting with 10% B for 20 min, then a linear gradient to 60% B over 25 min and finally a linear gradient

to 100% B over 5 min.; solvent A: 25 mM NH_4HCO_3 in water, solvent B: CH_3CN, flow rate 5.0 mL/min, detecting at 220 nm) in injections of 1.5 mL. The flow through of the HPLC was freeze dried and title compound was recovered as a white solid (recover in each injection 85 mg, 6%)

HRMS EI⁻ [M+2H] found 381.1237, $C_{15}H_{27}O_7P_2$ requires 381.1232, **^{1}H NMR** (250 MHz, D_2O) δ 5.38 (t, *J* = 7.0, 1 H, C=CH), 5.16 – 5.00 (m, 2 H, 2 x C=CH), 4.39 (t, *J* = 6.5, 2 H, CH_2OPP), 2.15 – 1.83 (m, 8 H, 4 x CH_2), 1.65 (s, 3 H, CH_3), 1.59 (s, 3 H, CH_3), 1.54 (s, 3 H, CH_3), 1.52 (s, 3 H, CH_3) **^{31}P NMR** $δ_P$ (121.6 MHz, D_2O) -5.82 (d, J_{PP} = 15.0), -9.53 (d, J_{PP} = 15.0).

6.12 Preparation of enzymes

6.12.1 General methods

LB media was prepared by dissolving tryptone (3 g), yeast extract purchased from Fluka and NaCl (30 g) in 3 L of deionised water.

Cell lysis buffer was prepared by dissolving of EDTA (5 mM), β-mercaptoethanol (5 mM) and tris-base (20 mM). The pH of the resulting solution was adjusted to 7.5 with 0.1 M aqueous solution of HCl. In the case of GDS detergent Tween® 20 (0.2%) was added to the buffer.

Dialysis buffer was prepared by dissolving tris-base (10 mM), NaCl (50 mM) and β-mercaptoethanol (5 mM) in deionised water. The pH was adjusted to 7.5 with 0.1 M aqueous solution of HCl. In the case of GDS detergent Tween® 20 (0.2%) was added to the buffer.

Binding buffer was prepared by dissolving tris base (50 mM), NaCl (500 mM), βME (10 mM) and imidazole (5 mM), The pH was adjusted to 8 with 0.1 M aqueous solution of HCl .

6.13 Preparation of AS

The expression of cDNA for AS and the purification of AS were performed according to published procedures.[308]

6.13.1 Transformation of *E. coli* BL21 (DE3) with cDNA for wild-type AS

A 100 µL sample of *E. coli* BL21(DE3) competent cells (from Dr. Veronica Garcia) in a Eppendorf vial was slowly defrost on ice. The vector containing a cDNA for AS and ampicillin resistance (1 µL, pZW04) was added to the cell solution. The preparation was left on ice for 30 min and then the mixture was thermally shocked by leaving the sample in a water bath at 42 ℃ for 45 s before returning the vial to the ice bath for 2 min. LB medium (1 mL) was added to the transformed cells and the solution was incubated with shaking (150 rpm) at 37 ℃ for 1 h. The cells were harvested by centrifugation (6000 rpm) for 1 min. The cells were re-suspended in the minimum amount of LB media and the mixture was spread onto an agar plate containing ampicillin. The plate was incubated at 37 ℃ for 12 h.

6.13.2 Over expression of AS

Ampicillin (53 mg) was added to sterilised LB media (100 mL) and the resulting LB-amp solution was inoculated with a single colony of pZW04 transformed *E. coli* BL21(DE3). The culture was inoculated at 37 ℃ overnight with shaking. The resulting culture was used to inoculate LB (4 x 500 mL, 5mL to each flask), containing ampicillin (53 mg each flask). The growth of bacteria was monitored by checking the OD of 1 mL of media at 600 nm until it reached 0.71, then the culture was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG, 63 mg each flask). The induced cultures were incubated at 37 ℃ for 3 h. Cells were harvested by centrifugation at 5000 rpm for 20 min, the supernatant solution was discarded and the pellets were stored at – 20 ℃.

6.13.3 Base extraction and refolding of aristolochene synthase

Combined pellet from culture were dissolved in cell lysis buffer (50 mL). The mixture was put in an ice bath and sonicated for 15 min (40% amplitude for 3 min with 5 s on/10 s off cycles). The resulting mixture was centrifuge at 5000 rpm for 10 min. The supernatant solution was discarded and the pellet was resuspended in fresh cell lysis buffer (70 mL). The stirred suspension was cooled to 0 ℃ and taken to pH 11.5 by the addition of aqueous

solution of NaOH (0.1 M). The solution was stirred for 20 min at this temperature. To the resulting stirred solution at 0 ºC β-mercaptoethanol (25 µL) was added and then pH was reduced to 8 at 0 ºC by the addition of aqueous HCl solution (0.1 M). The mixture was then stirred at this temperature for 30 min. The solution was centrifuge at 15000 rpm at 4 ºC for 30 min. The pellet was discarded and the supernatant was stored at 4 ºC.

6.13.4 Purification of AS by ion-exchange chromatography

The crude protein was purified by anion exchange chromatography on a Q-Sepharose™ (Amersham Pharmacia Biotech™) High Performance (2.5 × 20 cm) column. The absorbance of the eluate was measured at 280 nm to identify fractions containing protein. The cell free extract was loaded onto the column and then cell lysis buffer (150 mL) was used to remove any unbound protein. Protein was eluted with lysis buffer containing NaCl solution (500 mL gradient from 0.1 to 0.6 M) and then the column was washed with high salt lysis buffer solution (200 mL, 1 M, NaCl) to elute any remaining protein in the column. The presence of protein in fractions was confirmed by SDS-PAGE.

6.13.5 Dialysis and concentration of AS solutions

Fractions containing protein of the correct molecular weight as judged by SDS-PAGE were pooled and dialysed using Spectrum™ Spectra/Por molecular porous dialysis membrane (MW = 35000 cut off). The protein solution was dialysed in 3 litre of dialysis buffer at 4 ºC for 1 day.

The resulting protein solution was concentrated at 3 bar in a Amicon™ ultrafiltration apparatus containing a millipore 44.5 mm ultrafiltration membrane. The sample was concentrate to 4-5 mL and was stored at -20 ºC.

6.14 Preparation of GDS

Transformation of *E. coli* BL21 (DE3) with cDNA for wild-type GDS-His$_6$, Over expression, base extraction and refolding were performed following the same procedure as for AS.

6.14.1 Purification of GDS

The solution containing the protein was dialysed overnight. Then the solution was loaded onto a Ni Sepharose^{TM} 6 Fast Flow column (GE Healthcare, 12 mL, the column was eluted under gravity controlled drip flow). After 30 min, the column was washed with 4 CV of binding buffer followed by a gradient from 50 to 500 mM imidazole in binding buffer (20 CV) then a wash with 500 mM imidazole in binding buffer (10 CV). The protein eluted around 50-100 mM imidazole. Fractions were analysed by an SDS-PAGE. The fractions corresponding to a molecular weight of 63800 (GDS) were pooled, dialysed overnight and then concentrated to a final volume of ~ 5 m. Glycerol was added to the solution of purified enzyme (10% v/v) and the enzyme was stored at -20 °C.

6.15 Preparation of DCS and GAS

The preparation of these two enzymes was performed in the same manner as in the case of AS by Dr Veronica Gonzalez and Dr Sabrina Touchet.

6.16 Determination of the enzyme concentration (Bradford protein assay)

Bradford protein assay was used to determine the concentration of the enzyme in solution.[309]

6.17 Chemoenzymatic flow chemistry

6.17.1 General methods

Concentrations of sesquiterpenoids were measured using gas chromatography with flame ionisation detection (GC-FID) employing a manual injector and a VF-1ms column (15 m x 0.25 mm (0.25 mm). Carrier gas was helium (flow rate: 1 mL min^{-1}, split ratio 10:1) and chromatograms were started at 50 °C (injector temperature: 250 °C) held for 5 min at that temperature, then rising at 10 °C min^{-1} to 250 °C (held for 5 min) with the detector at 250 °C. For quantification of incubation products, the pentane used to overlay the incubations was spiked with α-humulene (35 µM, 1 mL) as an internal standard. FID integrations relative to the internal standard were used to calculate percentage conversions from the substrate. The linear response of the instrument against α-humulene was checked in the range between 5 and 100 µM (Figure 60).
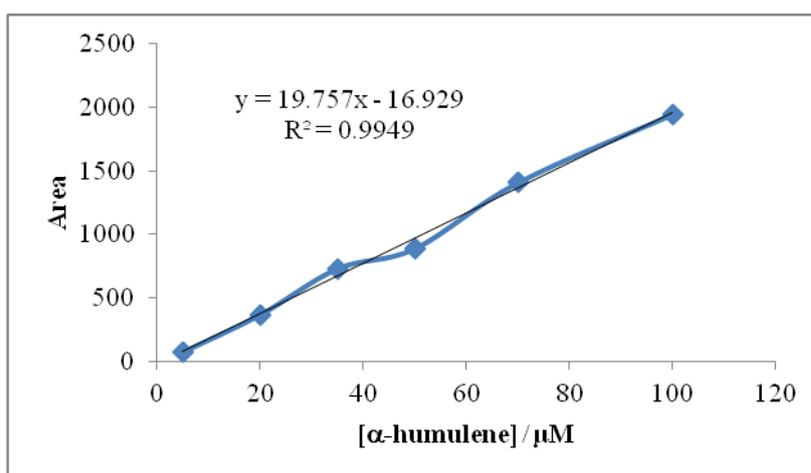
Figure 60. Linear response of the GC-FID against α-humulene.

All batch reactions were performed in glass vials and kept at 30 ℃ in an Eppendorf shaker thermomixer confort at 300 rpm. Volumes of organic material were measured using Hamilton syringe and aqueous volumes were measured using automatic pipettes. Solutions of standard were prepared in a volumetric flask.

Enzymatic transformations were performed in a flow reactor apparatus illustrated in Figure 61. Apparatus includes: 1 mL Hamilton syringe (organic phase), 1 mL disposable plastic syringe (aqueous phase), 2 syringe pumps KD-Scientific KDS-200-CE, tube with adaptors

for syringes, T-connector, tube reactor, temperature controlled water bath Buchi B481 and for experiments including sonication: ultrasonic bath Grant XB2 (50-60).
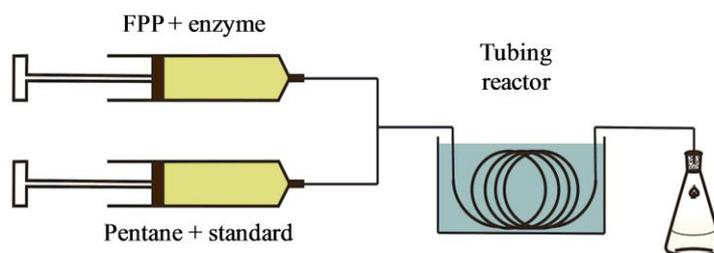
Figure 61. Schematic of the flow reactor apparatus used.

All experiments were performed in suitable aqueous incubation buffer for each enzyme, containing FDP and variable enzyme concentration. Pentane (1 mL), which was previously spiked with 35 µL of α-humulene solution (1 mM) as internal standard except in the case of (-)-germacrene D where pentane was spiked with 35 µL of a 10 mM α-humulene solution, was used to extract the organic material in the aqueous incubation media.

Standard batch experiments were performed in a glass GC-MS vial. To an enzyme solution in aqueous incubation buffer (500 µL), FDP (17.5µL of solution 10 mM, 0.35 mM) was added. The resulting mixture was overlayed with pentane solution containing internal standard (1 mL). The vial was sealed and incubated for 30 min at 30 ℃ with gentle agitation. Then, the biphasic mixture was vortexed for 1 min and the resulting emulsion was frozen with liquid nitrogen. After defrosting, the resulting biphasic mixture was separated and the pentane was analysed by GC-FID.

In a standard flow experiment, the aqueous incubation mixture was prepared in an identical manner to a batch experiment. The aqueous incubation was loaded in a disposable syringe and connected to the flow reactor. Pentane solution was load in a Hamilton syringe and connected to the flow reactor. The two solutions were passed through the flow reactor and collected in the collecting vial at the end. The reaction loop was evacuated by the injection of water at the same flow rate. The organic material was separated from the biphasic mixture and analysed by GC-FID.

The conversion was calculated by comparison of the integrals of the standard and the enzymatic product using the equation shown in Figure 62.

$$Conversion = \frac{C_{st} \times I_p}{I_{st} \times C_p} \times 100$$

Figure 62. C_{st}: concentration of standard , I_p: integral for the product, I_{st}: integral for the standard, C_p: concentration of product in organic layer assuming full conversion.

6.17.2 Representative GC-FID traces gas chromatograms

6.17.3 (+)-Aristolochene synthase (AS)

Gas chromatogram of the pentane extractable products isolated from the flow reaction with AS using a internal reactor diameter of 0.8 mm with sonication. This enzyme generates a small portion of intermediate (-)-germacrene A. The conversion achieved in this case was 88%.
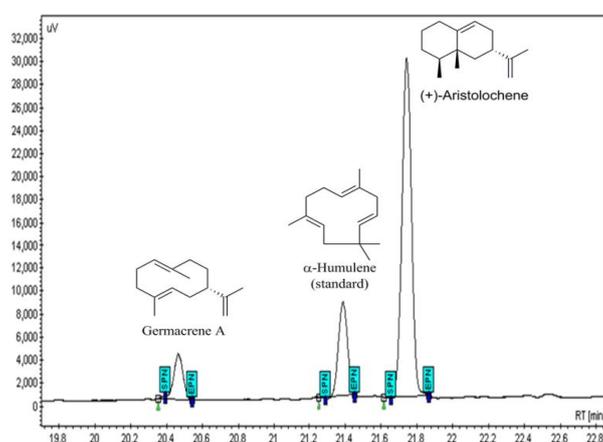
Index	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
1	UNKNOWN	20.46	9.54	3952.3	239.1	9.539
2	UNKNOWN	21.39	18.47	8110.2	463.0	18.473
3	UNKNOWN	21.74	71.99	29465.3	1804.5	71.989
Total			100.00	41527.9	2506.6	100.000

Figure 63. Gas chromatogram of the pentane extractable products arising from the incubation of AS and FDP in flow.

Gas chromatogram of pentane extractable products of the control experiment in batch with sonication, achieving in this case a conversion of 43%.
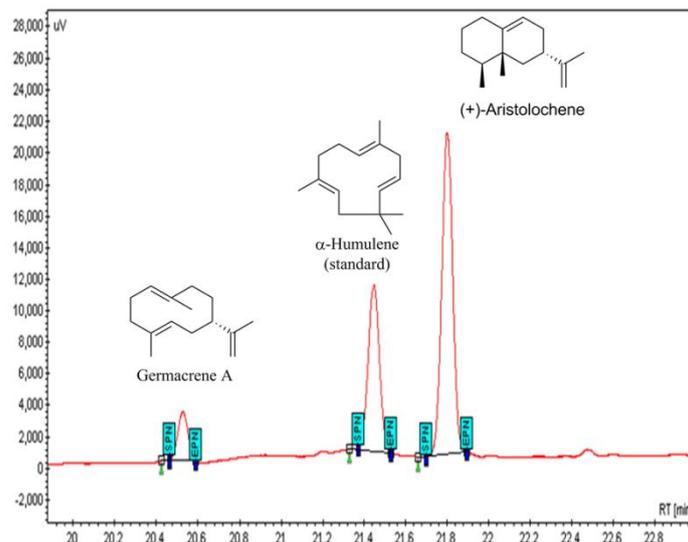
Index	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
1	UNKNOWN	20.53	8.52	3080.6	176.9	8.520
2	UNKNOWN	21.45	31.92	10513.3	662.9	31.920
3	UNKNOWN	21.80	59.56	20308.4	1237.0	59.560
Total			100.00	33902.4	2076.9	100.000

Figure 64. Gas chromatogram of the pentane extractable products arising from the incubation of AS and FDP in batch.

14Me FDP has been shown before to give the intermediate 14-methyl-germacrene A when incubated with AS. GC-FID chromatogram of the products isolated from the flow reaction of 14Me-FDP with AS using 0.8 mm internal reactor diameter under sonication. The conversion achieved in this case was 11%.
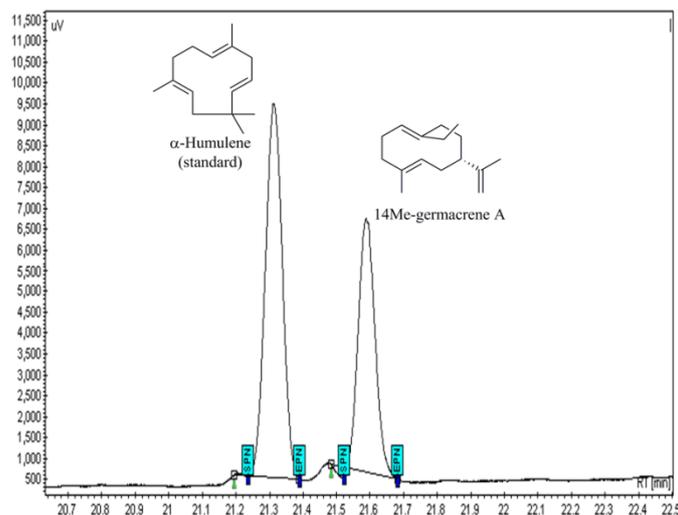
Index	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
1	UNKNOWN	4.94	99.89	31505693.6	1204129.8	99.891
2	UNKNOWN	21.30	0.05	10821.7	599.9	0.050
3	UNKNOWN	21.58	0.06	13281.1	718.2	0.060
Total			100.00	31529796.4	1205447.9	100.000

Figure 65. Gas chromatogram of the pentane extractable products arising from the incubation of 14Me-FDP and AS in batch.

Gas chromatogram of the pentane extractable products of the control experiment in batch with sonication, achieved in this case a conversion of 6%.
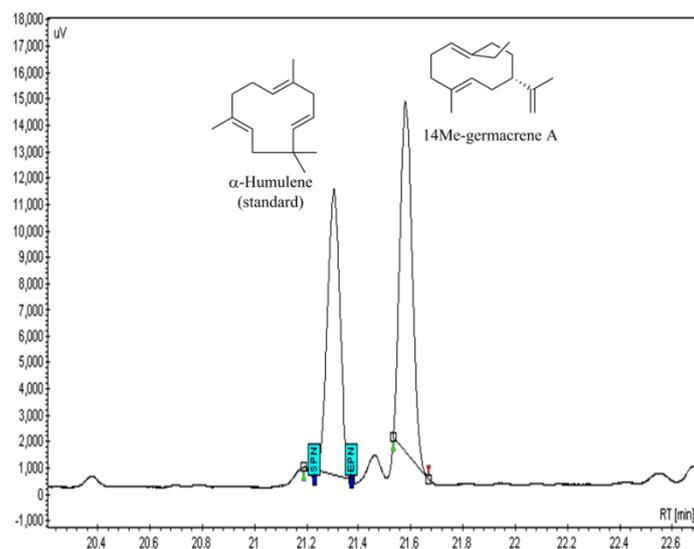
Index	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
1	UNKNOWN	4.83	99.98	46884429.9	4300752.0	99.980
2	UNKNOWN	21.31	0.01	8963.2	527.4	0.012
3	UNKNOWN	21.59	0.01	6070.4	340.1	0.008
Total			100.00	46899463.5	4301619.5	100.000

Figure 66. Gas chromatogram of the pentane extractable products arising from the incubation of 14Me-FDP and AS in batch.

Gas chromatogram of the pentane extractable products isolated from the flow reaction of 12Me_FDP with AS using 0.8 mm internal reactor diameter under sonication. The conversion achieved in this case is 63%. The identity of the products contain in the mixture obtained in this case is unknown.
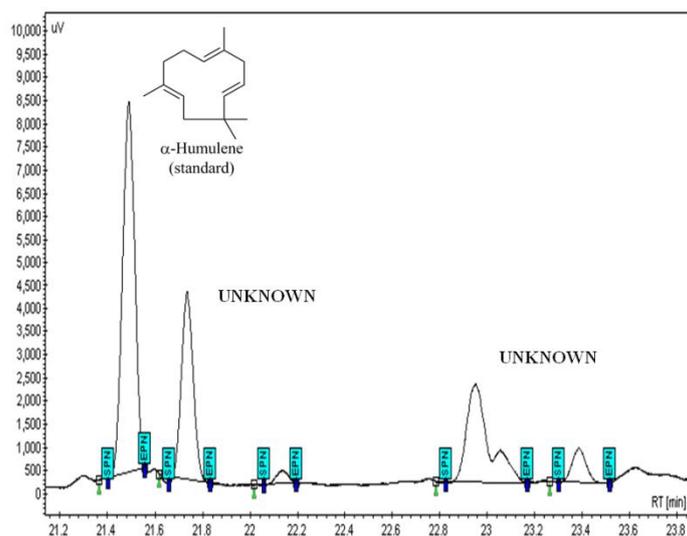
Index	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
1	UNKNOWN	5.39	99.97	23915498.6	7216395.3	99.973
2	UNKNOWN	21.49	0.01	8204.0	481.7	0.007
3	UNKNOWN	21.74	0.01	12572.9	751.5	0.010
4	UNKNOWN	22.97	0.01	7071.9	713.3	0.010
Total			100.00	23943347.4	7218341.8	100.000

Figure 67. Gas chromatogram of the pentane extractable products arising from the incubation of 12Me-FDP and AS in flow.

Gas chromatogram of pentane extractable products the control experiment in batch with sonication, achieving in this case a conversion of 22%.

Figure 68. Gas chromatogram of the pentane extractable products arising from the incubation of 12Me-FDP and AS in batch.

Gas chromatogram of the pentane extractable products isolated from the flow reaction of 15Me-FDP with AS using 0.8 mm internal reactor diameter under sonication. The conversion achieved in this case was 15 %. The product was identified as 15-Me-aristolochene.
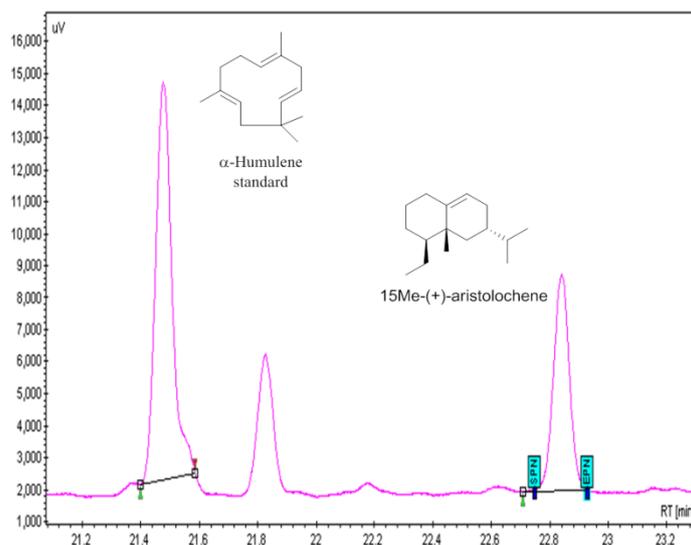
Index	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
1	UNKNOWN	5.29	99.99	26832773.7	9040626.2	99.986
2	UNKNOWN	21.48	0.01	12395.6	803.8	0.009
3	UNKNOWN	22.84	0.00	6750.0	422.3	0.005
Total			100.00	26851919.3	9041852.4	100.000

Figure 69. Gas chromatogram of the pentane extractable products arising from the incubation of 15Me-FDP and AS in flow

Gas chromatogram of the pentane extractable products of control experiment in batch with sonication, achieving in this case a conversion of 9%.
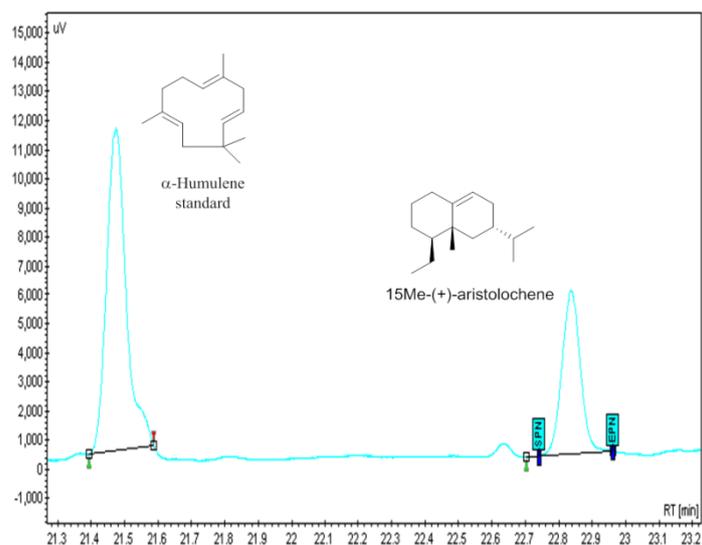
Index	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
1	UNKNOWN	5.34	99.99	23865720.9	7704310.1	99.986
2	UNKNOWN	21.48	0.01	11083.7	748.7	0.010
3	UNKNOWN	22.84	0.00	5614.9	357.3	0.005
Total			100.00	23882419.5	7705416.0	100.000

Figure 70. Gas chromatogram of the pentane extractable products arising from the incubation of 15Me-FDP and AS in batch

6.17.4 (+)-δ-Cadinene synthase (DCS)

Gas chromatogram of the pentane extractable products isolated from the flow reaction with DCS using 0.45 mm internal reactor diameter with sonication. The conversion achieved in this case was 56%.
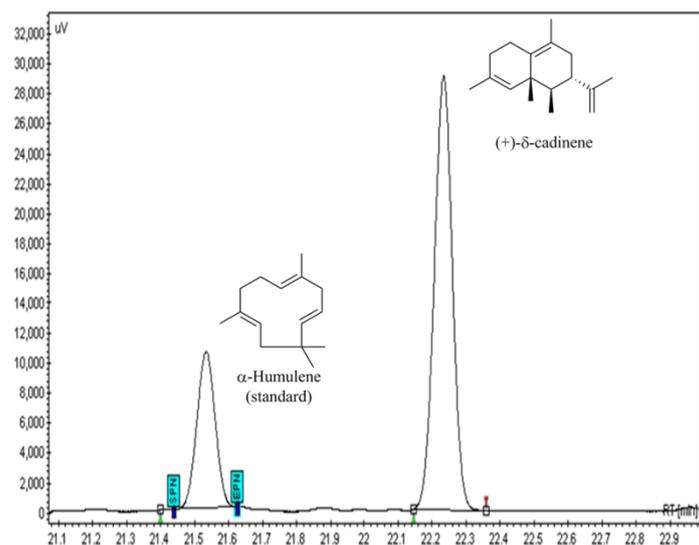
Index	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
1	UNKNOWN	21.53	26.64	10387.3	648.5	26.644
2	UNKNOWN	22.23	73.36	29010.1	1785.5	73.356
Total			100.00	39397.4	2434.0	100.000

Figure 71. Gas chromatogram of the pentane extractable products arising from the incubation of DCS and FDP in flow.

Gas chromatogram of the pentane extractable products from control experiment in batch with sonication, achieving in this case a conversion of 30%.
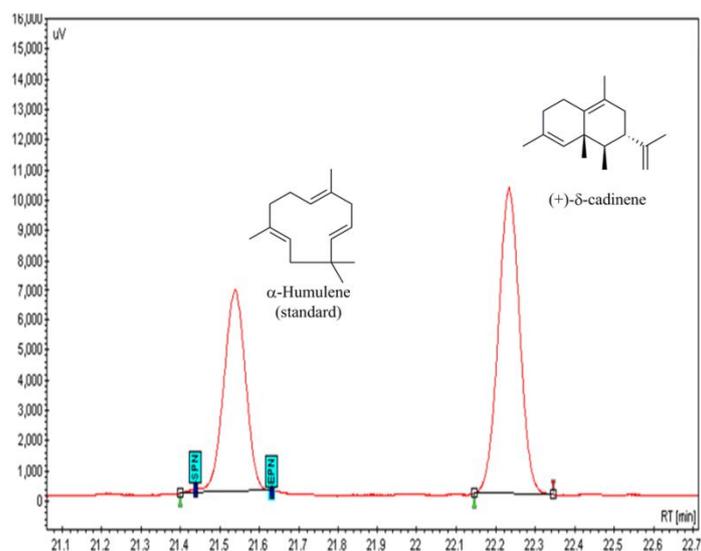
Index	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
1	UNKNOWN	21.54	40.28	6666.1	427.9	40.281
2	UNKNOWN	22.23	59.72	10192.4	634.3	59.719
Total			100.00	16858.5	1062.2	100.000

Figure 72. Gas chromatogram of the pentane extractable products arising from the incubation of DCS with FDP in batch.

Gas chromatogram of the pentane extractable products isolated from the flow reaction of 14Me-FDP with DCS using 0.45 mm internal reactor diameter under sonication. The conversion achieved in this case was 14%. The structure of the product obtained has not yet been determined.
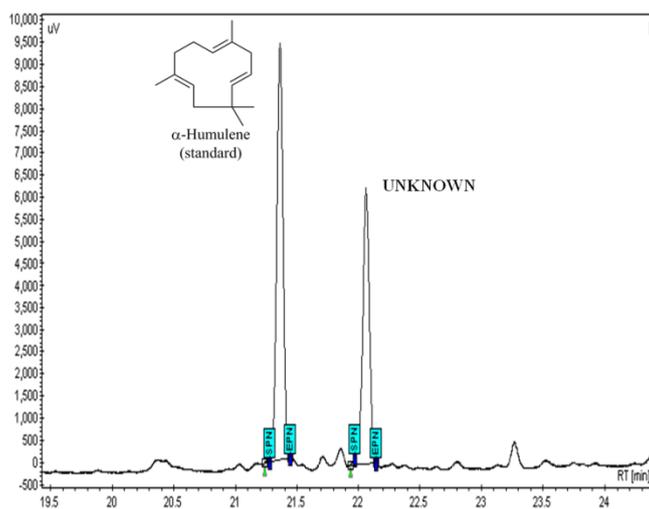
Index	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
1	UNKNOWN	5.12	99.99	29538830.9	8664987.3	99.989
2	UNKNOWN	21.36	0.01	9384.3	554.9	0.006
3	UNKNOWN	22.06	0.00	6214.2	387.2	0.004
Total			100.00	29554429.4	8665929.4	100.000

Figure 73. Gas chromatogram of the pentane extractable products arising from the incubation of 14Me-FDP and DCS in flow.

Gas chromatogram of the pentane extractable products isolated from control experiment in batch with sonication, achieving in this case a conversion of 9%.
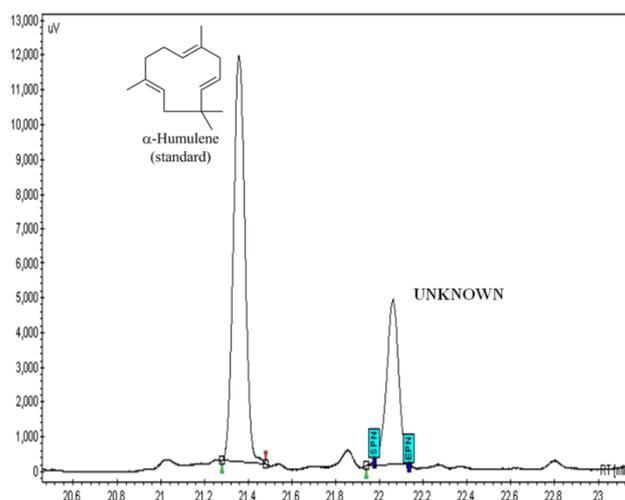
Index	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
1	UNKNOWN	21.36	70.16	11687.1	714.7	70.160
2	UNKNOWN	22.06	29.84	4740.5	304.0	29.840
Total			100.00	16427.6	1018.7	100.000

Figure 74. Gas chromatogram of the pentane extractable products arising from the incubation of 14Me-FDP and DCS in batch.

6.18 Identification of enzymatic products

6.18.1 General considerations

TLC plates, used for purification, were previously deactivated by immersion in 10% Et$_3$N solution in diethyl ether and allowed to dry for 48 h. Berberine.HCl was used as visualisation agent. Preparative TLC plates were sprayed with a solution of berberine.HCl 5% in diethyl ether and allowed to dry at least for 1 h.

Samples were applied to the preparative TLC plate using a glass pipette. The level of the crude compound in the TLC plate was homogenised by eluting the plate a few centimetres using diethyl ether as eluent, after allowing the ether to evaporated the plate was fully eluted with resolving solution. After the plate was eluted the product bands, were visualised with UV light and the bands corresponding to the product was scratched from the plate. The silica

was extracted with diethyl ether. The diethyl ether used in this procedure was previously distilled from sodium to remove stabilisers that would contaminate the desired products.

The concentration of pentane solutions of sesquiterpenoids used as samples for biological testing, were measured using a Varian 3900 GC-flame ionisation detection (FID) system with same temperature program described in the previous section. Samples necessarily could not contain an internal standard and so an external standard was used in these cases.

Samples of unknown concentration were injected (25 µL) and the area resulting from the integration of the sesquiterpene peak was used to estimate concentration. The area was used in an equation extrapolated from a calibration curve obtained with aromadendrene and 10F-farnesine mixture (calibration curves were performed by Dr Sabrina Touchet).

6.18.2 General procedure for qualitative analytical incubations

FDP (or FDP analogue) (9 µL, 10 mM) was diluted with 250 µL of optimised incubation buffer. Reaction was initiated by addition of enzyme solution (15 µL, 50 µM) was added followed by pentane (1 mL) and the mixture was gently agitated on a shaker at room temperature overnight. The olefin products were extracted by vortexing the biphasic mixture for 30 seconds. The organic extract was analysed by gas chromatography-mass spectrometry (GC-MS) as described in general synthetics procedures. The identification of germacrene A (and its homologues) by GC-MS was made by increasing the temperature of the injection port from 50 ℃ to 250.

6.18.3 General procedure for preparative incubations of FDP and analogues with sesquiterpene synthases.

All preparative incubations were performed using this method unless otherwise stated. Preparative incubations were performed in an ACE sealed tube. To a freshly prepared suitable incubation buffer (without β-mercaptoethanol) for each enzyme (*vide supra*, section 3.1) (30 mL,), stock solution of enzyme was added to achieve a final enzyme concentration of 12 µM. To the enzyme solution, farnesyl diphosphate (or analogue) stock solution (10 mM) was added as evenly as possible using an automatic pipette to achieve a final concentration of

0.4 mM. CDCl₃ (previously deactivated on basic alumina, 4 mL) was carefully deposited on the bottom of the sealed tube with a pipette to minimise contact with the aqueous incubation media. The sealed tube was closed and incubated in a shaker at 250 rpm for 24 h at 30 ℃. The biphasic mixture was rotated at 4 ℃ in a rotator over night extract sesquiterpenes. The resulting emulsion was frozen in liquid nitrogen and allowed to defrost at room temperature whereupon a biphasic mixture was formed. The biphasic mixture was separated with a phase separator (IST) and passed through a short plug of MgSO₄ and another of neutral alumina. The sealed tube was washed with an additional portion of CDCl₃ (2 mL). The organic layer was collected in an NMR tube and excess of solvent was removed under gentle stream of nitrogen until a suitable volume for analysis (0.4 mL) was obtained.

6.19 (+)-Germacrene A synthase (GAS)

6.19.1 Incubation of 14Me-FDP (137) with GAS
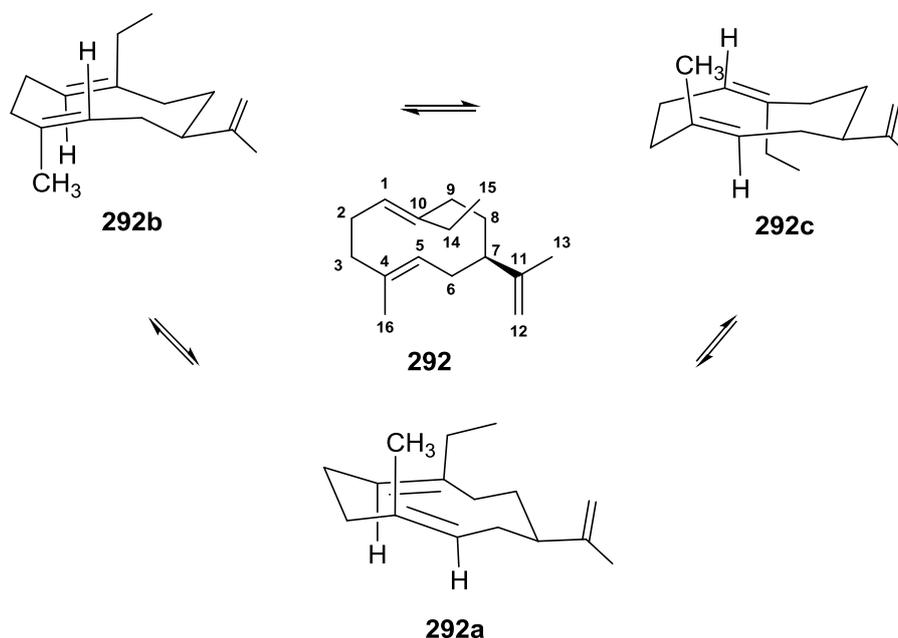
Incubation of GAS (30 mL DCS incubation buffer solution, 12 μM, 23 mg) with 14Me-FDP (1,2 mL, stock solution of 10 mM, 5.4 mg) was performed following the general procedure described above. The final solution of (R)-14Me-germacrene A (292) in CDCl₃ was analysed

265

by variable temperature ^{1}H NMR spectroscopy without further purification. The data was identical to the previously isolated (*S*)-14Me-germacrene A (**320**) *vide infra*.

^{1}H NMR 500 MHz, CDCl$_3$, -50 °C (δ, m, *J* in Hz)

Conformer	H-12	H-12'	H-13	H-15	H-16	H-1	H-5
292^a	**4.64** (s)	**4.56** (s, 1H)	**1.70** (bs)	**1.47** (s)	**0.88** (t, 7.5)	**4.71** (dd, 12.0, 4.0)	**4.53** (bs)
292B	**4.64** (s)	**4.60** (s,)	**1.70** (bs)	**1.43** (s)	**0.85** (t, 7.5)	**4.91** (dd,18.5, 10.5)	**5.01** (dd, 9.0, 4.0)
292C	**4.64** (s)	**4.53** (bs)	**1.70** (bs)	**1.44** (s)	**0.94** (t, 7.5)	**4.91** (dd,18.5, 10.5)	**5.22** (t, 7.5)

Table 15. Assignment of resonances in the ^{1}H NMR spectrum of **292**.

6.19.2 Incubation of 10F-FDP (290) with GAS

10F-FDP analogue was provided by Dr Juan A. Faraldos. Incubation of GAS (30 mL DCS incubation buffer solution, 12 μM, 23 mg) with 10F-FDP (1.2 mL, stock solution of 10 mM, 5.4 mg) was performed following the general procedure. **300** was purified by preparative TLC.

300
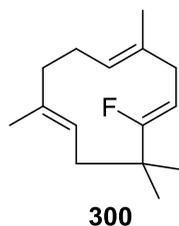
^{1}H NMR (500 MHz, CDCl$_3$) δ 4.97 (dt, *J* = 40.5, 8.0, 1H). **^{19}F NMR** (565 MHz, CDCl$_3$) δ 109.9 (d, *J* = 41.5, C<u>F</u>=C). *m/z* (EI$^+$): 222.2 (50%, M$^+$), 179.1 (8%, M$^+$-C$_2$F), 154.1 (17%, M$^+$-C$_5$H$_8$), 139.1 (98%, M$^+$-C$_6$H$_{11}$), 125.1 (31%, M$^+$-C$_7$H$_{13}$), 111.1 (100%).

6.20 Aristolochene synthase (AS)

6.20.1 Incubation FDP (17) with AS

56
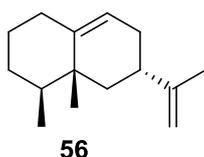
AS incubation buffer solution (300 mL, 12 μM AS, 140 mg) was added to an oven dried glass trap equipped with glass stopper (typically used as a liquid nitrogen trap in a high vacuum apparatus), aqueous enzyme solution (580 μM,, 4 mL) was then added followed by the addition of aqueous FDP solution (10 mM, 12 mL). CHCl$_3$ (30) mL was deposited in the bottom of the trap and the biphasic mixture was incubated for 12 h. Workup of the incubation was done as indicated in the general procedure. Once the aqueous layer media was separated from the organic material, extraction process was repeated two additional times with fresh aliquots of CHCl$_3$. Each aliquot was concentrated separately under reduce pressure (to ca. 5 mL) and the final volume of solvent was removed under stream of nitrogen in an NMR tube. Each extract was weighted (i. e. compounds were concentrated to dryness to measure yield in this instance) and analysed separately by NMR spectroscopy: first extraction 11 mg (45 %), second extraction 7 mg (28%), third extraction 4 mg (16%) and combined material after solvent removal 18 mg (73%) of pure **56**.

^{1}H NMR (300 MHz, CDCl$_3$) δ 5.32 (dd, J = 3.5, 2.0, 1 H, C=CH), 4.71 (s, 2 H, C=CH$_2$), 2.29 – 1.77 (m, 7 H, 3 x CH$_2$, HC(CH$_3$)C=CH$_2$), 1.74 (s, 3 H, CH$_3$), 1.31 (m, 5 H, 2 x CH$_2$, HCCH$_3$), 0.97 (s, 3 H, CH$_3$), 0.84 (d, J = 6.5, 3 H, , HC(CH$_3$)C=CH$_2$).**^{13}C NMR** (75 MHz, CDCl$_3$) δ 150.8 (C=CH$_2$), 144.6 (C=CH), 118.9 (C=CH), 108.4 (C=CH$_2$), 44.3 (CHCH$_3$), 43.4 (CH$_2$), 38.9 (CCH$_3$), 37.9 (HCC=CCH$_3$), 32.7 (CH$_2$), 31.5 (CH$_2$), 31.3 (CH$_2$), 28.0 (CH$_2$), 21.0 (H$_2$C=CCH$_3$), 18.2 (CH$_3$), 15.8 (CH$_3$). m/z (EI$^+$): 204.2 (14%, M$^+$), 189.167

(100%, M$^+$-CH$_3$), 161.134 (26%, M$^+$-C$_3$H$_7$), 147 (20%, M$^+$-C$_4$H$_9$), 133 (26%, M$^+$- 2xCH$_3$, isoproprenyl), 105.1 (54%), 91 (22%).

6.20.2 Incubation of 14Me FDP (137) with AS
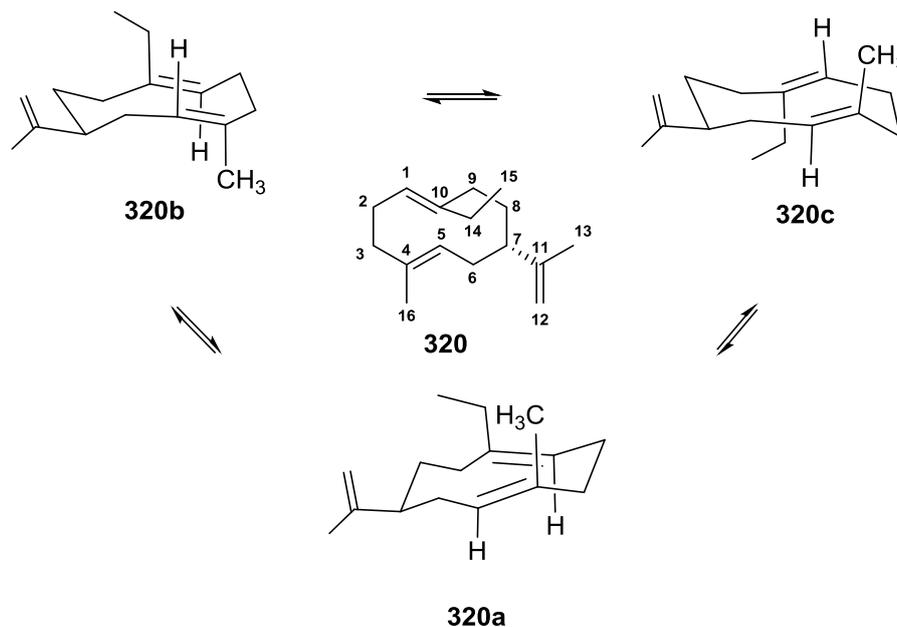
To a general AS incubation buffer (200 mL 20mM Tris, 5mM β-ME, 5mM MgCl$_2$, 15% glycerol) in a 500 mL glass bottle, 14Me-FDP was added (76 mg, 0.17 mmol) the mixture was stirred until solution. Enzyme solution (390 μM, 5 mL) was added and the aqueous incubation mixture was overlaid with pentane (15 mL). The cloudy solution was slowly stirred for 12 h. After this time, a further batch of enzyme solution (390 μM, 2 mL) was added and the mixture was stirred for an additional 12 h. The organic layer was separated and organic material was extracted from the aqueous solution with pentane (3 x 30 mL). The pooled organic extracts were dried over MgSO$_4$, filtered and then the solvent was concentrated under reduce pressure until volume was approximately of 5 mL. Final removal of the solvent was done under stream of nitrogen. The sample was purified by preparative TLC with pentane as eluent obtaining **320** as pale yellow oil (6 mg, 17%). Assignments of the protons down-field were made by comparison with previous reported spectrum of (+)-

germacrene A[310] and assignments of the protons up field were made by extrapolation of the relative abundance of each conformer taken from the signals down-field.

^{1}H NMR 500 MHz, CDCl$_3$, -50 ºC (δ, m, J in Hz)

Conformer	H-12	H-12'	H-13	H-15	H-16	H-1	H-5
320[a]	**4.64** (s)	**4.56** (s, 1H)	**1.70** (bs)	**1.47** (s)	**0.88** (t, 7.5)	**4.71** (dd, 12.0, 4.0)	**4.53** (bs)
320b	**4.64** (s)	**4.60** (s,)	**1.70** (bs)	**1.43** (s)	**0.85** (t, 7.5)	**4.91** (dd,18.5, 10.5)	**5.01** (dd, 9.0, 4.0)
320C	**4.64** (s)	**4.53** (bs)	**1.70** (bs)	**1.44** (s)	**0.94** (t, 7.5)	**4.91** (dd,18.5, 10.5)	**5.22** (t, 7.5)

Table 16. Assignment of resonances in the ^{1}H NMR spectrum to **320**.

Assignment of each signal to a specific conformer allowed calculation of the conformer distribution: conformer **320a** 39%, conformer **320b** 29% and conformer **320c** 32%.

6.20.3 Incubation of 14,15diMe-FDP (139) with AS
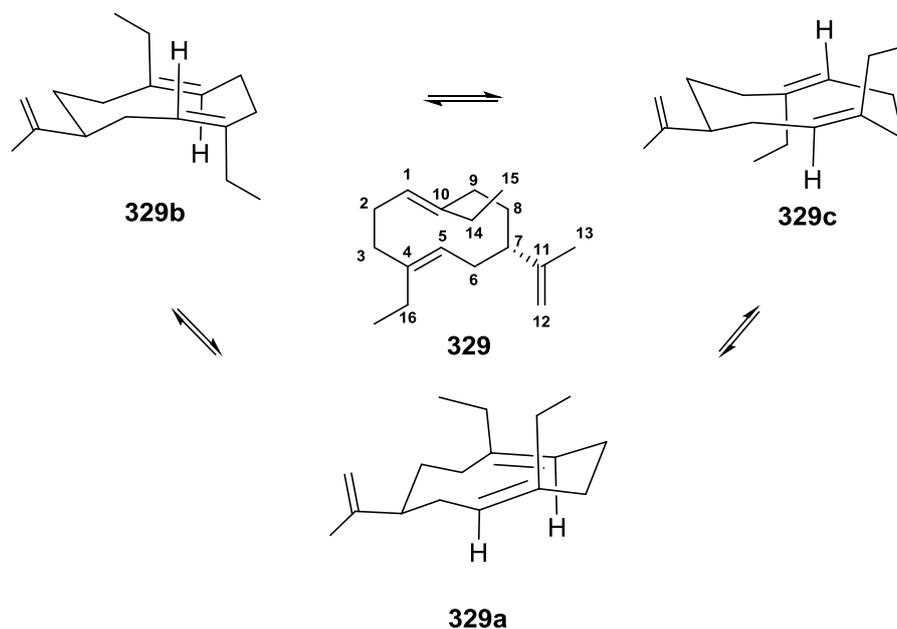
Incubation was performed following the general incubation procedure with 14,15diMe-FDP (1.2 mL, stock solution of 10 mM, 5.2 mg) and AS (30 mL AS incubation buffer solution, 12μM, 14 mg). Final solution was studied by variable temperature ^{1}H NMR spectroscopy without further purification. Assignments of 14Me-germacrene A (**329**), protons down field were assigned by comparison with previous reported spectrum of (+)-germacrene A[310] and assignment of the protons up-field were assigned by extrapolation of the relative abundance of each conformer taken from the signals down field.

¹H NMR 500 MHz, CDCl₃, -50 ºC (δ, m, *J* in Hz)

Conformer	H-12	H-12'	H-13	H-15	H-17	H-1	H-5
329a	4.64 (bs)	4.53 (s)	1.69 (d, 3.5)	0.97 – 0.81 (m)		4.71 (dd, 11.5, 4.0)	4.47 (dd, 9.5, 3.5)
329b	4.64 (bs)	4.59 (s)	1.69 (d, 3.5)	0.97 – 0.81 (m)		4.93 – 4.82 (m)	4.96 (dd, 9.5, 4.5)
329C	4.64 (bs)	4.55 (s)	1.74 (s)	0.97 – 0.81 (m)		4.93 – 4.82 (m)	5.17 (dd, 8.5, 7.0)

Table 17. Assignment of resonances in the ¹H NMR spectrum to **329**.

Assignment of each signal to a conformer allowed calculation of the conformer distribution: conformer **329a** 39%, conformer **329b** 29% and conformer **329c** 32%.

6.21 (-)-Germacrene D synthase (GDS)

6.21.1 Incubation of FDP (17) with GDS
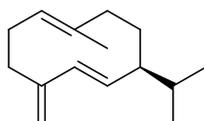
78

Incubation of GDS (30 mL DCS incubation buffer solution, 12 μM, 23 mg) with FDP (1,2 mL, stock solution of 10 mM, 5.2 mg) was performed following the general procedure. After NMR spectroscopic analysis the sample was concentrated to dryness under stream of N₂. **49** was isolated as pale yellow oil (1.7 mg, 61%)

¹H NMR (400 MHz, CDCl₃) δ 5.78 (d, *J* = 16.0 , 1 H, H̲C=CHCH̲), 5.25 (dd, *J* = 16.0, 10.0 , 1 H, HC=CH̲CH̲), 5.13 (dd, *J* = 11.5, 5.0, 1 H, CH̲=CCH₂CH₃), 4.79 (s, 1 H, H₂C=C), 4.74

(s, 1 H, H$_2$C=C), 2.47 – 2.32 (m, 2 H, CH$_2$), 2.30 – 2.15 (m, 2 H, CH$_2$), 2.14 – 1.95 (m, 3 H, CH$_2$, CHCH<u>H</u>CH$_2$), 1.51 (s, 3 H, CH$_3$), 1.44 (m, 3 H, CH$_2$, CHC<u>H</u>(CH$_3$)$_2$), 0.86 (d, J = 6.7 , 3 H, CH(C<u>H</u>$_3$)$_2$), 0.81 (d, J = 6.8, 3 H, CH(C<u>H</u>$_3$)$_2$). **^{13}C NMR** (126 MHz, CDCl$_3$) δ 149.1 (H$_2$<u>C</u>=C), 135.7 (H<u>C</u>=C), 134.1 (<u>H</u>C=CH), 133.7 (<u>H</u>C=CH), 129.9 (H<u>C</u>=C), 109.2 (H$_2$<u>C</u>=C), 53.1 (CH$_2$<u>C</u>HCH=C), 40.9 (CH$_2$), 34.7 (CH$_2$), 32.9 (<u>C</u>H(CH$_3$)$_2$), 29.4 (CH$_2$), 26.7 (CH$_2$), 20.9 (CH$_3$), 19.5 (CH$_3$), 16.1 (CH$_3$). m/z (EI$^+$): 204 (64%, M$^+$), 189 (9%, M$^+$-CH$_3$), 146 (15%, M$^+$-CH$_3$ and isopropyl), 133 (45%), 119 (75%), 105 (81%), 91 (76%)

6.21.2 Incubation of 14Me-FDP (137) with GDS
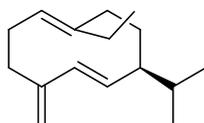
338

Incubation of GDS (30 mL DCS incubation buffer solution, 12 μM, 23 mg) with 14Me-FDP (1,2 mL, stock solution of 10 mM, 5.4 mg) was performed following the general procedure. Analysis of the sample by GC-FID determined the final concentration of **338** finding a total concentration of 1.3 mg/mL in 0.9 mL of CHCl$_3$.

^{1}H NMR (500 MHz, CDCl$_3$) δ 5.71 (d, J = 16.0, 1 H, <u>H</u>C=CH), 5.12 (dd, J = 16.0, 10.0, 1 H, HC=C<u>H</u>), 4.99 (dd, J = 11.0, 5.0 , 1 H, C<u>H</u>=CCH$_2$CH$_3$), 4.69 (bs, 1 H, H$_2$C=C), 4.71 (bs, 1 H, H$_2$C=C), 2.48 (m, 2 H, CH$_2$), 2.30 (dt, J = 15.0, 7.3, 2 H,CHC<u>H</u>CH$_2$), 2.17 – 1.91 (m, 4 H, 2 x CH$_2$), 1.71 (dq, J = 19.5, 10.0, 4.6 , 1 H, CHC<u>H</u>(CH$_3$)$_2$), 1.59 – 1.35 (m, 4 H, 2 x CH$_2$). 0.87 (t, J = 7.5, 3 H, C<u>H</u>$_3$CH$_2$), 0.73(d, J = 6.5, 3 H, (C<u>H</u>$_3$)$_2$CH), 0.79 (d, J = 6.5, 3 H, (C<u>H</u>$_3$)$_2$CH).**^{13}C NMR** (126 MHz, CDCl$_3$) δ 148.9 (H$_2$<u>C</u>=C), 139.3 (H<u>C</u>=C), 135.5 (<u>H</u>C=CH), 134.9 (<u>H</u>C=CH), 130.0 (H<u>C</u>=C), 109.4 (H$_2$<u>C</u>=C), 52.8 (CH$_2$<u>C</u>HCH=C), 37.1 (CH$_2$), 34.6 (CH$_2$), 32.8 (CH$_2$), 28.9 (<u>C</u>H(CH$_3$)$_2$), 26.9 (CH$_2$), 21.4 (CH$_2$), 20.7 (CH$_3$), 19.3 (CH$_3$), 12.6 (CH$_3$)

6.21.3 Incubation of 6F-FDP (289) with GDS
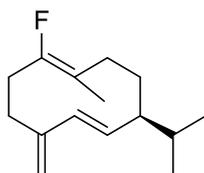
289

Incubation of GDS (30 mL DCS incubation buffer solution, 12 μM, 23 mg) with 6F-FDP (1,2 mL, stock solution of 10 mM, 5.4 mg) was performed following the general procedure. Analysis of the sample by GC-FID determined the final concentration of **289** finding a concentration of 1.2 mg/mL in 0.9 mL of $CHCl_3$.

^{1}**H NMR** (600 MHz, $CDCl_3$) δ 6.13 (d, J = 16.0, 1 H, <u>H</u>C=CH), 5.82 (dd, J = 16.0, 9.4, 1 H, HC=C<u>H</u>), 4.85 (bs, 1 H, H_2C=C), 4.73 (bs, 1 H, H_2C=C), 2.79 (bs, 2 H, CH_2), 2.41 – 2.27 (m, 1 H, CHC<u>H</u>CH_2), 2.27 – 2.03 (m, 2 H, CH_2), 1.89 (bd, J = 37.5, 2 H, CH_2CF), 1.64 (m, 3 H, CH_2, CHC<u>H</u>$(CH_3)_2$), 1.44 (s, 3 H, CH_3), 0.88 (dd, J = 20.0, 6.0, 6 H, CH(C<u>H</u>$_3)_2$). ^{13}**C NMR** (150 MHz, $CDCl_3$, resonances measured indirectly by HSQC) δ 15.4 (CH_3) 17.2 (CH_3), 20.4 (CH_3), 27.1 (CH_2), 28.9 (CH_2), 29.4 (CH), 29.5 (CH_2), 30.7 (CH_2), 31.0 (CH_2) 48.9 (CH), 112.3 (C=<u>C</u>H_2), 115.2 (CH_3<u>C</u>=CF), 128.3 (CH_2=C<u>C</u>H=<u>C</u>H), 138.5 (CH_2=C<u>C</u>H=CH), 148.2 (<u>C</u>=CH_2), 154.7 (d, J_{CF} 248.5, C=<u>C</u>F); ^{19}**F NMR** (565 MHz, $CDCl_3$) δ -106.0 (d, J = 42.0 , C<u>F</u>=C). m/z (EI$^+$): 222.2 (28%, M$^+$), 202.2 (22%, M$^+$-HF), 179.1 (59%, M$^+$-C_3H_7 (isopropyl)), 159.1 (100%, M$^+$-HF-C_3H_7).

6.22 (+)-δ-Cadinene synthase (DCS)

6.22.1 Incubation of 8OH-FDP with DCS

Incubation of DCS (30 mL DCS incubation buffer solution, 12 μM, 23 mg) with 8OH-FDP (1,2 mL, stock solution of 10 mM, 5.4 mg) was performed following the general procedure but the organic extract in this case was not passed through a short plug of neutral alumina. Two different compounds were isolated from the incubation of this analogue and separated by preparative TLC with hexane: ethyl acetate (20:1) as eluent and characterised as compound ketone **346** and alcohol **349**.

346
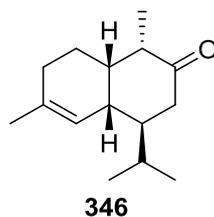
¹H NMR (600 MHz, CDCl₃)δ 5.54 (bd, *J* = 4.0 , 1 H, CH₃C=C<u>H</u>), 2.75 (p, *J* = 6.5 , 1 H C<u>H</u>CH₃), 2.31 (dd, *J* = 11.0, 5.5, 2 H, C=CHC<u>H</u>), 2.22 − 2.12 (m, 2 H, CH₃CHC<u>H</u>, 1 H x C=OC<u>H</u>₂), 2.09 (t, *J* = 13.0, 1 H, 1 H x C=OC<u>H</u>₂), 2.03 (dtd, *J* = 10.0, 7.0, 3.0, 1 H, <u>H</u>C(CH₃)₂), 1.99 − 1.94 (bd, 2 H, *J* = 6.5, C<u>H</u>₂CH₂CH), 1.83 − 1.75 (m, 1 H, <u>H</u>CHC(CH₃)₂), 1.66 (s, 5 H, C<u>H</u>₃CH, CH₂C<u>H</u>₂CH), 1.01 (d, *J* = 7.0, 3 H,C<u>H</u>₃CH), 0.89 (d, *J* = 7.0, 3 H (C<u>H</u>₃)₂CH), 0.86 (d, *J* = 7.0, 3 H, (C<u>H</u>₃)₂CH). **¹³C NMR** (151 MHz, CDCl₃) δ 214.5 (C=O), 134.8 (CH₃<u>C</u>=CH), 123.2 (CH₃C=<u>C</u>H), 48.0 (CH₃<u>C</u>HCH), 47.5 (<u>C</u>HCH(CH₃)₂), 43.9(CH₃CH<u>C</u>H), 40.2 (C=O<u>C</u>H₂), 39.2 (C=CH<u>C</u>H), 31.0 (<u>C</u>H₂CH₂CH), 27.2 ((CH₃)₂<u>C</u>H), 23.8 (<u>C</u>H₃C=CH), 21.4((<u>C</u>H₃)₂CH), 18.9 (CH₂<u>C</u>H₂CH), 15.1 ((<u>C</u>H₃)₂CH), 11.5 (<u>C</u>H₃CHC=O).

349
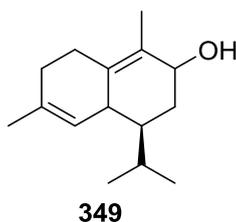
The signals on ¹H NMR spectrum were assigned by comparison with the ketone and by comparison with previously published NMR spectroscopic analysis of δ-cadinene[311,312] and δ-cadinene derivatives.[313]

¹H NMR (600 MHz, CDCl₃) δ 5.45 (bs, 1 H, C=CH), 5.30 (s, 1 H, OH), 3.97 (bd, *J* = 7.3, 1 H, C<u>H</u>OH), 2.71 − 2.62 (m, 1 H, *J* = 3.5, 13.0 CH₂C<u>H</u>₂C=C(CH₃)), 2.46 (d, *J* = 11.0 , 1 H, C=CHC<u>H</u>CH), 2.16 − 2.09 (m, 1H, C<u>H</u>(CH₃)₂), 2.03 (bd, *J* = 8.0 , 1 H, C<u>H</u>₂CH₂C=C(CH₃)),

1.98 – 1.88 (m, 1 H, C$\underline{H}$CH(CH$_3$)$_2$), 1.80 (s, 3 H, C=C(C$\underline{H}_3$)CHOH), 1.68 (s, 3 H, (C$\underline{H}_3$)C=CH), 0.98 (d, J = 6.9 , 3 H, CH(C$\underline{H}_3$)$_2$), 0.79 (d, J = 6.9, 3 H, CH(C$\underline{H}_3$)$_2$).

Apendix I

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Apendix I

Publications

A 1,6-Ring Closure Mechanism for (+)-δ-Cadinene Synthase?

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Ⓢ *Supporting Information*

ABSTRACT: Recombinant (+)-δ-cadinene synthase (DCS) from *Gossypium arboreum* catalyzes the metal-dependent cyclization of (E,E)-farnesyl diphosphate (FDP) to the cadinane sesquiterpene δ-cadinene, the parent hydrocarbon of cotton phytoalexins such as gossypol. In contrast to some other sesquiterpene cyclases, DCS carries out this transformation with >98% fidelity but, as a consequence, leaves no mechanistic traces of its mode of action. The formation of (+)-δ-cadinene has been shown to occur via the enzyme-bound intermediate (3R)-nerolidyl diphosphate (NDP), which in turn has been postulated to be converted to cis-germacradienyl cation after a 1,10-cyclization. A subsequent 1,3-hydride shift would then relocate the carbocation within the transient macrocycle to expedite a second cyclization that yields the cadinenyl cation with the correct cis stereochemistry found in (+)-δ-cadinene. An elegant 1,10-mechanistic pathway that avoids the formation of (3R)-NDP has also been suggested. In this alternative scenario, the final cadinenyl cation is proposed to be formed through the intermediacy of trans,trans-germacradienyl cation and germacrene D. In addition, an alternative 1,6-ring closure mechanism via the bisabolyl cation has previously been envisioned. We report here a detailed investigation of the catalytic mechanism of DCS using a variety of mechanistic probes including, among others, deuterated and fluorinated FDPs. Farnesyl diphosphate analogues with fluorine at C2 and C10 acted as inhibitors of DCS, but intriguingly, after prolonged overnight incubations, they yielded 2F-germacrene(s) and a 10F-humulene, respectively. The observed 1,10-, and to a lesser extent, 1,11-cyclization activity of DCS with these fluorinated substrates is consistent with the postulated macrocyclization mechanism(s) en route to (+)-δ-cadinene. On the other hand, mechanistic results from incubations of DCS with 6F-FPP, (2Z,6E)-FDP, neryl diphosphate, 6,7-dihydro-FDP, and NDP seem to be in better agreement with the potential involvement of the alternative biosynthetic 1,6-ring closure pathway. In particular, the strong inhibition of DCS by 6F-FDP, coupled to the exclusive bisabolyl- and terpinyl-derived product profiles observed for the DCS-catalyzed turnover of (2Z,6E)-farnesyl and neryl diphosphates, suggested the intermediacy of α-bisabolyl cation. DCS incubations with enantiomerically pure $[1-^2H_1](1R)$-FDP revealed that the putative bisabolyl-derived 1,6-pathway proceeds through (3R)-nerolidyl diphosphate (NDP), is consistent with previous deuterium-labeling studies, and accounts for the cis stereochemistry characteristic of cadinenyl-derived sesquiterpenes. While the results reported here do not unambiguously rule in favor of 1,6- or 1,10-cyclization, they demonstrate the mechanistic versatility inherent to DCS and highlight the possible existence of multiple mechanistic pathways.
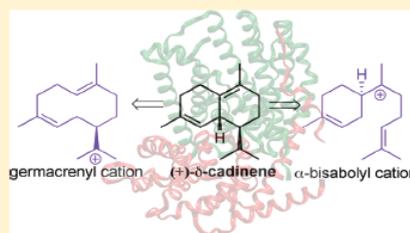
■ INTRODUCTION

Terpene synthases catalyze complex reaction cascades with high regio- and stereochemical precision involving cyclizations (alkylations), rearrangements, and deprotonations of highly reactive carbocations.[1] Only recently has it become possible to address experimentally the intricate mechanistic details of these reactions through the use of substrate analogues,[2,3] aza-analogues of putative carbocationic intermediates,[4] mutant enzymes,[5] and X-ray crystallography.[6] Fluorine-containing analogues of enzyme substrates have been shown to be instrumental in mechanistic investigations,[7] and in particular, fluoro prenyl derivatives[3] have provided crucial insights regarding the cationic mechanisms of terpene synthases.[8] While the small size of the fluorine atom does not appear to significantly compromise active-site binding,[3d,h,6g,h,8b,d] its intrinsic electronegativity is known to inactivate fluoro-containing double bonds toward protonation and electrophilic alkylation[3c,d,g] and to alter the stability of allylic cation

intermediates.[3h,8,9] Fluorine containing prenyl diphosphates have been used to study several terpene synthases including aristolochene synthases from the fungi *Aspergillus terreus* and *Penicillium roqueforti* (AT-AS and PR-AS),[3b,c,g,6h] tobacco 5-*epi*-aristolochene synthase (TEAS),[3d,h] trichodiene synthase (TS),[3e,8e,10] taxadiene synthase,[3a] limonene and (+)-bornyl diphosphate synthases,[3f,6g] and α-pinene synthase.[11]

δ-Cadinene synthase (DCS) from *Gossypium arboreum* is a sesquiterpene cyclase that catalyzes the metal-dependent conversion of farnesyl diphosphate (FDP, **1**) to the bicyclic hydrocarbon (+)-δ-cadinene (**6**) with a specificity of >98% (Scheme 1). This transformation is the first committed step in the biosynthesis of cotton phytoalexins such as gossypol.[12] Similar to all class I terpene cyclases,[6] DCS maintains the characteristic aspartate-rich D[307]DXXD motif on helix D that

Scheme 1. Reaction Mechanisms Proposed in the Literature for DCS Catalysis[15,20]
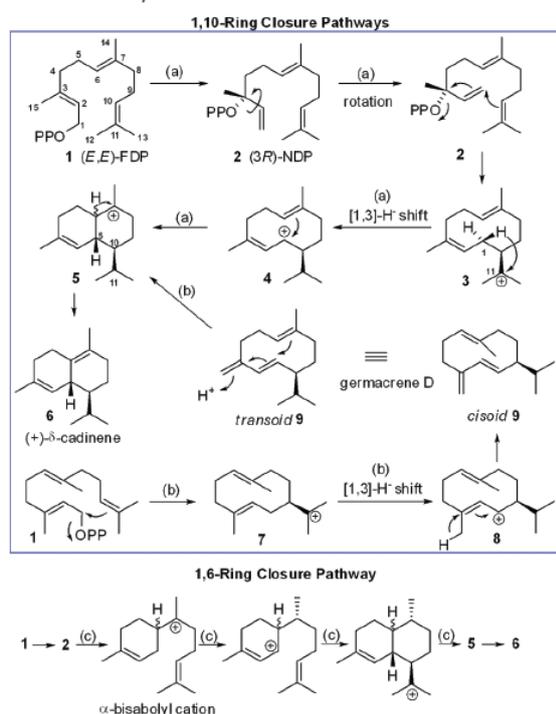
chelates two (A and B) of the three Mg^{2+} ions essential for catalysis.[13] However, amino acid sequence alignments as well as X-ray structural analysis[13] have revealed the absence of the conserved second metal-binding "NSE/DTE" motif[6] on helix H. Instead, DCS possesses a second aspartate rich motif (D^{451}DVAE), similar to those found in "isoprenoid chain elongation enzymes" such as farnesyl diphosphate synthase.[14]

Mechanistically, the consensus in the literature[12a,13,15] is in favor of the DCS-catalyzed reaction path a outlined in Scheme 1. This pathway goes through (3R)-nerolidyl diphosphate ((3R)-NDP, 2) as an enzyme-bound intermediate that after rotation around the C2,C3 σ-bond, leads to the correct orbital alignment for 1,10-macrocyclization and production of cis-germacradienyl cation (3).[1a] A subsequent C1 → C11 1,3-hydride shift of the original H-1_{si} of 1[15c,d] is followed by a 1,6-electrophilic ring closure reaction that generates cadinenyl cation (5), from which δ-cadinene (6) is formed after proton loss from C6. The tertiary diphosphate 2 has been shown to be a substrate of DCS,[15b,c] and its formation (or that of (2Z,6E)-FDP, 15)[16] within the active site of the enzyme has been inferred from [1,2-$^{13}C_2$]acetate as well as [2-^{14}C] and [4-^{14}C]mevalonate labeled feeding experiments.[17,18] In closely related biosynthetic studies, (3R)-NDP (2) has also been identified as an enzyme-bound intermediate in (+)-1-epi-cubenol biosynthesis,[19] and more recently, 2 has been suggested as an intermediate in (−)-δ-cadinene biosynthesis.[15d]

The biosynthesis of cadinane-type sesquiterpenes could also occur without the intervention of enzyme bound NDP (2) that ultimately allows the formation of the cis C2,C3-double bond (FDP numbering) present in 6.[20] In this alternative scenario (path b, Scheme 1), a 1,10-cyclization via the direct

displacement of the diphosphate group of 1 yields the transient trans,trans-germacradienyl cation (7), which further undergoes a 1,3-hydride shift followed by proton loss from C15 to generate the neutral hydrocarbon germacrene D (9) as the key biosynthetic intermediate.[21] A conformational change of enzyme-bound 9[20] gives rise to its reactive transoid conformer, which upon proton transfer to the exocyclic double bond and 1,6-ring closure produces the Z-configured cation 5, the final carbocationic intermediate common to pathways (a) and (b). Recently, pathway (b) via germacrene D has been shown to operate in a promiscuous sesquiterpene synthase from Medicago truncatula (MtTPS5).[22]

A third mechanistic possibility is also outlined in Scheme 1 (path c). In this pathway, the formation of [5-^{2}H]- and [11-^{2}H]-δ-cadinene from racemic [1-^{2}H]-farnesyl diphosphate was explained by an initial 1,6-electrophilic ring closure that leads to α-bisabolyl cation as the key biogenetic precursor of hydrocarbon 6.[15b] In support of this proposal, it has been reported previously that NDP (2), a reaction intermediate on path a, is converted by DCS to (E)-β-farnesene and β-bisabolene in addition to δ-cadinene.[15c] This observation is not easily explained if a 1,10-cyclization mechanism (e.g., path a) is followed. In addition, when [4,4,15,15,15-^{2}H$_5$]-nerolidyl diphosphate was used, no isotope loss to the solvent was observed,[15b,c] a result that makes unlikely the alternative 1,10-cyclization mechanism[22] via germacrene D (9) since it involves protonation/deprotonation at C15.

Here, we describe results obtained through the use of deuterated and fluorinated farnesyl diphosphates as mechanistic probes to examine the proposed DCS catalyzed reaction cascades summarized in Scheme 1. Enzymatic incubations with fluorinated FDP analogues (Figure 1) bearing fluorine at C2
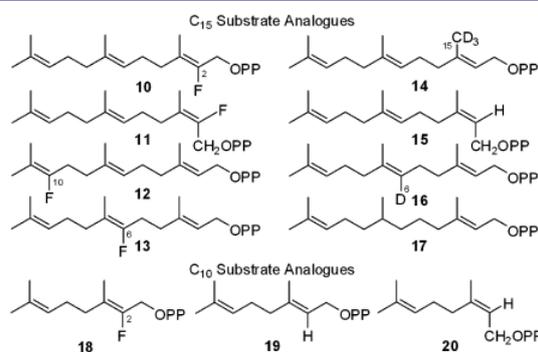
Figure 1. Farnesyl and geranyl diphosphate analogs used in the present study.

(10 and 11) and C10 (12) led to the formation of 2F-germacrenes (1,10-ring closure) and 10F-humulene (1,11-ring closure) as single products, results that are consistent with a macrocyclization reaction (paths a and b) as the biosynthetic means of (+)-δ-cadinene production. Conversely, however, the strong inhibition of DCS displayed by 6F-FDP (13), together with the exclusive bisabolyl- and terpinyl-derived product profiles observed from incubations with (2Z,6E)-farnesyl (15) and neryl (20) diphosphates and the absence of germacrene production (i.e., expected 1,10-cyclization activity) upon incubation with 6,7-dihydro-FDP (17) are in better agreement with a 1,6-ring closure mechanism (path c) by way of the (6R)-α-bisabolyl cation[23] (37). While these apparently conflicting

mechanistic interpretations of the experiments described here do not unambiguously show whether 1,6- or 1,10 cyclization is the pathway followed during the DCS catalyzed turnover of FDP (**1**) to (+)-δ-cadinene (**6**), they imply a high degree of mechanistic versatility of DCS and may suggest the existence of multiple pathways to **6**.

■ RESULTS AND DISCUSSION

Incubation of DCS with (2Z,6E)-2F-FDP (10), (2E,6E)-2F-FDP (11), and [15,15,15-²H₃]-FDP (14). The catalytic mechanism of DCS is thought to go by way of (3R)-NDP (**2**) involving a coupled ionization-isomerization-1,10-cyclization reaction (**1** → **3**, pathway a, Scheme 1).[15] Hence, the presence of a fluorine atom on C2 should prevent (or slow down), via depletion of electron density,[9] the heterolytic diphosphate ester cleavage reaction that secures the supply of farnesyl cation. Indeed, under steady-state kinetic conditions (2Z,6E)-2F-FDP (**10**) and (2E,6E)-2F-FDP (**11**) were found to inhibit the enzyme, albeit with reduced binding affinities (K_i = 70 μM and K_i = 30 μM for **10** and **11**, respectively) when compared to FDP (K_M = 3.2 μM),[13] indicating a rather modest competitive inhibition toward DCS (Supporting Information). Remarkably, the C2-fluorinated 'cis' isomer **11**[3h] binds to the active site of DCS approximately twice as tightly as its corresponding 'trans-configured' analogue **10**, which is structurally a better mimic of all-trans FDP (**1**). These kinetic results together with the better resemblance of diphosphate **15** (and the fluorinated analogue **11**) to the presumed reaction intermediate **2** support the potential intermediacy of (E,Z)-farnesyl diphosphate (**15**) or its corresponding cation in the biosynthesis of **6**.[17,18,20] This suggestion is in agreement with the observation that (3R)-NDP (**2**) binds to DCS with 10-fold greater affinity than FDP (**1**).[15c]

Despite the inhibitory properties of diphosphates **10** and **11** against DCS, GC−MS analyses of individual incubations of **10** and **11** revealed that the enzyme was able to turn over these analogues, giving in both cases single but different fluorinated hydrocarbons characterized by their molecular ions of m/z 222. The product generated from (2Z,6E)-2F-FDP (**10**) was readily identified by GC−MS as 2-fluorogermacrene A (**21**, Figure 2),

2F-germacrene A (**21**)   2F-helminthogermacrene A (**22**)
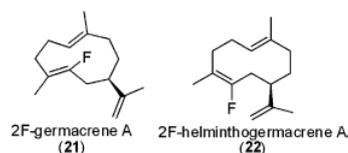
Figure 2. Structures of 2F-germacrene A (**21**) and 2F-helmintho-germacrene A (**22**).

a fluorinated cyclodecadiene sesquiterpene isolated during previous mechanistic studies on aristolochene synthase (PR-AS) catalysis (Supporting Information).[3c] For comparison, analytical incubations of (2E,6E)-2F-FDP (**11**) were carried out with DCS and PR-AS. Similarly, both enzymes were able to turn over **11** to the same product **22** as judged by GC−MS analysis (Supporting Information). Compared to 2-fluoroger-macrene A (**21**), the product formed from **11** was characterized by a shorter GC retention time and a much higher reluctance to undergo a thermal Cope rearrangement. On the other hand, **21** and **22** displayed almost identical MS fragmentation patterns. These results, together with ¹H- and ¹⁹F-NMR spectroscopic analysis, indicated that **22** was indeed 2-fluoro-helminthoger-

macrene A, the C2,C3 cis isomer of **21**. The ¹H NMR spectrum of **22** showed no major changes when a CDCl₃ solution of **22** was cooled to −50 °C and then gradually warmed to +50 °C, a result that is in good accordance with the observation that the parent hydrocarbon helminthogermacrene A exists almost exclusively as one conformer at room temperature in C₆D₆.[24] In contrast, all-trans-germacrene sesquiterpenes exist as several interconvertible conformational isomers in solution, a dynamic effect that had been observed previously among others[25] for 2F-germacrene A,[3c] 6F-germacrene A,[3d] and (+)-germacrene A.[26]

These results indicate that the fluorinated diphosphates **10** and **11** were able to efficiently suppress the native isomerization of **1** to **2** (path a). Indeed, the DCS-generated 2-fluorogermacrenes **21** and **22** (Figure 2) were shown to preserve the original C2,C3-double bond geometry of their corresponding substrates **10** (trans) and **11** (cis) after the enzymatic 1,10-cyclization reaction. The relative ease with which **21** and **22** are formed is consistent with the possible involvement of the 1,10-macrocyclization(s) pathway assumed to operate in δ-cadinene biosynthesis. In particular, the maintenance of the substrate's Z/E-geometry of the C2,C3 double bond during the enzymatic reaction weighs in favor of the 1,10-pathway (b, Scheme 1) that avoids the formation of a cisoid NDP through the direct generation of trans, trans-germacrenyl cation[27] (**7**) and then germacrene D as the biosynthetic intermediates.[19,21] In support of pathway b, it could be reasoned that after the enzymatic 1,10-ring closure, the electron-withdrawing effect of the vinylic 2-fluoro substituent might prevent the native C1 → C11 hydride shift (**7** → **8**, Scheme 1) that relocates the positive charge at C1, thus, explaining the observed accumulation of 2F-germacrene A (**21**) or 2F-helminthogermacrene (**22**) after deprotonation. However, since the formation of δ-cadinene along pathway b would require protonation of the exocyclic double bond of germacrene D (**9**),[22] the reaction cascade would be expected to show, in addition to proton exchange with the solvent,[2g,i,22] a dependence on pH, as observed for catalysis by TEAS,[28] the maize enzymes TPS6 and TPS11,[2i] the fungal cyclase Cop4,[2p] and some Cop4 mutants.[5o] However, in contrast to Cop4,[2p,5o] the catalytic cycle of DCS was not disrupted by higher values of pH (7.5 → 10.5) and no accumulation of germacrene D was observed (Supporting Information).[29] Moreover, incubations of [15,15,15-²H₃]-FDP (**14**) with DCS in aqueous buffer (H₂O) led to the release of [15,15,15-²H₃]-δ-cadinene with the deuterium content intact (Supporting Information). These observations are in agreement with those obtained with [4,4,15,15,15-²H₅]-NDP[15b] and seem to rule out pathway b[20] (Scheme 1).

Incubation of 10F-FDP (12) with DCS. Similar conclusions were reached from experiments with 10F-FDP (**12**). The reduced electron density of the C10,C11-double bond in **12**, coupled to the destabilizing effect of the 10-fluoro-substituent on the developing positive charge on C11 during DCS catalysis, was anticipated to prevent the proposed 1,10-cyclization reaction (Scheme 1). Consequently, the formation of 10F-farnesenes or 10F-bisabolenes arising from the putative (3R)-10F-NDP was expected. Diphosphate **12** acted as a competitive inhibitor (K_i = 16.5 μM) of DCS, a result that suggests a potentially high energy barrier for the isomerization of **12** to (3R)-10F-NDP during DCS catalysis in support of the DCS-catalyzed 1,10-pathway. DCS was also able to turn over diphosphate **12** yielding a single (>95% by GC−MS)

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fluorinated hydrocarbon (**26**) that failed to undergo thermal Cope rearrangements even at temperatures as high as 250 °C, thus, most likely ruling out a germacrene hydrocarbon as the enzymatic product.[30] The mass spectrum of this fluorinated-hydrocarbon was different from that of an authentic sample of enzymatically generated (*E*)-10F-β-farnesene (**25**) (Supporting Information).[31a] In addition, despite the striking similarity between the EI MS spectra of this fluorinated hydrocarbon and (*Z*)-α-farnesene (Supporting Information), the chemical generation of an authentic 3:1:1 mixture of 10F-farnesenes[31b] (**23**–**25**, Figure 3) and subsequent GC/MS comparisons with
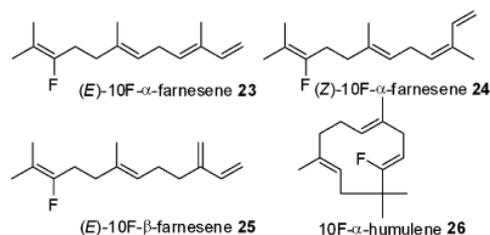

**Figure 3.** Structures of 10F-farnesenes (**23**–**25**) and 10F-α-humulene (**26**).

the DCS-generated product ruled out any of the three possible fluorinated farnesenes. This result indicates that DCS does not seem to convert **12** to its (3*R*)-10F-NDP isomer which likely precludes the enzymatic formation of 1,6-cyclized products from **12**. Diphosphate **12** has been used previously to explore the 1,6-ring closure mechanism catalyzed by trichodiene synthase (TS). Surprisingly, 10F-FDP acted as a potent inhibitor of TS,[8a] thus, suggesting the potential existence of a alternative/degenerate mode of catalysis by TS.

While an unambiguous full structure determination of the product generated from 10F-FDP (**12**) is outstanding, preliminary ^{1}H NMR (500 MHz, CDCl₃) spectroscopic data are in agreement with a α-10F-humulene hydrocarbon structure (**26**, Figure 3) arising from an unprecedented DCS-mediated 1,11-macrocyclization. This suggestion is supported by the observation of a downfield doublet of triplets signal (J_{H-F} = 40.4 Hz and J_{H-H} = 8.1 Hz) at 5.00 ppm, which strongly resembles the characteristic doublet of triplets absorbance at 5.59 ppm (J_{H-H} = 15.8. Hz, and J_{H-H} = 7.3 Hz) assigned to the H-9 olefinic proton of α-humulene.[32] The relative upfield H-9 shift (ca. 0.6 ppm) observed for **26** is consistent with the γ-shielding effect of the vinylic fluoro substituent on C9. In contrast to the characteristic ^{19}F-NMR triplet signal (J_{H-F} = 24.6 Hz) at −114.0 ppm of diphosphate **12**, the ^{19}F-NMR (282 MHz) spectrum of the reaction product generated from **12** displayed a doublet (J_{H-F} =41.6. Hz) centered at −119.9 ppm, which further supports the identification of this enzymatic hydrocarbon as 10F-α-humulene (**26**) (Supporting Information).

Incubations of 6F-FDP (13) and 6,7-DihydroFDP (17) with DCS. 6-Fluoro-FDP (**13**) has been used previously to probe the catalytic cycles of PR-AS[3c] and TEAS.[3d] The kinetic data obtained for TEAS revealed that the presence of the vinylic fluorine had negligible effects on binding affinity, diphosphate ionization, and the initial 1,10-cyclization reaction.[3d] For DCS, irrespective of which 1,10-mechanism is followed (Scheme 1), the presence of the 6-fluoro substituent should destabilize the transition state(s) leading to cadinenyl cation **5**. Therefore,

incubations of DCS with **13** were expected to yield, via 6F-3, fluorinated germacrene hydrocarbons (e.g., **27**–**29**, Scheme 2)

Scheme 2. Structures of 6F-Germacrenes (27–29) from a Putative 6F-NDP (6F-2)[a]
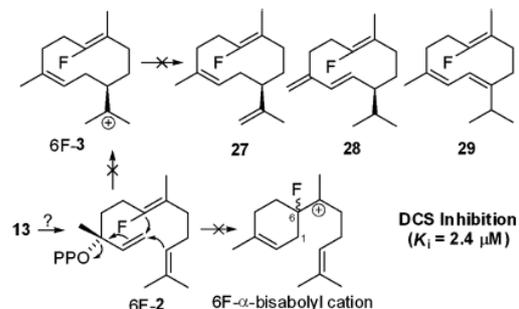
[a]The detection of the possible enzymatic release of 6F-2 in the aqueous phase was not attempted.

through premature deprotonation reactions. Intriguingly, diphosphate **13** was not a substrate of DCS, and even after prolonged enzymatic incubations, no turnover was observed. Steady-state kinetic studies demonstrated that 6F-FDP (**13**) was a potent competitive inhibitor of DCS with an inhibition constant (K_i = 2.4 μM, Supporting Information) comparable to the Michaelis−Menten constant measured for FDP (K_M = 3.2 μM).[13]

Assuming that the C6,C7-double bond of **1** (or **13**) is not likely to participate in the production of (3*R*)-NDP (**2**) (path a, Scheme 1),[15,17,18] the strong inhibition displayed by **13** suggests that the native DCS-catalyzed reaction cascade leading to **6** might proceed through the alternative 1,6-ring closure pathway c (Scheme 1), as 1,10-macrocyclization-derived products would be expected for this analogue from path a.[3c,d] Hence, in this scenario, δ-cadinene (**6**) is formed by nucleophilic attack of the central C6,C7-double bond of **2** on C1 to generate the α-bisabolyl cation (Scheme 1), which has been reported to be central to other sesquiterpene synthase-catalyzed cyclizations of FDP.[23] The destabilizing effect of the vinylic 6-fluoro substituent toward the proposed electrophilic 1,6-alkylation would explain the inability of DCS to use 6F-FDP (**13**) as a substrate.

The possibility of a 1,6-electrophilic ring closure in DCS catalysis was further supported by incubations with racemic 6,7-dihydroFDP (**17**).[33] Indeed, in agreement with the results previously obtained with the 1,6-cyclase TS,[33a,b] DCS incubations of diphosphate **17** yielded (*Z*)-α-6,7-dihydrofarnesene[34] as the main (64%) hydrocarbon. Conversely, 6,7-dihydrogermacrene is the exclusive product detected with the 1,10-cyclase aristolochene synthase from *A. terreus* (AT-AS).[35]

Incubation of DCS with Geranyl (19), Neryl (20), and 2F-Geranyl (18) Diphosphates. It has been documented that sesquiterpene synthases that can isomerize the C2,C3-double bond of (*E,E*)-FDP (**1**) often produce cyclic monoterpenes from GDP (**19**).[2h,i,p,36] In contrast, rigorously 'trans' enzymes such as TEAS[2h] or the germacrene A synthase NS1,[2p] produce exclusively acyclic monoterpenes when they encounter **19**. Thus, for 'cis' sesquiterpene cyclases[3h] that utilize a coupled ionization-isomerization-1,6-cyclization mechanism,[1b,37] neryl cation and α-terpinyl cation[1b,37] (Scheme 3) can act as good mimics of the corresponding C₁₅ *cis*-farnesyl and α-bisabolyl

Scheme 3. Monoterpenes Produced by DCS from Geranyl (19) and Neryl (20) Diphosphates
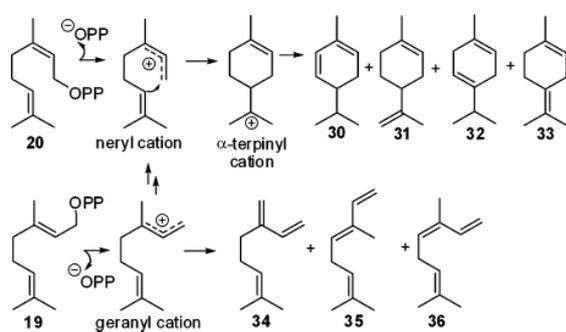
cations, respectively. On the basis of these considerations, and to assess the feasibility of a 1,6-ring closure, the ability of DCS to convert **19** to cyclic monoterpenes was tested. DCS was indeed able to catalyze the conversion of **19** to an approximate 2:3 mixture of cyclic (α-phellandrene (**30**), limonene (**31**), γ-terpinene (**32**), and α-terpinolene (**33**)), and acyclic (myrcene (**34**), (*E*)-β-ocimene (**35**), and (*Z*)-β-ocimene (**36**)) monoterpenes (Supporting Information). Interestingly, neryl diphosphate (**20**) produced only cyclic monoterpenes (Scheme 3)[38] suggesting that the observed acyclic olefins (**34−36**, Scheme 3) arise exclusively from the transoid geranyl cation, and that their enzymatic release occurs before isomerization to neryl cation.[1b,37] In accordance with this mechanistic picture, 2-fluorogeranyl diphosphate (**18**) was shown to efficiently prevent the ionization-isomerization step essential for the accumulation of cyclic products; indeed, **18** was a potent competitive inhibitor of the DCS (K_i = 8 μM) (Supporting Information). These results reflect on the ability of DCS to use an isomerization-1,6-cyclization step and sustain a possible reaction via α-bisabolyl cation (Scheme 1) from FDP.

Incubation of DCS with (2*Z*,6*E*)-FDP (15). The possibility of either a 1,10- or a 1,6-ring closure mechanism in DCS catalysis was further evaluated with (2*Z*,6*E*)-FDP (**15**) (Scheme 4). It had been recognized previously that diphosphate **15** could be considered an effective 'preisomerized' form of (*E,E*)-FDP (**1**).[2n,3h] Hence, its ionization by DCS should supply an ion pair of cisoid farnesyl cation and diphosphate anion ready for the enzymatic reaction cascade to
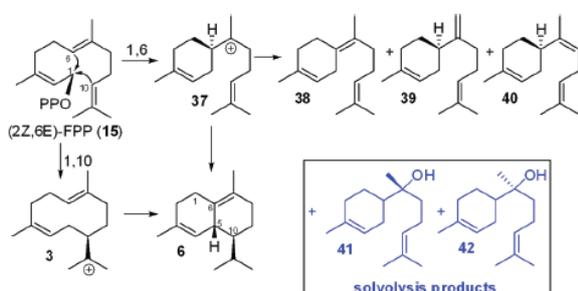
proceed along path a (Scheme 1). GC−MS analysis of the pentane extractable products from incubations of **15** with DCS showed that the enzyme generated two hydrocarbon products (Supporting Information). The MS fragmentation pattern and GC retention time of the minor product (33%) matched that of δ-cadinene (**6**).[15] To identify the major product (67%), a preparative-scale incubation of **15** with DCS was carried out. The hydrocarbon (50% of total) and alcohol (50% of total) products were initially separated by silica gel column chromatography. Each fraction was then purified further by preparative TLC to yield two hydrocarbon fractions (A and B) and one alcohol fraction. ^{1}H NMR spectroscopic measurements of fraction A revealed the presence of δ-cadinene (**6**) of ca. 85% purity.[15d,39] Fraction B (corresponding to the major GC peak with shorter retention time) was found to be composed of a 3:3:2 mixture of (*Z*)-γ-bisabolene (**38**), β-bisabolene (**39**), and (*Z*)-α-bisabolene (**40**) as judged by ^{1}H NMR spectroscopy and comparison with the literature values previously reported for these compounds.[40,41] In addition, ^{1}H NMR spectroscopy of the alcohol fraction revealed the presence of an inseparable 2:1:1 mixture of nerolidol and the two epimeric α-bisabolols **41** and **42**,[42] which most likely arise from the nonspecific addition of water to α-bisabolyl cation. It is worthy of note that this product distribution (Scheme 4) is remarkably similar to that previously observed from DCS incubations using NDP (**2**) as the substrate.[15c]

The accumulation of the bisabolyl-derived olefins **38−40**, accounting for ca. 67% (GC−MS) of all cyclic hydrocarbons, is easily explained with an initial DCS-catalyzed 1,6-ring closure reaction that leads to the (6*R*)-α-bisabolyl cation (**37**), which is then either deprotonated to the observed hydrocarbons **38−40**, or trapped by a molecule of water to generate the tertiary alcohols **41** and **42** (Scheme 4). However, the observation of considerable amounts of alcohols **41** and **42** from incubation of **15** in the absence of recombinant DCS established, for the most part, their nonenzymatic origin. It is notable that not only **6**, but also the DCS-generated hydrocarbon (*Z*)-γ-bisabolene (**38**) as well as the α-bisabolols are terpene volatiles found in cotton plants.[15c]

Mechanistically, the formation of δ-cadinene (**6**) together with a variety of bisabolyl-derived hydrocarbons (**38−40**) supports the possibility of a 1,6-ring closure mechanism. However, the result is intriguing. Either DCS possesses the ability to mediate parallel 1,10- and 1,6-ring closure reactions along energetically similar pathways (Scheme 4), or all cyclic enzymatic products including **6** arise from a predominant conformation of farnesyl cation (A, Scheme 5) that allows the 1,6-cyclization mechanism observed with the surrogate diphosphate **15**. The possibility of a DCS-catalyzed 1,6-ring closure pathway has been suggested previously[15b] and the feasibility of the required 1,3-H and 1,5-H shifts involving **37** has been discussed.[43] However, a detailed description of a 1,6-mechanism consistent with both the 5,10-cis stereochemical relationship of cadinenyl-derived hydrocarbons and the deuterium distribution found in **6** using racemic and chiral [1-²H₁]-FDPs[15] has not been provided before. In agreement with our proposal (vide infra), Tantillo and Hong have provided computational evidence from gas-phase calculations in support of 1,3- and 1,5-H shifts that could interconnect, via cation **37**, the biosynthesis of amorphadiene (1,6-ring closure) and amorphene (1,10-ring closure) derived sesquiterpenes. These gas-phase calculations energetically favored a 1,6-ring

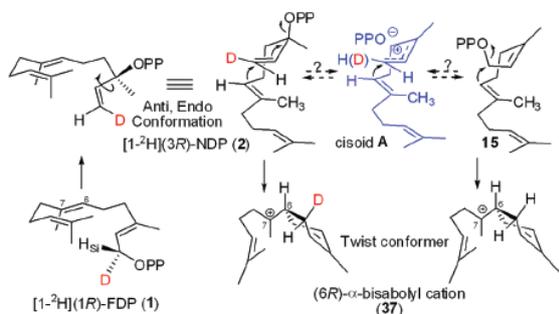**Scheme 4. Sesquiterpenes Generated by DCS from (2*Z*,6*E*)-FDP (15)**[a]

[a]The (*R*)-configuration of **39−40** is assumed based on the intermediacy of a putative (6*R*)-configured α-bisabolyl cation (**37**). Alcohols **41** and **42** are nonenzymatic products.

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Scheme 5. DCS-Catalyzed Conversion of $[1\text{-}^2H_1](1R)$-FDP $((1R)\text{-}1)$ and 15 to the $(6R)\text{-}\alpha$-Bisabolyl Cation (37) via $[1\text{-}^2H_1](3R)$-NDP (2) or the Corresponding cis-Farnesyl Cation/PPO⁻ Ion Pair

Scheme 6. Conversion of Cation 37 to (+)-6 Involving 1,3- and 1,5-Hydride Shifts of the H-1_{Si} Proton of $[1\text{-}^2H_1](1R)$-FDP

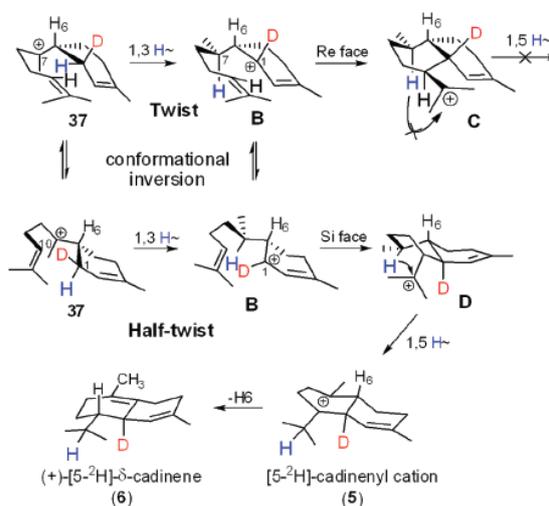closure rather than the accepted 1,10-cyclization mechanism in amorphene biosynthesis.[44]

Incubation of $[6\text{-}^2H_1]$-FDP (16) and $[1\text{-}^2H_1](1R)$-FDP $((1R)\text{-}1)$ with DCS. In the present study, the stereochemical course of the DCS-catalyzed reaction was followed by individual incubations with $[6\text{-}^2H_1]$-FDP (16) and $[1\text{-}^2H_1](1R)$-FDP $((1R)\text{-}1)$, which resulted in the formation of unlabeled 6 and $[5\text{-}^2H_1]$-6, respectively (Supporting Information), as evidenced by GC/MS analysis. In contrast to what was observed with 6 (base peak at m/z 161), the GC-mass spectra of the product ($[5\text{-}^2H_1]$-6) generated from $(1R)\text{-}1$ had a base peak m/z of 162 (assigned to $[M^+\text{-}C_3H_7]$), thus, indicating that the original H-1_{Re} proton of FDP was retained on C1 during the formation of $[5\text{-}^2H_1]$-6.[15d] Hence, the loss of an undeuterated isopropyl side chain from $[5\text{-}^2H_1]$-6 confirms the migratory properties of the original H-1_{Si} of diphosphate 1. The formation of unlabeled 6 from incubations with $[6\text{-}^2H_1]$-FDP (16) indicates the loss of the H-6 of FDP during catalysis.

While these (and other[15]) limited labeling experiments alone cannot distinguish between a 1,6- and a 1,10-mechanism (Schemes 1, 5 and 6), the experimental observations reported here support an electrophilic 1,6-alkylation reaction as the predominant pathway to $(+)\text{-}\delta$-cadinene (6).[43] Moreover, the 1,6-ring closure pathway, illustrated in Schemes 5 and 6 with the deuterium labeled substrate $(1R)\text{-}1$ and unlabeled 15, is in good agreement with previous feeding experiments with $[1,2\text{-}^{13}C_2]$-acetate and $[2\text{-}^{14}C_1]$- and $[4\text{-}^{14}C_1]$-mevalonate in cotton plants, from which a plausible role for diphosphate 15 was inferred,[17,18] and with more recent studies that revealed the (3R)-enantiomer of nerolidyl diphosphate (2, Scheme 5) as the active substrate of recombinant (+)-DCS.[15c]

Accordingly, we propose that the enzyme-generated tertiary diphosphate 2 (Scheme 5) reacts in the active site pocket of (+)-DCS in the anti, endo conformation typically found in mono- and sesquiterpene cyclases that bring about 1,6-cyclizations.[1a–f,36] Since biomimetic cyclizations of the related linalyl and neryl derivatives are chemically effected mainly from this conformation,[9,45] it seems plausible to suggest that the prenyl chain of 15 adopts a helical orientation similar to that of NDP (2), the enzyme bound intermediate generated from FDP. With this chirality, the 6,7-double bond of 2 (and the double bond of 15) is ideally positioned to effect the presumed anti 1,6-cyclization that ensures the formation of the α-bisabolyl cation (37) with the proposed (R)-configuration at C6 (Scheme 5). An identical R-stereochemistry would be expected

considering the cisoid farnesyl cation (A) as the reactive active site intermediate from which cyclic 37 is formed.

After formation of the $(6R)\text{-}\alpha$-bisabolyl cation (37), the remaining mechanistic steps to (+)-6 (Scheme 6) resemble those precedented in amorpha-4,11-diene synthase (AMDS).[2h,46] However, in contrast to AMDS catalysis, MM2 molecular modeling studies indicate that after the unique C1 → C7 hydride shift to the Si face of 37 involving the original H-1_{Si} of FDP,[2h,46] the second cyclization step (B → D, Scheme 6) requires a conformational ring inversion (twist B → half twist B) to obtain the exo 1,10-electrophilic cyclization onto the Si face of carbocation B that ultimately guarantees the correct 5,10-cis stereochemistry diagnostic of cadinene sesquiterpenes. As shown in Scheme 6, the required conformational change could also occur at the bisabolyl cation (37) stage, but since the C1 → C7 hydride shift occurs to the same C7 Si face of cation 37, the transient 7R-stereochemistry displayed by B (and E, see Scheme 7) would be expected.

Scheme 7. Conversion of Cation E to Amorpha-4,11-diene (42) and Amorphenes (43), respectively[44]
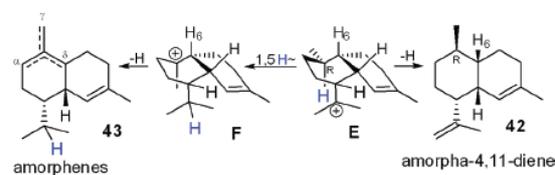
The proposed exo cyclization (Scheme 6) generates the second cyclohexane ring of D in a high-energy boat conformation that nevertheless allows the critical 1,5-H shift to cadinenyl cation 5 with concomitant release of strain. Finally, proton loss from C6 of the trans-fused cadinenyl cation 5 (Scheme 6) accounts for the formation of $(+)\text{-}[5\text{-}^2H_1]\text{-}\delta$-cadinene, or δ-cadinene, when $(1R)\text{-}1$ or $[6\text{-}^2H_1]$-FDP (16) are used as the respective substrates. It is worth noting that while a similar 1,10 electrophilic cyclization onto the C1 Re face of

cation B (twist B → C, Scheme 6) could equally explain, via cation C, the 5,10-cis stereochemistry present in **6** (Scheme 6), the anti stereochemical relationship between H7 and C11 in C precludes the proposed 1,5-hydride shift and hence the installment of the correct deuterium label in **6** observed with *d*-labeled FDPs.[15c,d]

A corollary from this stereochemical analysis stems from the fact that an endo 1,10-cyclization onto the Re-face of carbocation B (twist conformer) would give rise to the *cis*-fused amorphenyl cation E, the direct precursor of amorpha-4,11-diene **42**.[2h,44,46] Similarly, the high-energy boat conformation of cation E (Scheme 7) allows the presumed 1,5-H shift that accounts for amorphene (**43**) biosynthesis.[44]

In summary, the mechanism of the DCS-catalyzed cyclization of FDP (**1**) to (+)-δ-cadinene (**6**) was studied by incubations using a variety of substrate analogues. In agreement with a mechanism via (3R)-NDP (**2**),[15c] prenyl diphosphates with vinylic fluoro substituents at C2 (**10**, **11**, and **18**) were shown to act as competitive inhibitors that prevent the initial cationic ionization-isomerization step. Surprisingly, FDP analogues **10** and **11** with fluorine at C2 also acted as substrates of DCS and yielded fluorinated germacrenes arising from 1,10-cyclizations with the stereochemistry of the C2,C3 double bond remaining intact. The isolation of 2F-germacrenes (**21**, **22**), despite inhibition and stereochemical arguments, seems to favor the 1,10-macrocyclization mechanism. Similarly, the inhibition displayed by 10F-FDP (**12**), together with the formation of a 10F-α-humulene (**26**) via a DCS-mediated 1,11-cyclization of **12**, suggests a 1,10-pathway (path a, Scheme 1) as the most plausible biosynthetic pathway. On the other hand, 6-fluoro-FDP (**13**) served as a potent competitive inhibitor of DCS, an observation that is difficult to explain assuming a 1,10-macrocyclization mechanism (Scheme 1). In addition, diphosphate **13** was the only fluorinated C_{15} diphosphate that was not turned over by the enzyme, suggesting an early involvement of the central C6,C7-double bond of **1** during DCS catalysis. Incubations of DCS with (2Z,6E)-FDP (**15**) produced, in addition to δ-cadinene, a mixture of bisabolenes and bisabolols arising exclusively from a 1,6-ring closure, and resembling the product distribution previously observed when NDP (**2**) was used as the substrate. Taken together, these results are best explained with the involvement of a 1,6-cyclization of (E,E)-FDP (**1**) in DCS chemistry to generate (6R)-bisabolyl cation.[23] Further support for the possible involvement of this mechanism was provided by the observations that C_{10} GDP (**19**) and neryl diphosphate (**20**) generated a variety of 1,6-ring closure products and that 6,7-dihydroFDP (**17**) yielded the acyclic *cis*-6,7-dihydro-α-farnesene as the main product of the enzymatic reaction rather than the expected 6,7-dihydrogermacrene.

CONCLUSION

The results presented here demonstrate for the first time the remarkable mechanistic versatility of DCS that can use an array of distinct cyclizations to generate single reaction products from FDP and its analogues. In addition, the present study supports the possible existence of a 1,6-ring closure mechanism in δ-cadinene biosynthesis[15,43,44] and highlights the high regio- and stereochemical precision of DCS-catalyzed cyclizations. Indeed, with the exception of the inhibitor 6-fluoro-FDP (**13**), from which no products were formed, DCS was able to turn over diphosphates **10**, **11**, and **12**, respectively, to the single fluorinated sesquiterpenes **21**, **22**, and possibly 10F-α-

humulene (**26**) via unprecedented 1,10- and 1,11-cyclizations, in which the geometry of the C2,C3 double bond of the starting diphosphates remained unchanged. This observation contrasts with the native cyclization of FDP, in which DCS must convert the initial trans C2,C3 π-bond via NDP (**2**) to cis in δ-cadinene. While a 1,11-cyclization mechanism en route to δ-cadinene seems unlikely, the observation of 1,10-cyclizations from **10** and **11** and the possible dual 1,6- and 1,10-ring closures from **2** and **15** are in agreement with the mechanistic versatility of cadinane-producing sesquiterpene synthases,[43] which do not appear to depend on a common biosynthetic mechanism in spite of their shared ancestral origin.[47]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental procedures, gas chromatograms, mass spectra and/or NMR spectra of key compounds, as well as inhibition kinetics studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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Chemoenzymatic preparation of germacrene analogues†

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A small library of novel germacrenes was generated using a combination of two plant enzymes, germacrene A synthase, and D synthase and modified farnesyl diphosphate (FDP) analogues. This chemoenzymatic approach allows the preparation of potentially valuable volatiles for biological studies.

Terpenoids represent a valuable class of bioactive fine chemicals[1] and are therefore attractive targets for synthetic modification; modulation of their natural properties may lead to new medicinal and agrochemical compounds with improved properties. However, the complexity of the hydrocarbon skeletons and the often significant chemical instability of many terpenoids can present a formidable challenge to the synthetic chemist.[2] Synthetic biology approaches have focused on the preparation of natural terpenoids in living organisms,[3a] but they operate with whole biochemical pathways using fundamental biosynthetic building blocks (*e.g.* isopentenyl diphosphate) and can therefore not easily be applied to generate modified terpenes. One attractive synthetic approach that complements current terpene synthetic biology[3b,c] and circumvents the difficult task of engineering full metabolic pathways to generate alternative substrates *in vivo*, could rely on the chemical preparation of FDP analogues as substrates of recombinant terpene synthases to create modified terpenoids. Modified FDPs[4,5] have been used extensively to study the mechanisms of the cationic reactions mediated by (sesqui)terpene synthases.[4–7] However, despite the fact that several unnatural FDPs are indeed turned over by these enzymes,[4,5,8] only a few reports have explored the synthetic utility of terpene synthases toward the production of valuable novel terpenoids.[9]

Germacrene A and germacrene D synthases (GAS and GDS) are two plant sesquiterpene synthases that catalyze the Mg^{2+}-dependent conversion of FDP (**1a**) to germacrene A (**3a**) and germacrene D (**5a**), respectively (Fig. 1). These two macrocyclic sesquiterpenes have been shown to act as semiochemicals affecting the olfactory response of insects.[10] While a synthesis of the rather unstable germacrene D (**5a**) has been reported,[11] the extreme thermal and photochemical instability

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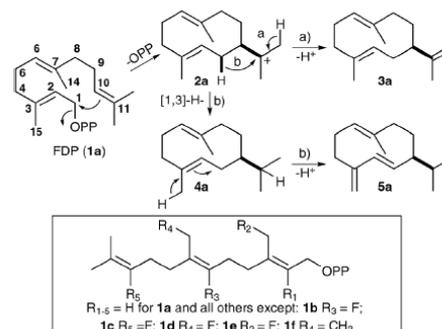
Fig. 1 Proposed biosynthesis of germacrenes A (**3a**) and D (**5a**). Modified substrate analogues of GAS and GDS (framed).

of the acid labile germacrene A (**3a**) has so far hampered the development of a satisfactory chemical synthesis.[12] Nevertheless, fluorinated germacrene A analogues with improved stabilities have previously been produced enzymatically from fluorinated FDP analogues.[4a,c,5b]

Thus, based on the biological and potential economic significance of compounds **3a** and **5a**, germacrene A and D synthases from *Solidago canadensis*[13] were selected to investigate their capability to produce non-natural germacrenes from modified FDPs. To this end, recombinant GAS and GDS were overproduced in *E. coli* and purified as previously described.[9b,14] Several fluorine and methyl modified FDPs were screened by GC-MS on an analytical scale for substrate activity. Germacrene A analogues were readily identified through their ability to undergo thermal Cope rearrangements to the corresponding β-elemene analogues under GC-MS conditions;[14] germacrene D analogues were identified from their mass spectra since the presence of the more stable (*i.e.* more abundant, 100%) $[M - 43]^+$ fragment in the EI^+-MS is diagnostic of the parent **5a**. Only modified FDP analogues (Fig. 1, framed) that gave a relatively strong ion count in the total ion chromatogram (GC-MS) as compared with the natural substrate **1a** were considered suitable for this study (*vide infra*).

Interestingly, in contrast to what has been observed with other sesquiterpene synthases,[4a,d,9a] 2-fluoro-FDP was not turned over significantly by GAS or GDS. In addition, the H/F and in particular the H/CH_3 substitution at the C15 position of FDP

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was not tolerated by either enzyme. The full details and the mechanistic implications of these observations are beyond the scope of this manuscript and will be published elsewhere.

Optimal reaction conditions for the preparation of modified germacrenes: initial preparative incubations using both enzymes were shown to be inefficient and hence an optimisation of the reaction conditions was carried out. After some experimentation, conversions were found to be optimal at concentrations of Mg^{2+}, FDP and enzyme of 10 mM (5 mM for GAS), 0.35 mM and 6 µM, respectively (ESI†). Higher concentrations of Mg^{2+} and/or enzyme led to the formation of insoluble/inactive FDP–Mg^{2+}-complexes and/or enzyme aggregation, which in turn resulted in less efficient turnovers. The concentration of GDS (but not GAS) could be increased to 12 µM simply by inclusion of 1% of the non-denaturing detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) (CHAPS) in the assay buffer. The nature of the organic solvent, the reaction vessel itself and the extractive work up were of fundamental importance for optimal conversions.[9b] In the final optimised conditions, *d*-chloroform was used as the organic layer, the incubations were carried out in sealed tubes with gentle agitation and enzymatic products were extracted overnight using an automated rotator. The filtered and dried *d*-chloroform solutions were then analysed by GC-MS and NMR-spectroscopy. Under these conditions, the enzymatic conversions of **1a** to germacrene D (**5a**) and germacrene A (**3a**) were 76% and 40%, respectively (Fig. 2).

Incubations of FDP analogues with GAS: fluorinated germacrene A analogues were obtained from incubations of GAS with 6F-FDP (**1b**) and 14F-FDP (**1d**) (Fig. 2). These compounds were identified by GC-MS through co-elution with authentic material previously isolated from experiments with aristolochene synthase from *Penicillium roqueforti* (PR-AS, see ESI†).[4c]

Upon incubation with GAS, 10F-FDP (**1c**) produced efficiently (30% rel.) a single fluorinated hydrocarbon that co-eluted in the GC-MS column with an authentic sample of α-10F-humelene, a known compound prepared previously using δ-cadinene synthase (DCS).[4d] This result demonstrates that with diphosphate **1c**, both enzymes are able to catalyze an anti-Markovnikov 1,11-macrocyclisation *via* π-donation from the vinylic fluorine atom into the distal C10,C11-double bond of **1c**.

14Me-FDP (**1f**) was also readily turned over by GAS yielding a mixture of at least seven hydrocarbons. The major

product (retention time 28.5 min, approx. > 50% total hydrocarbons, ESI†) underwent a thermal rearrangement, thereby suggesting a germacrene A analogue as the major enzymatic product from substrate **1f**. In addition, in a parallel study with PR-AS, the same compound mixture was generated from **1f** (ESI†). Purification by preparative TLC and subsequent ^{1}H-NMR analysis of this sample unambiguously confirmed the structure of the major GAS- and PR-AS-generated products as 14Me-germacrene A (**3f**). Hydrocarbon **3f** displayed the well documented conformational flexibility exhibited by germacrene A (**3a**).[12] Indeed, ^{1}H-NMR spectra comparisons (ESI†) with those previously obtained for (**3a**) at different temperatures[12] suggested that **3d** exists as an interconverting mixture of the same three conformers observed for **3a**, albeit with different relative populations (ESI†). As with the parent hydrocarbon **3a**, the most abundant conformation corresponds to the 'crossed up–up' (UU) configuration,[12] but in contrast to **3a**, the combined 'parallel down–up' DU and 'up–down' UD conformations of **3f** dominates (61%) over the individually more stable (*i.e.* more abundant) UU conformation (39%). The present conformational distribution relates to the apparent increase in steric bulk on C14 of **3f** relative to **3a**, which likely raises the energy of the UU conformer with respect to the UD and DU conformations. For a diagram and further explanation regarding the conformations of **3f** see ESI.†

Incubations of FDP analogues with GDS: 6F-FDP (**1b**) and 14Me-FDP (**1f**) were turned over efficiently by GDS each giving a single product displaying the more abundant and stable $[M - 43]^+$ fragment in their EI^+-MS spectra, which is characteristic of germacrene D (**5a**) through loss of the isopropyl group within the MS-detector. Indeed, preparative incubations followed by direct ^{1}H- and ^{19}F-NMR spectroscopy analysis confirmed their identity as the expected germacrene D analogues (ESI†).

Two products (10% rel.) in an approximate 3 : 1 ratio were observed in the pentane extracts from incubation of 10F-FDP (**1c**) with GDS (ESI†). GC-MS analysis of a mixture of 10-fluoro-farnesenes, prepared in a previous study,[4d] unambiguously identified the major product as (*E*)-β-10F-farnesene (ESI†). Interestingly, the minor component (25%) of this mixture was also identified by co-elution as the fluorinated α-10F-humulene previously observed in incubations of **1c** with GAS or DCS.[4d]

Substrate	Substituent					3 GAS		5 GDS	
	R_1	R_2	R_3	R_4	R_5	Conv. (%) rel.[a]		Conv.(%) rel.[b]	
1a	H	H	H	H	H	40	100	76	100
1b	H	H	F	H	H	30	74	38	50
1d	H	H	H	F	H	19	47	33	43
1e	H	F	H	H	H	—		37	49
1f	H	H	H	Me	H	23	57	45	59

Fig. 2 Incubations of modified FDP (**1b, d–f**) under optimized conditions: [a] GAS (6 µM), Mg^{2+} (5 mM) and FDP (0.35 mM); [b] GDS (12 µM), Mg^{2+} (10 mM) and FDP (0.35 mM). Conversions were determined by GC-FID (ESI†) in pentane. Relative conversions (rel.) denote percentage with respect to **1a**.

Both 15F-FDP (**1e**) and 14F-FDP (**1d**) were converted by GDS as judged by GC-MS to a well-defined product under analytical conditions; each product (**5e** and **5d**) displayed the major $[M - 43]^+$-fragment suggesting that they were indeed germacrene D derivatives. However, prolonged preparative incubations led to the formation of a second product apparently arising from the initial GDS-generated product (ESI†). Although the presence of this minor product hampered a full NMR interpretation of the spectrum of the original enzymatic product (**5e**), the observation (^{1}H NMR, 500 MHz) of a relatively downfield (approx. 2 ppm with respect to **5a**) wide doublet at $\delta_H = 6.53$ ppm ($^2J_{H-F} = 86.0$ Hz, C=C*H*F) instead of the diagnostic broad doublet at $\delta_H = 4.77$ ppm ($^2J_{H-H} = 13.0$ Hz, C=C*HH*, *exo* methylene group) of **5a**^{13c} (ESI†) is consistent with the major product being 15F-germacrene D (**5e**, Fig. 2). Surprisingly, the ^{19}F-NMR spectrum of this mixture displayed three absorbances, two identical doublets ($^2J_{H-F} = 86.0$ Hz) at $\delta_F = -136.1$ (minor) and -138.6 (major) ppm, respectively, plus a downfield triplet ($^2J_{H-F} = 46.0$ Hz) at -184.8 ppm due to the very minor peak observable by GC-MS. Thus, the major (and only) enzymatic product is most likely produced by GDS as a mixture of two geometric 15F-germacrene D isomers (**5e**) (Fig. 2) that is not resolved by GC-MS. This observation implies that the corresponding tightly bound carbocation (**4e**) possesses sufficient mobility within the active site of GDS to allow a not completely specific proton-loss to generate the observed isomeric mixture of **5e**.

In summary, the results presented here provide insight into aspects of the reaction mechanisms employed by GAS and GDS and describe a general chemoenzymatic approach for the synthesis of non-natural terpenoids that are otherwise not easily accessible by classical chemical synthesis or synthetic biology. Indeed, these results show that GAS and GDS can turn over a variety of modified FDPs to germacrene A and D analogues often with synthetically acceptable conversions and in sufficient amounts for biological testing as semiochemicals.

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Efficient Terpene Synthase Catalysis by Extraction in Flow

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Sesquiterpenes are an important family of natural products that play many roles in nature. They provide the hydrocarbon scaffolds for terpenoids with medicinal and agrochemical applications. Chemical synthesis of the structurally complex hydrocarbons is usually not a viable option. In nature sesquiterpenes are biosynthesized by terpene synthases from the water-soluble substrate farnesyldiphosphate (FDP) **1** (Scheme 1). Catalysis takes place with high regio- and stereo-
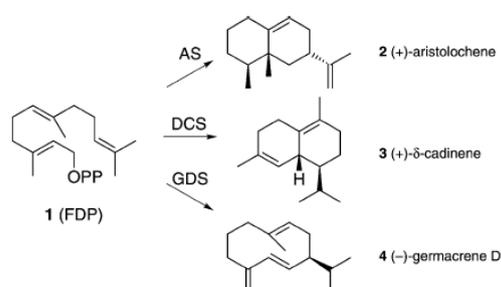
Scheme 1. Sesquiterpenes obtained with terpene synthases used in this study.

selectivity to afford enantiomerically pure bioactive compounds; the turnover numbers of these enzymes are however usually rather low.[1] Examples are *aristocholene synthase* (AS), which is responsible for the production of the PR-toxin precursor aristolochene **2** in *Penicillium roqueforti*,[2] δ-*cadinene synthase* (DCS) from *Gossypium arboreum* synthesizing δ-cadinene **3** as the precursor of the cotton phytoalexine gossypol,[3] and *germacrene D synthase* (GDS) from *Solidago canadensis*. (−)-Germacrene D **4** has been reported to act as an alarm pheromone for several species of aphids.[4]

The synthetic application of terpene synthases such as α-pinene synthase or germacrene synthase targeting incubation and work-up conditions has been reported earlier.[5] Recently, high-throughput screening essays of terpene synthases have been developed.[6] The overall rate of the enzymatic reaction is limited by the release of the hydrophobic product into

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the aqueous solvent; pre-steady-state kinetic studies of trichodiene synthase revealed that the enzymatic steps are about 40 times faster than product release.[7] The solubility of monoterpenes in water is typically very low (10–30 mg L^{-1}).[8] Therefore, the aqueous incubation media will be saturated almost from the beginning of the enzymatic reaction and the rate of product formation is controlled by the mass transfer ratio between aqueous and organic phases.

The high current interest in continuous-flow processes performed in microfluidic systems[9] is driven by their advantages over classical batch approaches. Miniaturisation of flow devices allows an enhanced mass and heat transfer owing to a very large surface-to-volume ratio. Apart from organic synthetic protocols, biocatalytic reactions have been facilitated by biphasic flow. Esterifications in ionic liquid/heptane mixtures,[10] hydrolysis of esters in water/decane solvents,[11] and reductions of carbon–carbon double bonds[12] or of aldehydes[13] are accelerated in biphasic flow systems because of efficient product removal from the aqueous phase. Microwave- and ultrasound-facilitated biphasic reactions have already been reported.[14]

In this study, we present a new incubation methodology, using continuous flow with sonication that speeds up the enzymatic reaction by increasing the mass transfer between two phases of the biphasic mixture. The method has been tested with the three terpene synthases GDS, AS, and DCS. These three terpene synthases were produced in *E. coli* and purified to apparent homogeneity as described previously.[5b, 15] Subsequently, the method was also used for the enzymatic conversion of FDP analogues into the corresponding modified products.

The sesquiterpene synthases used in this study require Mg^{2+} for activity. In batch experiments, the optimal metal concentrations for these enzymes were found to be in the range of 3–10 mM. These data are in agreement with previous kinetic experiments with radiolabeled FDP.[16] With FDP concentrations above 0.4 mM, an insoluble, unreactive precipitate was observed in the presence of Mg^{2+}[17] and hence a substrate concentration of 0.35 mM was selected for this study.

Initial flow experiments were performed to evaluate optimal protocols for this study. In initial flow experiments, the substrate FDP, enzyme (GDS), and pentane were injected into the flow reactor as three separate solutions. The experiment was repeated after premixing the enzyme and substrate solutions. Identical conversions to germacrene D were found in both experiments indicating that the reaction rate is solely ruled by the removal of product from the aqueous phase and that the substrate conversion in the enzyme solution is negligible. Another reaction was performed with ethylenediamine tetraacetic acid (EDTA) in the collecting vial to quench any reaction after the incubation time. The conversion in this case was also iden-

tical with the original experiment performed without quenching indicating that any improvement observed in flow is not due to extra incubation time in the loading syringe or in the collecting vial.

The first set of reactions in flow was performed with a GDS solution at a fixed enzyme concentration of 9 μM and a substrate concentration of 0.35 mM using pentane as the organic solvent for product extraction. The diameter of the tubing reactor was changed while the tubing reactor volume (length of tubing) and the flow rate were adjusted to a 30 minute reaction time (see the Supporting Information). In reactions performed in tubing with a small internal diameter (0.175 mm) enzyme inactivation was observed. A precipitate was formed and a conversion of < 1% suggested enzyme denaturation. No precipitate was observed when bigger tubing diameters were used indicating that the turbulences generated inside the tubing are able to extract the product and are mild enough to avoid enzyme denaturation. A conversion of 17% was observed with a tubing reactor of 0.45 mm internal diameter. To further improve the mass transfer between the organic and aqueous phase, the tubing reactor was immersed in a sonication bath. The conversion was increased to 52%.[18] However, when the flow rate was decreased and the reaction time increased to 1 hour, a large decrease in the conversion to only 10% was observed. The decrease in the turbulences generated inside the tubing resulting from the decrease in the flow rate lead to a greatly diminished extraction of the product and, therefore, to lower conversion. A reaction performed in a tubing reactor with 0.8 mm internal diameter led to almost the same conversion (47%) whereas a further increase in diameter to 1.6 mm resulted in a less efficient extraction and, therefore, in a decreased conversion (22%) as shown in Figure 1.
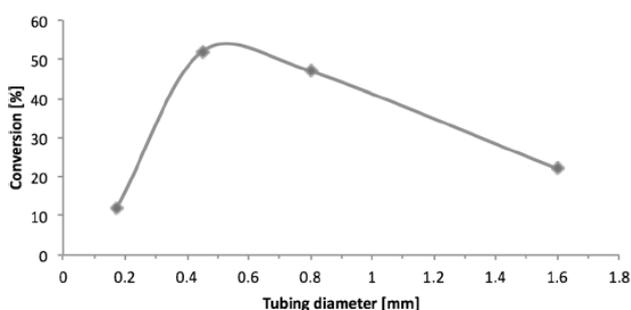

Figure 1. Conversions with different tubing internal diameters (30 min reaction time).

The general applicability of this methodology with other terpene synthases was established by extending the study to DCS and AS. These two enzymes were tested at different concentrations in batch and in flow. All experiments (reaction time: 30 min at 30 °C) were performed without sonication to determine the optimal enzyme concentration for batch and flow reactions as shown in Table 1. A setup sketched out in Figure 2 was used for the flow reactions.

All these experiments revealed an improved conversion by using a flow reactor in comparison with batch experiments.

Table 1. Conversions in batch and flow at different enzyme concentrations.

Entry	Concentration [μM] (enzyme)	Conversion batch [%]	Conversion flow [%][a]
1	6 (AS)	15	21
2	9 (AS)	27	34
3	12 (AS)	43	49[b]
4	6 (DCS)	6	14
5	9 (DCS)	11	26
6	12 (DCS)	15	42
7	9 (GDS)	24	48[c]
8	12 (GDS)	n.d.	52[c]

[a] Tubing internal diameter: 0.45 mm. [b] 21% conversion when performed at 40 °C, enzyme precipitation observed. [c] Reaction performed with sonication. n.d. = not determined.
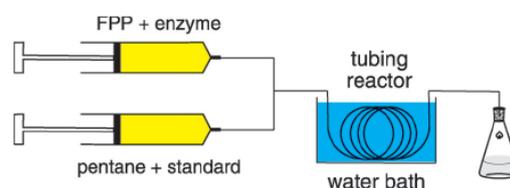

Figure 2. Flow setup for enzymatic reactions.

With AS producing (+)-aristocholene, the improvement was rather modest (Table 1, entries 1–3) whereas in the case of synthesizing (+)-δ-cadinene with DCS, the use of flow more than doubled the conversion (Table 1, entries 4–6). The batch experiments also showed enzyme aggregation at higher concentrations (Table 1, entries 5 and 6). In previous batch studies,[2] the conversion after 24 hours incubation was decreased owing to inactivation of the enzyme by aggregation. The flow approach now allows much shorter incubation times where the (slow) aggregation process is negligible. Further experiments were performed at an enzyme concentration of 12 μM using sonication and varying the tubing internal diameters, which gave the highest conversions with sonication (Table 2). For AS, almost quantitative conversion (88%) was observed with 0.8 mm tubing, meanwhile DCS performed optimally (56% conversion) with tubing of 0.45 mm internal diameter.

Engineering metabolic pathways in yeast have already achieved large-scale production of valuable sesquiterpene products. However, this approach uses the yeast metabolic pathways for the generation of **1** and

Table 2. Comparison of batch and flow reactions with sonication.

Entry	Conc. [μM] (enzyme)	Conversion batch [%]	Conversion flow 0.45 mm[a] [%]	Conversion flow 0.8 mm[a] [%]
1	12 (AS)	43	66	88
2	12 (DCS)	30	56	27

[a] Tubing internal diameter.

therefore only the production of natural compounds can be achieved; this is because the alkyl chains R^1–R^3 (Scheme 2) derive from acetyl-CoA.[19] Some unnatural FDP analogues have been shown to act as substrates of these enzymes generating

5 (12-Me-FDP) R^1=Et, R^2=Me, R^3=Me
6 (14-Me-FDP) R^1=Me, R^2=Et, R^3=Me
7 (15-Me-FDP) R^1=Me, R^2=Me, R^3=Et

Scheme 2. FDP analogues as substrates for terpene synthases.

a range of unnatural sesquiterpenes. Three methyl-substituted analogues were tested here with our approach to generate a library of potentially bioactive unnatural compounds. The syntheses of the FDP analogues **6** (14-Me-FDP) and **7** (15-Me-FDP) were performed as described previously.[7] The synthesis of the novel compound **5** (13-Me-FDP) is described in the Supporting Information. Using the reaction conditions identified above, the enzymes DCS and AS were used to transform the three FDP analogues **5**–**7** shown in Scheme 2. The results of these reactions are summarized in Table 3 and show that the methodology is also applicable to the conversion of FDP analogues

Table 3. Conversions in batch and flow at 12 μM enzyme concentration.

Entry	Substrate (enzyme)	Conversion batch [%]	Conversion flow [%]
1	**5** (AS)	22	63[a]
2	**6** (AS)	6	11[a]
3	**7** (AS)	9	15[a]
4	**5** (DCS)	2.5	5[b]
5	**6** (DCS)	9	14[b]
6	**7** (DCS)	1	3.5[b]

[a] Tubing internal diameter: 0.45 mm. [b] Tubing internal diameter: 0.8 mm.

into aristocholene and δ-cadinene derivatives. The difficulty with such FDP analogues in batch reactions is largely overcome because the reactions are about two to three times more efficient when performed in flow.

In summary we have shown that the use of a biphasic continuous flow system increases the enzymatic conversion of terpene synthases through improved extraction of the hydrophobic cyclic reaction products. This effect is not limited to a specific enzyme and is especially useful when the overall conversion is low as in the case of substrate analogues.

Experimental Section

Enzymatic conversion of 1 into (+)-aristolochene 2 in flow

Incubation buffer (20 mM Tris, 5 mM 2-mercaptoethanol, 15% glycerol, 3 mM MgCl$_2$, pH 7.5, 422.5 μL) with enzyme solution in buffer (Tris 20 mM, pH 7.5; 100 μM, 60 μL) and a solution of **1** (10 mM,

17.5 μL) were premixed to a final total volume of 500 μL. The mixture was placed in a 1 mL syringe. Pentane (1 mL) was spiked with a solution of α-humulene (1 mM, 35 μL) and placed in a 1 mL syringe. Both syringes were placed separately in two syringe pumps and connected via a T-piece to a tubing reactor (length: 4 m, internal diameter: 0.8 mm). The loop was immersed in a thermocontrolled sonication water bath at 30 °C. Flow rates were adjusted to a total flow rate of 66 μL min^{-1} (22 μL min^{-1} for the aqueous phase and 44 μL min^{-1} for the organic phase) leading to a residence time of 30 min. Direct analysis of the organic phase with GC analysis allowed the determination of the conversion by comparison of the product peak area with the area of the standard.
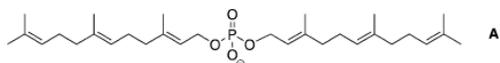
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