DESIGN AND SYNTHESIS OF FARNESYL DIPHOSPHATE ANALOGUES FOR MODULATING THE CHEMISTRY OF ARISTOLOCHENE SYNTHASE

by

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DEDICATED TO MY PARENTS

遊子吟

－唐·孟郊

慈母手中線，遊子身上衣。
臨行密密縫，意恐遲遲歸。
誰言寸草心，報得三春暉。

ABSTRACT

A variety of farnesyl pyrophosphate (FPP) analogues were prepared by both published protocols and novel methodology, which was developed by modification and improvement of the traditional Suzuki-Miyaura coupling. These compounds were incubated with aristolochene synthase in order to probe its mechanism of action and resulted in many interesting results.

The synthesis 12,13-difluoro farnesyl pyrophosphate was achieved in 13 steps using Suzuki-Miyaura chemistry. It proved to be a potent inhibitor of aristolochene synthase (AS), which revealed that the initial cyclisation to germacryl cation occurs in a concerted fashion.

Analogue 2-fluoro FPP was synthesized and upon incubation with aristolochene synthase was converted to a single pentane extractable product according to GC/MS analysis. On the basis of NMR-analyses and GC-MS experiments this product was identified as 2-fluorogermacrene A. This work suggests that after an initial concerted cyclisation of FPP to germacryl cation, deprotonation leads to the formation of germacrene A and provides compelling evidence that germacrene A is indeed an on-pathway product of catalysis by aristolochene synthase.

Analogue 6-fluoro FPP was prepared using the Weiler's chain extension method in 10 steps, and has been postulated to be give 6-fluoro germacrene A as product, which is consistent with published results on Epi-aristolochene synthase.

10-Fluoro FPP was made with sulfonylation-alkylation-desulfonylation methodology and it was found not to act as substrates of the AS. This result is fully consistent with the conclusions drawn from the results with 12,13-difluoro FPP.

FPP analogues with one fluoro substituent at position C14 and C15 were made using methodology employing the Horner-Emmons Wittig condensation as a key step. These two compounds were tested with AS and both gave one major extractable terpene product according to GC/MS analysis. These two products are postulated as two different compounds - 14-fluoro aristolochene and 15-fluoro germacrene A respectively, because of the destabilizing effect of the β-substituted fluorine atom on the carbocation in their vicinity.

Analogues of farnesyl pyrophosphate containing phenyl substituents in place of methyl groups have been prepared in syntheses that feature use of Suzuki-Miyaura reactions as key steps. These analogues were found not to act as substrates of the aristolochene synthase. However, they were potent competitive inhibitors of AS, which indicate that the active sites of terpene synthases are sufficiently flexible to accommodate even substrate analogues with large substituents suggesting a potential way for the generation of non-natural terpenoids.

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ABBREVIATIONS

aq.	Aqueous
Amp	Ampicillin
APS	Ammonium peroxodisulfate
Ar	Unspecified aryl substituent
AS	Aristolochene synthase
Bn	Benzyl
b.p.	Boiling point
BSA	Bovine serum albumin
Bu	Butyl
BuLi	Butyllithium
cat.	Catalytic
conc.	Concentrated
COSY	Correlation Spectroscopy
Cys	Cysteine
Da	Daltons
DAST	Diethylaminosulfur trifluoride
DCM	Dichloromethane
DIBAL-H	Diisobutylaluminium hydride
DME	1,2-Dimethoxyethane
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dppp	1,3-*bis*(Diphenylphosphino)propane
E. coli	*Escherichia coli*
EDTA	Sodium diaminoethanetetraacetate
ES	Electron Spectroscopy
EAS	5-epi-aristolochene synthase
Et	Ethyl

equiv. or eq.	Equivalent
FPP	Farnesyl diphosphate
g	Grams
GC	gas chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
Gly	Glycine
HMBC	Heteronuclear Multiple-Bond Correlation
HMQC	Heteronuclear Multiple-Quantum Correlation
HPLC	High Pressure (Performance) Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
HSQC	Heteronuclear Single-Quantum Correlation
i	*iso*
in vitro	*Latin* occurring or made to occur outside an organism
in vivo	*Latin* occurring or made to occur within a living organism
IPP	Isopentenyl diphosphate
IPTG	Isopropyl-thio-β-galactopyranoside
IR	Infra Red
J	Coupling constant (in Hz)
LB	Luria-Bertani
LDA	Lithium Di*iso*propylamine
LRMS	Low Resolution Mass Spectrometry
M	Molar
m-CPBA	*meta*-Chloroperbenzoic acid
Me	Methyl
min	Minutes
m.p.	Melting point
mRNA	messenger-Ribonucleic Acid
MRSA	Methicillin Resistant Staphylococcus Aureus
MS	Mass Spectrometry
MsCl	Methanesulfonyl chloride
MVA	Mevalonic acid

n	*normal*
NBS	*N*-Bromosuccinimide
NMR	Nuclear Magnetic Resonance
noe	Nuclear overhouser effect
NOSEY	Nuclear Overhauser effect spectroscopy
p	*para*
PAGE	Polyacrylamide gel electrophoresis
PCC	Pyridinium chlorochromate
Ph	Phenyl
P_i	Inorganic phosphate
PP_i	Inorganic diphosphate
PTSA	*para*-Toluenesulfonic acid
R	Specified substituent
RNA	Ribonucleic acid
rRNA	ribosomal-Ribonucleic Acid
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
Ser	Serine
t	time
TBAF	Tetra *n*-butylammonium Fluoride
tert	*tertiary*
Tf	Trifluoromethanesulfonyl
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
TMS	Trimethylsilyl
Tris	Tris-(hydroxymethyl)-amino-methane
tRNA	transfer-Ribonucleic Acid
TPP	Thiamine pyrophosphate
UV	Ultraviolet

v/v	Volume/volume
VFPP	Vinyl analogue of farnesyl diphosphate
WT	Wild type

Content Table

CHAPTER 1

INTRODUCTION

1.1 General introduction to terpenes

The name "terpene" derives from the word "turpentine", which is a fluid obtained by the distillation of resin obtained from trees, mainly pine trees. Terpenes are a large and varied class of hydrocarbons which are widespread in nature. They are small organic molecules that have an immense diversity of structure. So far more than 30,000 terpenoids have been identified, and plants produce more than half of them.[1]

The majority of terpenoids synthesised by plants are produced by secondary metabolism. Although for most of these compounds, the exact functions are unidentified, it has been known that they are not essential for viability of the organism. Instead, they play important roles, for instance, in communication or defence.[2] There is also a small group of terpenoids which are used in primary metabolism, such as the phytol side chain of chlorophyll.[3]

1.2 Importance and ecological role of terpenes

Apart from their huge array of structural diversity, terpenoids also have varied biological activity. In nature, their uses encompass employment as scents, oils, pheromones, phytoalexins.[4] For example, there are different groups of terpenoid molecules produced by plants that act in different roles to help them survive in nature. Some may be produced by plants under attack. Gossypol (**1**) from cotton,[5] which is toxic to the attacking organism is a good example. Some plants give out small volatile molecules as scents to attract insects that can help distribute pollen for reproduction and other compounds act as inhibitors to prevent the growth of competitor plants in a resource-limited area.[6, 7] The varied usage of terpenes in nature will become apparent in the

following sections.

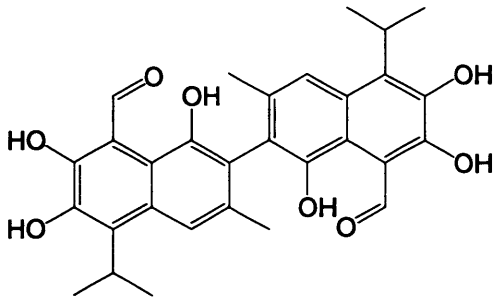

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Figure 1.1: *Structure of gossypol* (**1**).

Terpenes can be divided into groups according to the number of five-carbon units they contain. The group of compounds with 10 carbons is named monoterpenes, sesquiterpenes contain 15 carbons, diterpenes contain 20 carbons, and so on.[8]

Monoterpenoids are produced from geranyl pyrophosphate (GPP) (**2**).[4] They are nonnutritive dietary components and usually found in the essential oils of citrus and other plants.[9] They are largely responsible for the distinctive fragrance of many plants. However, recently, more medical and ecological functions have been allocated to this group of terpenoid compounds. β-Pinene (**3**) is a flavour and fragrance material[10] which has a structural isomer in nature, α-pinene (**4**). The latter compound has been proven as a sex pheromone attracting the female cotton boll weevil.[9] Its primary oxidation product is verbenone (**5**), which plays an important role in the control of the southern pine bark beetle. Recent publications have noted that verbenone may also have antimicrobial properties.[11] Another example of medicinal use of a monoterpene is δ-limonene (**6**). It is extracted from orange peel oil and has chemopreventive activity against some cancers.[9] Some other dietary monoterpenes have been found to have similar anti-cancer property when fed

to rat during the initiation phase.[9] Other monoterpenes are also used as antimicrobial agents such as camphor (**7**), which is found in wood of the Camphor laurel, a large evergreen tree found in Asia, and thymol (**8**), which is a monoterpene phenol derivative of cymene (**9**), isomeric with carvacrol (**10**), and is present in the oil of thyme, oregano and sage.[12]

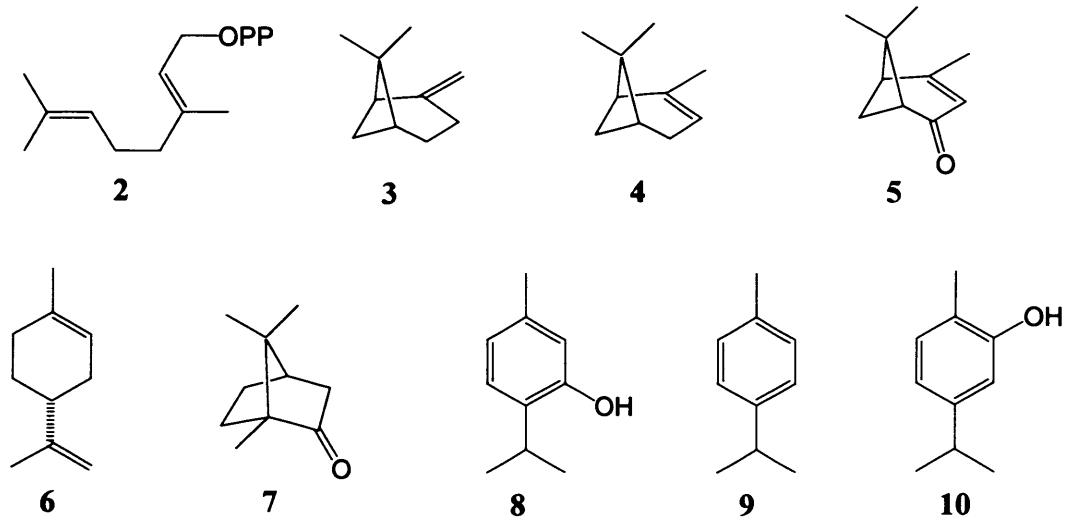
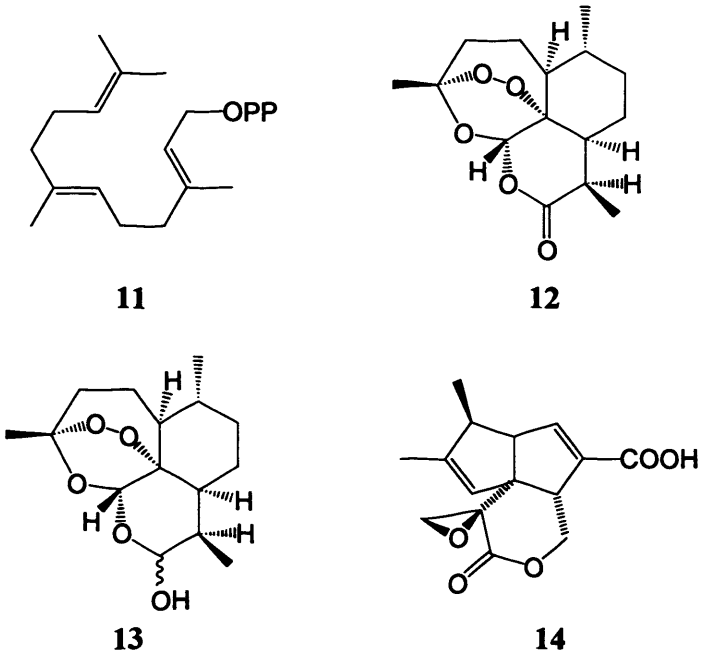


Figure 1.2: *Structures of geranyl pyrophosphate* (**2**), *β-pinene* (**3**), *a-pinene* (**4**), *verbenone* (**5**), *δ-limonene* (**6**), *camphor* (**7**), *thymol* (**8**), *cymene* (**9**) *and carvacrol* (**10**).

All sesquiterpenes, of which more than 300 are known, are derived from the same starting material - farnesyl pyrophosphate (FPP) (**11**).[13] Sesquiterpenoids or rather natural extracts containing sesquiterpenoids have been used as traditional medicines for thousands of years. More recently, along with such natural products, modified natural products have been used as therapeutic agents.[14] For example, artemisinin (**12**), an endoperoxide-containing sesquiterpenoid lactone from the Chinese-Vietnamese plant - *artemisia annua L.* (sweet wormwood) has been used in China (where it is known as Qing Hao Su) as a traditional antimalarial and antipyretic drug for hundreds of years.[15] Recent research has found that the characteristic peroxide lactone

structure is indispensable for anti-malarial activity, but other modifications of the parent structure have been found to retain or improve its pharmacological properties.[16] For example, dihydroartemisinin (**13**) is more potent than the parent material.[17] Sesquiterpenoids have also been found to have antibacterial properties - a good example is pentalenolactone (**14**), which is a member of the pentalenene-derived family of antibiotics.[18]



11 **12**

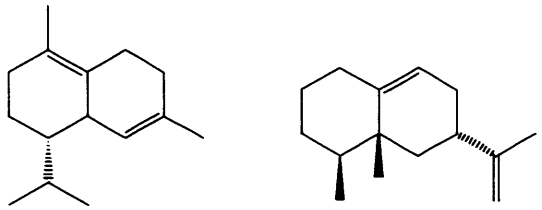
13 **14**

Figure 1.3: *Structures of farnesyl pyrophosphate* (**11**), *artemisinin* (**12**), dihydroartemisinin (**13**) *and pentalenolactone* (**14**).

As with monoterpenes, sesquiterpenoids are widespread in nature and exhibit the full range of biological properties illustrated above. For example, δ-cadinene (**15**) is the precursor of gossypol (**1**) which is a plant defensive phytoalexin in cotton.[5, 19]

In contrast to phytoalexins that are defensive compounds, some sesquiterpenoids are mycotoxins that are used to attack competing organisms. Aristolochene (**16**) from *Penicillium roqueforti* can

be derivatised to several fungal toxins that can seriously damage crop plantations with world-wide economic consequences.[20]



15 **16**

Figure 1.4: *Structure of δ-cadinene* **(15)** and *(+)-aristolochene* **(16)**.

As with other groups of terpenoids, diterpenoids have quite a few different uses both in nature and in medicine. When castor bean plants are under attack, much higher levels of casbene **(17)** have been observed in the seedlings.[21] With both antibacterial and antifungal properties, this diterpene has been considered as a phytoalexin.[21] As to the therapeutical properties of diterpenes, paclitaxel (taxol) **(18)** is a multi million dollar anti-cancer drug.[22] Taxol is extracted from the pacific yew tree and has remarkable antitumour and antileukemic activity. One common characteristic of most cancer cells is their rapid rate of cell division. In order to accommodate this, the cytoskeleton of a cell undergoes extensive restructuring. Paclitaxel is an effective treatment for aggressive cancers because it adversely affects the process of cell division by preventing this restructuring by binding to tubulin. It is most commonly used to treat ovarian, breast and non-small cell lung cancer.[22]

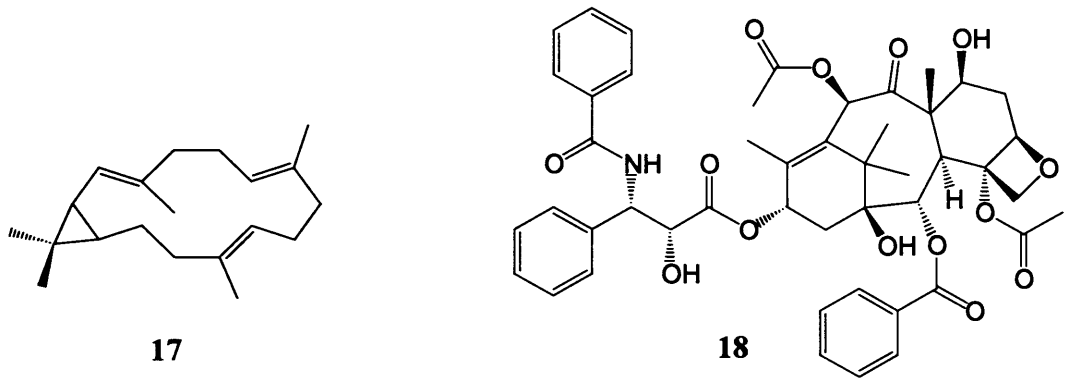


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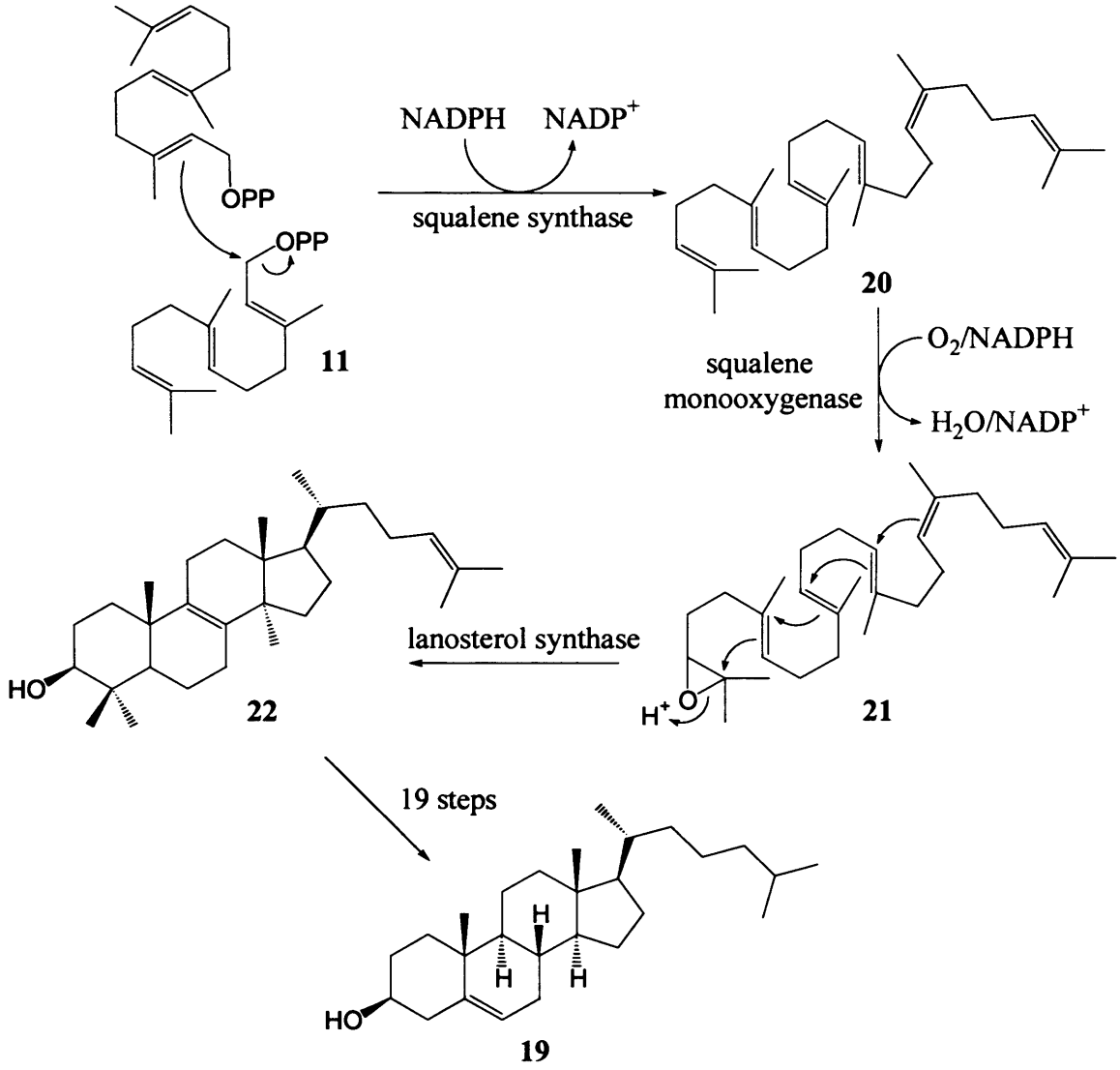
Figure 1.5: *Structures of casbene* (**17**) *and paclitaxel* (**18**).

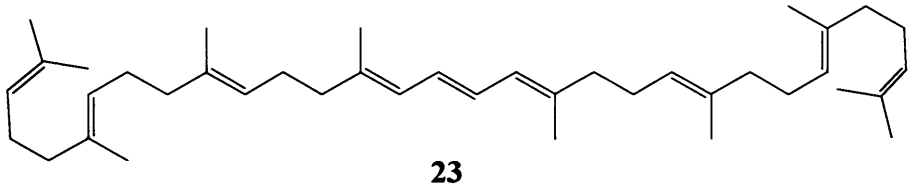
Cholesterol (**19**) and other steroids are considered as triterpenes even though they possess C27-C30 skeletons. This is because they are derived from the C30 linear precursor, squalene (**20**), which is derived from two molecules of FPP (**11**) (Scheme 1.1).[23] Oxidation by squalene monooxygenase of one of the terminal double bonds of squalene yields 2,3-squalene oxide (**21**) which undergoes enzyme-catalyzed cyclization to afford lanosterol (**22**), and is then elaborated into cholesterol and other steroids. Cholesterol is the source of sexual homones, bile acid and vitamin D in animals as well as having functions in controlling calcium and phosphorus metabolism. Another important function is the maintenance of membrane fluidity and permeability.[24]



Scheme 1.1: *Generation of cholesterol* (**19**) *from FPP* (**11**).

Phytoene (**23**), the C40 terpene precursor, is the parent molecule of carotenoids, the family of naturally occurring pigments.[25] Members of this group of molecules are deeply coloured compounds and are present in all photosynthetic organisms, in fungi and even in some animals. Due to their conjugated structures, carotenoids are central for the absorption of light in photosynthetic organisms. They can absorb the light at lower wavelengths than chlorophyll and this increases the range of wavelength of light that may be used in photosynthesis.[26] In this case,

the terpenoids are primary metabolites, as they are essential for survival. Phytoene can also be converted to vitamin A (**24**), which is an important compound in the vision process, for resistance to infectious disease and bone remodelling.[27-29]



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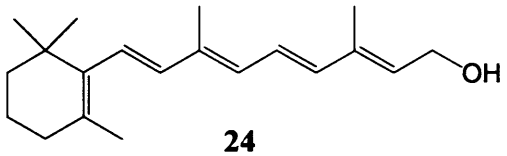

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Figure 1.6: *Structures of Phytoene* (**23**) *and vitamin A* (**24**).

1.3 The isoprene rule

The initially postulated "isoprene rule" by Wallach stated that most terpenoids could be hypothetically constructed by a repetitive joining of isoprene (2-methyl-1,3-butadiene) (**25**) units and have formula of C_5H_8 or $(C_5H_8)_x$.[30] In 1953, the "biogenetic isoprene rule" postulated by Leopold Ruzicka,[8] which ignores the precise character of biological precursors and assumes only that they are "isoprenoid" in structure. The carbon skeleton of terpenes is composed of isoprene units linked in a regular (head to tail) or an irregular arrangement. In most cases, the initially formed structure assembled from C5 units is enzymatically modified in a number of ways to generate the known terpenoid skeletal types. This indicates that all terpenes are related, regardless of their apparent structural differences. Terpenes can be thought of as arising from the five-carbon building block "isoprene" and oligomerisation of this motif, followed by cyclisation,

rearrangement and further oxidation of the carbon skeleton, which makes for an enormous diversity of structure.[8]

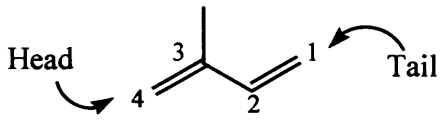

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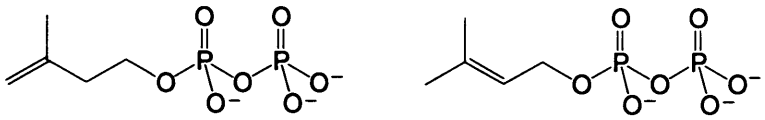
Figure 1.7: *Structure of isoprene* **(25).**

1.4 Biosynthesis of IPP and DMAPP

Isoprene is not the biological precursor of terpenes, even though the isoprene unit is the structural component of the terpenes. Rather, two biological equivalents of isoprene, isopentenyl pyrophosphate (IPP) (**26**) and dimethylallyl pyrophosphate (DMAPP) (**27**), are the building blocks of isoprenoids.[31]



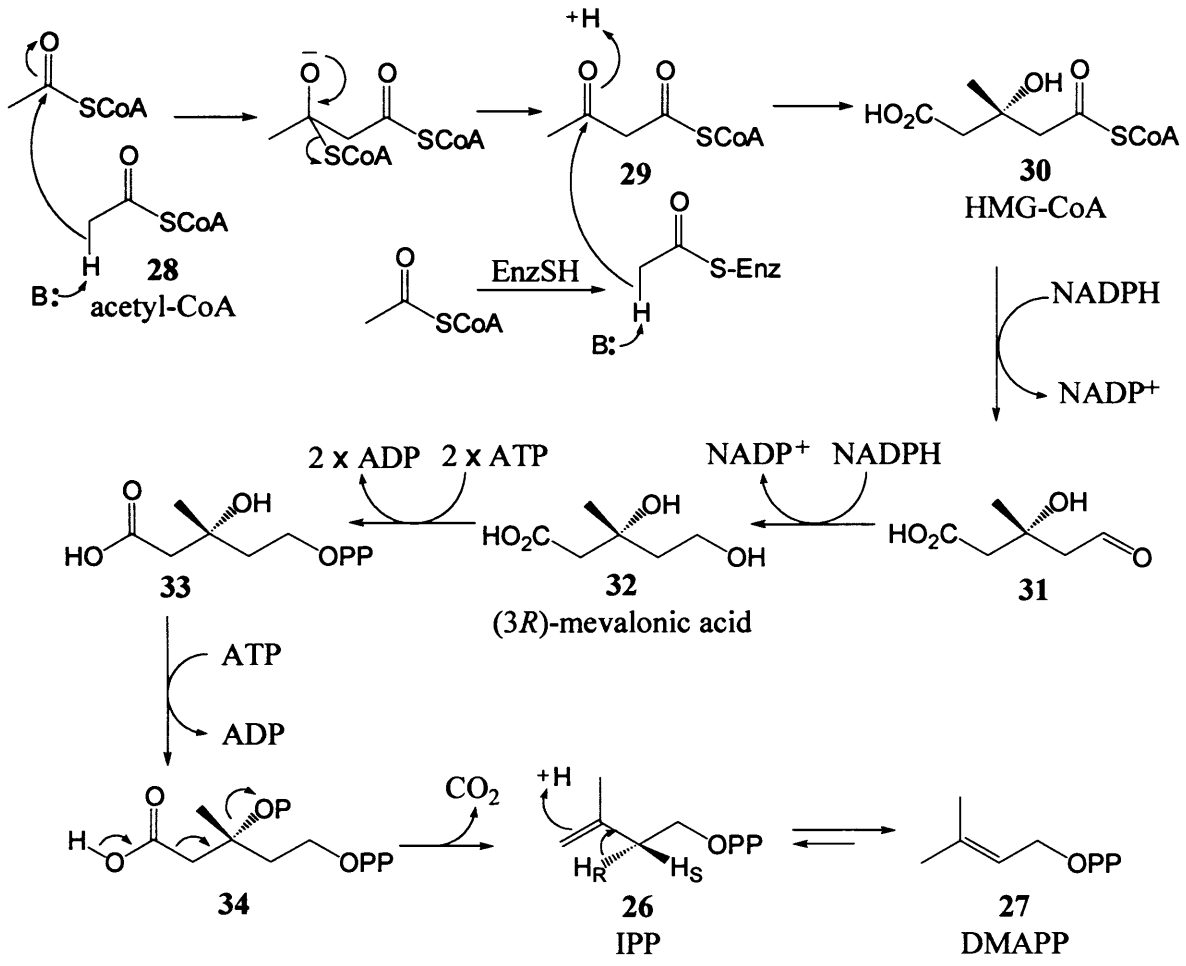
26 **27**

Figure 1.8: *Structures of isopentenyl pyrophosphate* **(26)** *and dimethylallyl pyrophosphate* **(27).**

There are two pathways used to produce the IPP and DMAPP in nature. They are the so-called mevalonic acid pathway and non-mevalonate pathway.[32] The mevalonic acid pathway takes place in the cytosol and is responsible for the formation of sesquiterpenes and triterpenes. The non-mevalonate pathway takes place in plastids and is responsible for the formation of monoterpenes, diterpenes and tetraterpenes.

1.4.1 Mevalonic acid (MVA) pathway

The mevalonic acid pathway is usually used by mammals, higher plants, fungi and even some bacteria to produce IPP and DMAPP.[33] This pathway starts with three units of acetyl-CoA (**28**). The first two units of acetyl-CoA react in a Claisen condensation catalysed by acetoacetyl-CoA synthase with the release of coenzyme A to afford the acetoacetyl-CoA (**29**). A third molecule of acetyl-CoA is added in an aldol-like reaction with the help of β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) synthase to afford the product, HMG-CoA (**30**).[33]



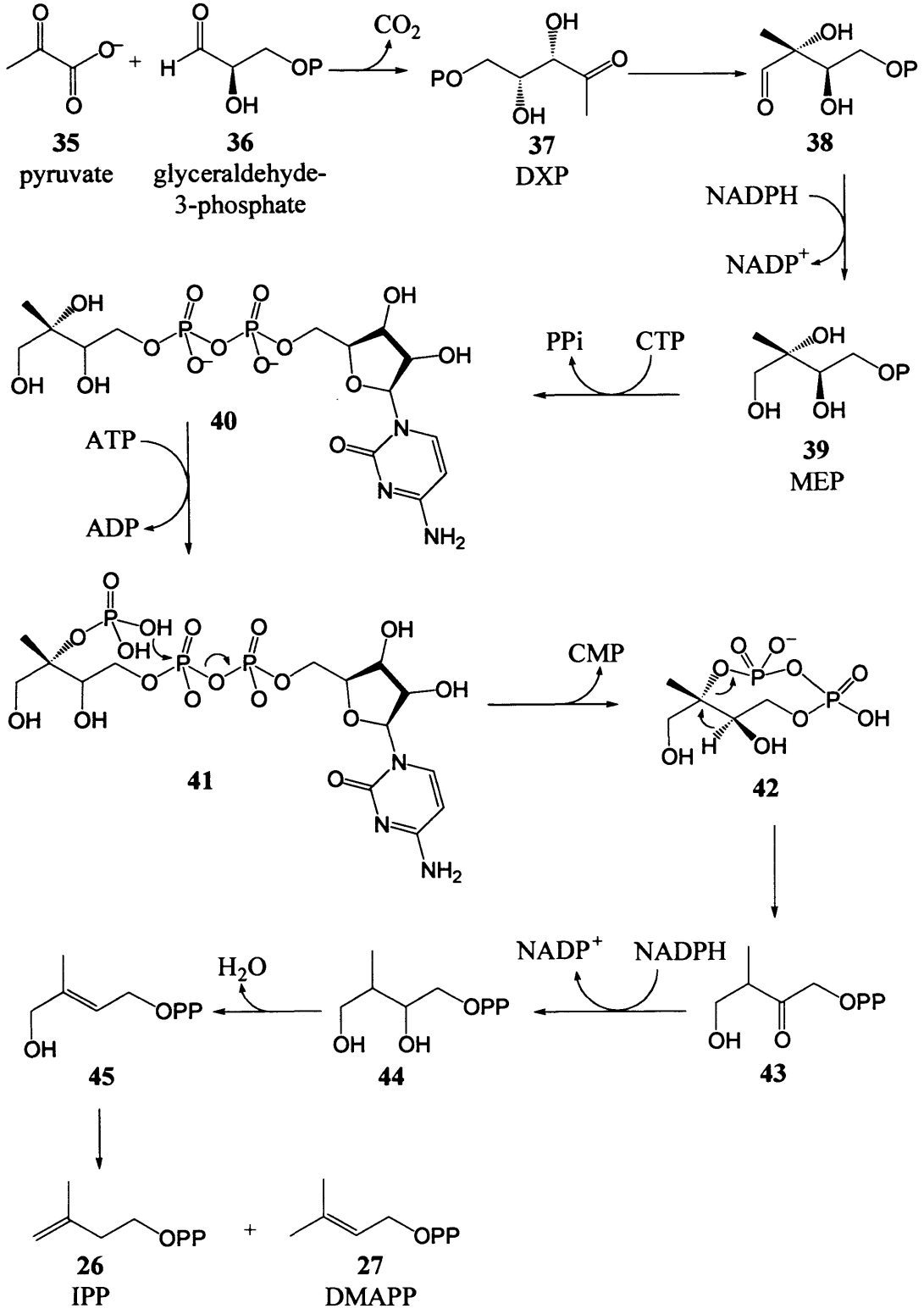
Scheme 1.2: *Mevalonic acid pathway to IPP and DMAPP.*

HMG-CoA reductase is the enzyme which catalyses the reduction of the thioester group of HMG-CoA to the primary alcohol **32**. The cofactors used in this reaction are two equivalents of NADPH. The first NADPH reduces the HMG-CoA to mevaldic acid (**31**), and the second performs a reduction to make (*3R*)-mevalonic acid (MVA) (**32**).[34]

The two hydroxy groups of (*3R*)-mevalonic acid are phosphorylated and pyrophosphorylated by three molecules of ATP to yield mevalonic acid triphosphate (**34**). The final product IPP (**26**) comes from the decarboxylation of mevalonic acid triphosphate. IPP (**26**) and DMAPP (**27**) are interconvertible by stereospecific isomerisation. It was shown by tritium-labelling experiment that only the pro-*R* proton on C2 of **26** is involved in the isomerisation process.[35]

1.4.2 Non-mevalonate (non-MVA) pathway

The non-MVA pathway (Scheme 1.3) was discovered at the end of the last century by Rohmer and coworkers.[36, 37] It is found in the chloroplasts of algae, cyanobacteria, eubacteria and apicomplexa. This pathway is also named as the pyruvate/glyceraldehyde-3-phosphate pathway or sometime as MEP pathway, due to the fact that pyruvate (**35**) and D-glyceraldehyde-3-phosphate (**36**) are the starting compound of this pathway and that 2-C-methyl-D-erythritol-4-phosphate (MEP) (**39**) is an important intermediate in the pathway.

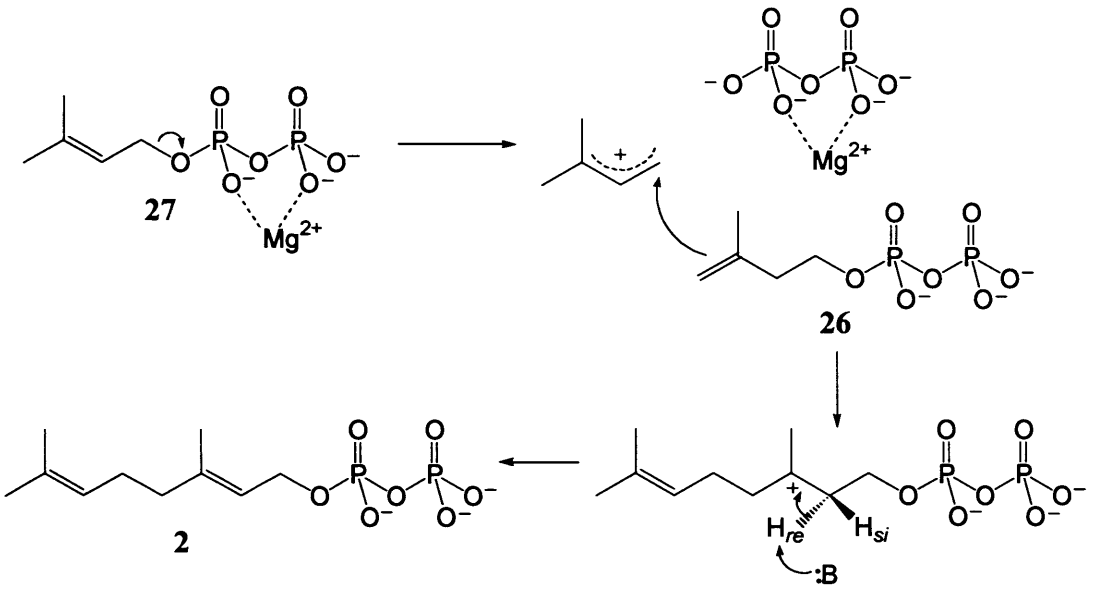


Scheme 1.3: *The non-mevalonate pathway to IPP and DMAPP.*

Thiamine pyrophosphate (TPP) mediated decarboxylation of pyruvate (**35**) affords an enamine that reacts with glyceraldehyde-3-phosphate (**36**) to generate 1-deoxy-D-xylulose-5-phosphate (DXP) (**37**). After a pinacol-like rearrangement of DXP, it is reduced by NADPH in DXP-reductoisomerase, giving 2-C-methyl-D-erythritol-4-phosphate (MEP) (**39**) as the product.[38] MEP reacts with cytidine triphosphate (CTP) and is then phosphorylated by one equivalent of ATP to yield 2-phospho-4-cytidinediphosphate-2-C-methylerythritol (**41**), which is converted to 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (**42**).[38] Following this, there are a reduction and a dehydration that are not yet precisely identified leading to the formation of IPP (**26**) and DMAPP (**27**).

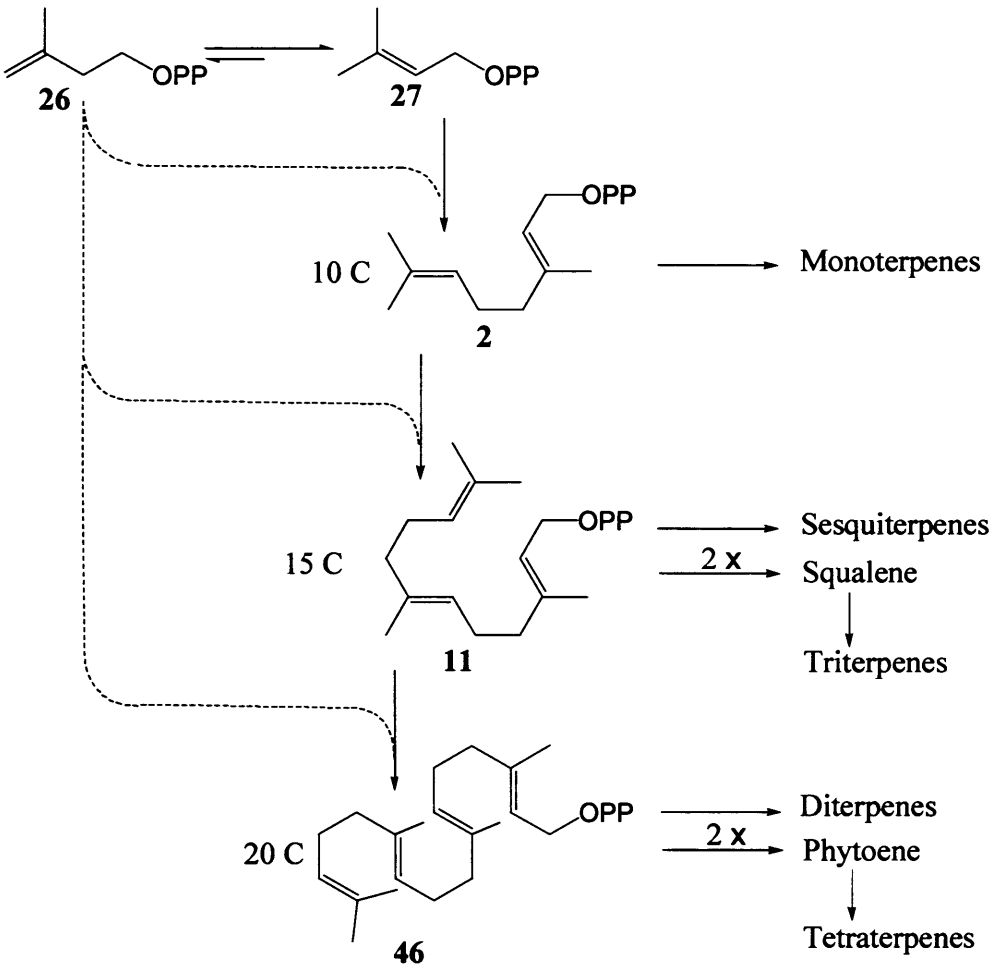
1.5 Biosynthesis of linear polyisoprenoid precursors

Condensation of IPP and DMAPP in a head to tail fashion is catalysed by isoprenyl pyrophosphate synthase leading to the formation of geranyl pyrophosphate (**2**) - the universal precursor of monoterpenes. Poulter and co-workers proposed the reaction mechanism, which comprises the ionisation of DMAPP (**27**), condensation of the isopentenyl cation with IPP (**26**), and then proton elimination (Scheme 1.4).[39]



Scheme 1.4: *Formation of geranyl pyrophosphate* (**2**) *through condensation of IPP and DMAPP.*

This reaction can be extended in the same manner with specific isoprenyl pyrophosphate synthases to produce further linear precursors of terpenes, as shown in Scheme 1.6. For example, the precursor of sesquiterpenes is farnesyl pyrophosphate (FPP) (**11**), and the precursor of diterpenes is geranyl geranyl pyrophosphate (GGPP) (**46**). There are even longer terpene precursors, such as squalene made from two molecules of FPP, which is the precursor of triterpenes, and phytoene, which is the precursor of tetraterpenes. Note that all double bonds in these linear precursor have specifically *E*-stereochemistry.[40]



Scheme 1.6: *Biosynthesis of the linear precursor of terpenes.*

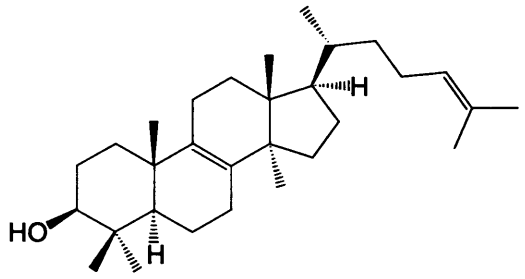
1.6 Introduction to terpene synthases

1.6.1 General introduction

Terpene cyclases are enzymes that chaperone the conversion of a universal polyisoprenoid precursor to one or more, complex cyclic terpene products. Terpene synthases are divalent metal cation dependent enzymes and have often two specific binding motifs for the metal ions. These metal ions are believed to coordinate to the pyrophosphate group and trigger what is proposed to be the first step in most cyclisation reactions - the loss of the pyrophosphate group.[41] A series of cyclizations, eliminations, methyl and hydride shifts and other rearrangements then occur to

produce the final products. The divalent metals are usually Mg^{2+} or Mn^{2+}, with Mg^{2+} being used most often.[42, 43] The levels of enzyme activities with different metals are variable and enzyme dependent. For example, activity of (*E*)-β-farnesene synthase is much higher in the presence of Mn^{2+} than Mg^{2+},[44] however, humulene synthase is less effective in the presence of Mn^{2+}, and even can be inhibitory when the concentration of Mn^{2+} is higher than 3 mM.[45]

Terpene synthases exhibit impressive accuracy in terms of stereo- and regio-specificity. A number of carbon-carbon bonds are formed during the catalytic reaction, and when this kind of reaction occurs around a prochiral centre, the terpene synthase displays very good ability to discriminate bond formation on one or another side of the prochiral centre.[46] A good example in nature is that of lanosterol (**22**) (Figure 1.9), which has seven chiral centres. In theory, it has 128 (2^7) possible steroisomers. However, in the biosynthesis of lanosterol only one steroisomer is produced.[47]



22

Figure 1.9: *Structure of lanosterol* (**22**).

Terpene synthases, especially the synthases from bacterial, fungal and plant sources, share little sequence similarity typically below 25-35%.[48] For example, the aristolochene synthase (AS) from *Penicillium roqueforti* and epi-aristolochene synthase (TEAS) from *nicotiana tabacum* only share

16% sequence similarity (Figure 1.10).[49] However, there is an interesting observation to be seen from all the crystal structures of sesquiterpene synthase up to date - they share a common fold, an α-barrel usually containing ten to twelve α-helices arranged in anti-parallel fashion. The active site is always nested deep within the α-barrel, and the contour of the active site varies between the different cyclases. This forms a template that stabilises the unique conformation of the substrate required to form the product.[50] Usually, the active site is surrounded by about 5 helices, which are connected by loops.[51] The loops are relatively short at the bottom of the active site and relatively long at the top, which makes the shape of the active site conical.[52]

```
AS      ------------------------------------------------------------
AT-AS   ------------------------------------------------------------
EAS     MASAAVANYEEEIVRPVADFSPSLWGDQFLSFSIDNQVAEKYAKEIEALKEQTRNMLLATG   61

AS      --MARSRERISSLAQP--FVHLENPINSPLVKETIRPRNDTTITPPPTQWSYLCHPRVKEV   57
AT-AS   -------------------MKKPNGTNG----------ASSSLEPPPSTFQPLCHPLVEEV   32
EAS     MKLADTLNLIDTIERLGISYHFEKEIDDILDQIYNQNSNCNDLCTSALQFRLLRQHGFNIS   122

AS      QDEVDGYFLENWKFPS------------------------------------------------   76
AT-AS   SKEVDGYFLQHWNFPN------------------------------------------------   51
EAS     PEIFSKFQDENGKFKESLASDVLGLLNLYEASHVRTHADDILEDALAFSTIHLESAAPHLK   183

AS      -------FKAVRTFLDAKFSEVTCLYFPLALDDR----IHFACRLLTVLFLIDDVLEHMSF   123
AT-AS   -------EKARKKFVAAGFSRVTCLYFPKALDDR----IHFACRLLTVLFLIDDLLEYMSF   98
EAS     SPLREQVTHALEQCLHKGVPRVETRFFISSIYDKEQSKNNVLLRFAKLDFNLLQMLHKQEL   244

AS      ADGEAYNNPLIPIS-------------------------RGDVLPDRTKPEEFILYDLWE   158
AT-AS   EEGSAYNEKLIPIS-------------------------RGDVLPDRSIPVEYIIYDLWE   133
EAS     AQVSRWWKDLDFVTTLPYARDRVVECYFWALGVYFEPQYSQARVMLVKTISMISIVDDTFD   305

AS      S-------------MRAHDAELANEVLEPTFVFMRAQTDRARLSIHELG---------HYL   200
AT-AS   S-------------MRAHDREMADEILEPVFLFMRAQTDRTRARPMGLG---------GYL   175
EAS     AYGTVKELEAYTDAIQRWDINEIDRLPDYMKISYKAILDLYKDYEKELSSAGRSHIVCHAI   366

AS      EYREKDVG-----------------KALLSALMRFSMGLRLSADELQDMKALEANCAKQLS   241
AT-AS   EYRERDVG-----------------KELLAALMRFSMGLKLSPSELQRVREIDANCSKHLS   216
EAS     ERMKEVVRNYNVESTWFIEGYTPPVSEYLSNALATTTYYYLATTSYLGMKSATEQDFEWLS   427

AS      ------VVNDIYSYDKEEEASRTGHKEGAFLCSAVKVLAEESKLGIPATKRVLWSMTR-EW   296
AT-AS   ------VVNDIYSYEKELYTSKTAHSEGGILCTSVQILAQEADVTAEAAKRVLFVMCR-EW   216
EAS     KNPKILEASVIICRVIDDTATYEVEKSRGQIATGIECCMRDYGISTKEAMAKFQNMAETAW   488

AS      ETVHDEIVAEKIASPDGCSEAA-------KAYMKGLEYQMSGNEQWSKTTRRYN------   342
AT-AS   ELRHQLLVAR-LSAEGLETPGL-------AAYVEGLEYQMSGNELWSQTTLRYSVVVD--   320
EAS     KDINEGLLRPTPVSTEFLTPILNLARIVEVTYIHNLDGYTHPEKVLKPHIINLLVDSIKI   548
```

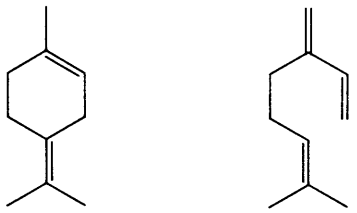
Figure 1.10: *Sequence alignment of AS from P. roqueforti and A. terreus and TEAS from N. tabacum. Identical residues are highlighted.*

There are several other interesting facts regarding the active sites of terpene synthases. Usually,the active site cleft is lined with mainly hydrophobic residues that can help to sequester the substrate from the solvent.[53, 54] This formation also stabilizes the unique conformation of the substrate needed to make the required product. During the formation of terpenoids in the enzyme's active site, it is proposed that the various intermediates, such as carbocations, are stabilized by specific amino acid residues in the vicinity of reacting parts of the substrate and intermediates.[55] These direct the cyclisation of the substrate towards the specific product required and prevent alternative cyclisations that otherwise would be of very similar energy. For example, aromatic amino acid residues can be found quite often in the active site. These aromatic residues can be very useful in electrostatic stabilization of carbocation intermediate in the pathway (see section 1.6.4 for more details).[56-59]

Compared to the insignificant sequence homology across the different kingdoms of life, the plant synthases show great similarity among themselves. For example, the δ-selinene synthase and γ-humulene synthase both from the gymnosperm *abies grandis* show 83% sequence similarity.[60] And even different groups of terpene synthases from plants have high degree of sequence homology: casbene synthase (diterpene cyclase) has 65% of sequence similarity with 5-epi-aristolochene synthase (sesquiterpene cyclase) and 53% of sequence similarity with limonene synthase (monoterpene cyclase).[61]

Substrate specificity varies among plant terpene synthases. Geranyl pyrophosphate (GPP) is the exclusive substrate accepted by monoterpene synthases, just as the geranylgeranyl pyrophosphate

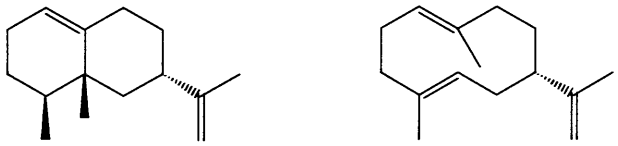
(GGPP) is the major substrate for diterpene synthase. However, this rule is not suitable for triterpene synthases and sesquiterpene synthases. For example, sesquiterpene (*E*)-β-farnesene synthase can use the monoterpene linear precursor GPP to synthesis monoterpenes limonene (**6**), terpinolene (**47**) and myrcene (**48**).[62]



47 **48**

Figure 1.11: *Structure of terpinolene* (**47**) *and myrcene* (**48**).

Some terpene synthases produce only one major product, for instance, δ-cadinene synthase makes δ-cadinene (**15**) as its sole product.[63] However, some other terpene synthases produce more than one product, aristolochene synthase from *P. roqueforti* produces three products for example. In addition to the major product aristolochene (**16**), there are two side products, valencene (**49**) and germacrene A (**50**).[64] γ-Humulene synthase from Grand Fir produces fifty-two different products.[60]



49 **50**

Figure 1.12: *Structure of valencene* (**49**) *and germacrene A* (**50**).

According to the tertiary structure and the general reaction mechanism, terpene synthases can be divided into two groups - class I terpene synthases and class II terpene synthases. Class I terpene synthases are more common and are predominantly helical.[65] Several residues conserved amongst terpene cyclases have been identified by sequence alignment, the most prominent of which are the amino acids of the metal binding motif, the specific DDXX(D/E) metal binding motif that is at the entrance of the active site, and another metal binding motif, (D/N)DXX(S/T)XXXE, on the opposite site.[41, 66] The metal ions attached to the metal-binding motifs coordinate the pyrophosphate group and trigger the first step in the cyclisation cascade, which is the loss of the pyrophosphate group from the substrate. Class I terpene synthases have a conical active site that contains about five helices.[51] However, class II terpene synthases initiate the whole reaction from protonation of a C-C double bond or the corresponding epoxide and contain a DXDD metal binding motif.[67] The squalene-hopene synthase from *alicyclobacillus* belongs to this class. It consists of a major and a minor domain, comprising six α-helices each, in regular barrel conformation.[51] Some terpene synthases have both the class I and class II characters. For example, the C-terminal catalytic domain of 5-epi-aristolochene synthase belongs to the class I terpenoid fold which consists of 281 residues, whilst the 240-residue N-terminal domain belongs to class II terpenoid fold but has unknown function.[51, 68] Another interesting example is an enzyme that is encoded by the SCO6073 gene in *Streptomyces coelicolor* A3(2), which can catalyses the cyclization of FPP to geosmin. Its C-terminal and N-terminal domains each have independently functioning active site. It has been demonstrated that the N-terminal protein catalyse the Mg^{2+}-dependent cyclization of FPP to germacradienol and germacrene D, and the C-terminal domain of SCO6073 converts germacradienol produced by N-terminal domain to geosmin.[69]

1.6.2 Crystal structures of terpene synthases

Recently, the 3D structures of the several terpene synthases have been solved, and half of these are sesquiterpene synthases. They are aristolochene synthase from *Penicillium roqueforti*[49] and *Aspergillus terreus*,[50] 5-epi-aristolochene synthase from *Nicotiana tabacum*,[68] bornyl diphosphate synthase from *Salvia officinalis*,[70] farnesyl pyrophosphate synthase from *Staphylococcus aureus*,[71] recombinant human squalene synthase,[23] pentalenene synthase from *Streptomyces* UC5319,[52] trichodiene synthase from *Fusarium sporotrichioides*[72] and limonene synthase from *Mentha spicata* (Figure 1.13).[73]

The 2.5-Å resolution crystal structure of aristolochene synthase (AS) from *Penicillium roqueforti* was the first of a fungal terpenoid cyclase. Until now, only the apo-enzyme has been crystallised. It shows that the enzyme has 11 α-helices in its structure, 6 of which form the hydrophobic active site cavity. The cone shape active site has been measured as 15 Å wide and 20 Å deep.[49] Aristolochene synthase from *Aspergillus terreus* has also been crystallized recently. The 2.2 Å resolution X-ray crystal structure of aristolochene synthase reveals a tetrameric quaternary structure. There are a total of 13 α-helices, 6 of which form the 20 Å active site cleft. At the same time, the 2.15 Å resolution crystal structure of the complex with $Mg^{2+}{}_3$-PP_i indicates that ligand binding only occurs in tetramer subunit D.[50] More details of aristolochene synthase will be discussed in the next section (1.7).

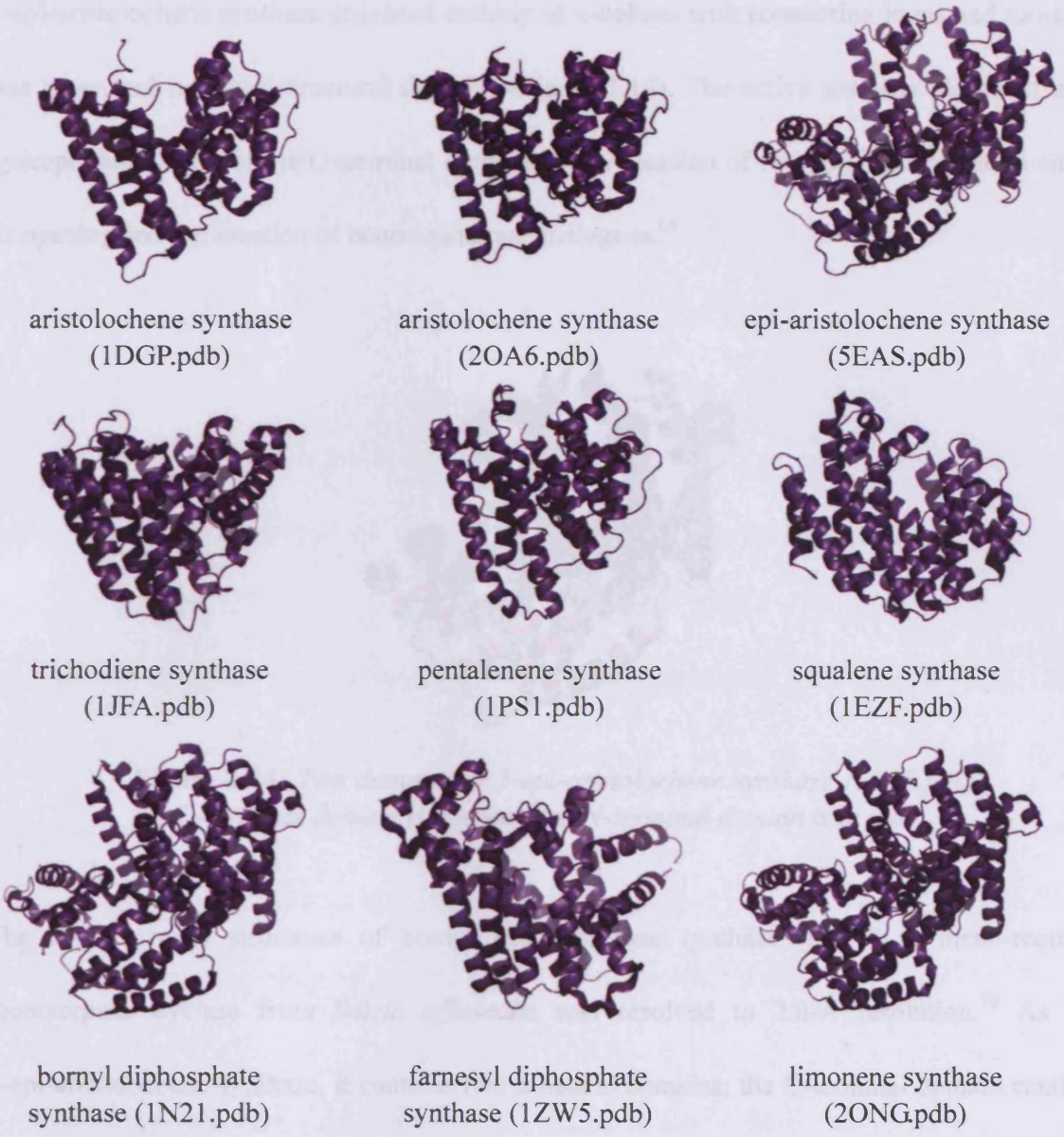


Figure 1.13: *Ribbon diagram representations of the crystal structures of various terpene synthases.*

The crystal structure of 5-epi-aristolochene synthase (EPS, from *Nicotiana tabacum*) alone as well as the crystal structure of 5-epi-aristolochene synthase complexed with two substrate analogues (farnesyl hydroxyphosphonate and trifluoro-farnesyl pyrophosphate) and two Mg^{2+} in the active site was analysed in 1997 by Starks *et al.* at 2.2 to 2.8 Å resolution.[68] It showed that

5-epi-aristolochene synthase consisted entirely of α-helices with connecting loops and turns, and was organized into two structural domains (Figure 1.14). The active site was identified in the hydrophobic pocket of the C-terminal domain by the location of two Mg^{2+} coordination sites at its opening and the location of bound substrate analogues.[68]

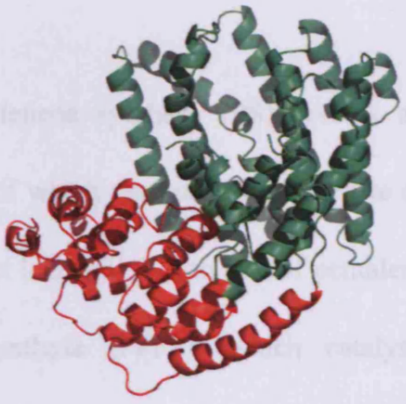


Figure 1.14: *Two domains of 5-epi-aristolochene synthase (1EAS.pdb); the C-terminal domain is in green and N-terminal domain is in red.*

The X-ray crystal structures of bornyl pyrophosphate synthase (BPPS), a metal-requiring monoterpene cyclase from *Salvia officinalis* was resolved to 2.0-Å resolution.[70] As with 5-epi-aristolochene synthase, it contains two α-helical domains; the C-terminal domain catalyzes the cyclization of geranyl pyrophosphate, orienting and stabilizing multiple reactive carbocation intermediates; however the N-terminal α-barrel domain has no established catalytic function. The C-terminal domain of BPPS contains 12 α-helices, 6 of which largely define a hydrophobic active site cleft. The crystal structure of the enzyme-ligand complexes with pyrophosphate (PP_i) and three Mg^{2+} ions has also been solved.[70]

The crystal structures of trichodiene synthase in both unliganded and complexed form were

obtained by Rynkiewicz *et al* in 2001.[72] The native trichodiene synthase structure is formed by 17 α-helices, 6 of which define a conical and hydrophobic active site cleft. The complexed crystal structure is similar to BPPS, which contains three bound Mg^{2+} ions and one molecule of inorganic pyrophosphate. The three Mg^{2+} ions are located in two metal-binding regions in the active site.[72]

The X-ray structure of pentalenene synthase (PS) reveals a tertiary structure defined by aggregation of 11 α-helices, 5 of which surround an active site cavity approximately 15 Å deep and 9 Å wide. A similar, but not identical fold to that of pentalenene synthase is also discovered in farnesyl pyrophosphate synthase (FPPS), which catalyses the synthesis of farnesyl pyrophosphate from IPP and DMAPP despite only 15% sequence identity between the two enzymes. It is found that the bottom of this cavity is hydrophobic and contains quite a few aromatic and aliphatic residues that confer a shape to the cavity that serves as a template for the binding of FPP in the correct conformation for cyclization to the product.[52]

Another enzyme that does not belong to sesquiterpene synthases is human squalene synthase (SQS). Squalene synthase catalyses the reductive dimerization of two molecules of FPP in a two-step reaction to form squalene, which is a key cholesterol precursor. The structure of SQS is entirely α-helical, with the helices arranged in three layers. Squalene synthase has a very similar core structure to farnesyl pyrophosphate synthase, pentalenene synthase and 5-epi-aristolochene synthase. The conserved feature in all the structures is an α-helical core surrounding a central active site cavity, of which one end is predominantly hydrophobic, and the other end is more

hydrophilic and contains polar and charged residues.[23]

1.7 Aristolochene synthase (AS) and aristolochene.

1.7.1 Aristolochene synthase

Aristolochene synthase (AS) is a Mg^{2+} dependent sesquiterpene cyclase enzyme that catalyses the formation of aristolochene from FPP. Aristolochene may be further transformed to produce a family of toxins that includes PR toxin.[74]

Aristolochene synthase was first isolated and purified from the fungus, *Penicillium roqueforti* in 1989[75] and the X-ray crystal structure of the apo-enzyme was solved in 2000.[49] Aristolochene synthase from *P. roqueforti* is a monomeric sesquiterpene cyclase consisting of 342 amino acid residues and has a molecular weight of 38000.[49] Like other sesquiterpene synthases, both Mg^{2+} and Mn^{2+} can be the cofactors of this reaction. However, Mn^{2+} also can act as inhibitor when it is in high concentration.[75] According to its tertiary structure and the catalytic reaction mechanism, *P. roqueforti* AS is a class I terpene synthase.[49] Even though no Mg^{2+} ions were identified in the crystal structure, one Sm^{3+} ion was cocrystallised and located near D115, which indicates that one metal binding motif is located between D115 and E119. The enzyme's structure consists of 11 α-helices, 6 of which form the hydrophobic active site cavity. All α-helices are connected by loops. The loops close to the entrance of the active site are on average 12 residues long. However, the loops which connect the other side of these helices are only 5 residues long, which makes the active site adopt a conical shape (Figure 1.15).[49] In order to infer the residues within the active site that may be involved in the catalytic mechanism, Caruthers *et al.* docked the substrate and

intermediates into the active site pocket manually and optimised the geometries of the complexes with energy minimization.[49] Because of the lack of metal ions in this manually docking, the pyrophosphate group was put in a particular position by fixing the substrate orientation manually. So that the pyrophosphate leaving group could interact with metal ions bound to the aspartate-rich sequence (as indicated by Sm^{3+} binding in the $SmCl_3$ derivative). According to the modelling result, it was identified that some of amino acid residues (Phe-178, Phe-112 and Trp-333) in the active site were involved in stabilising various cationic intermediates, and thus involved in the catalytic mechanism.[56-59]

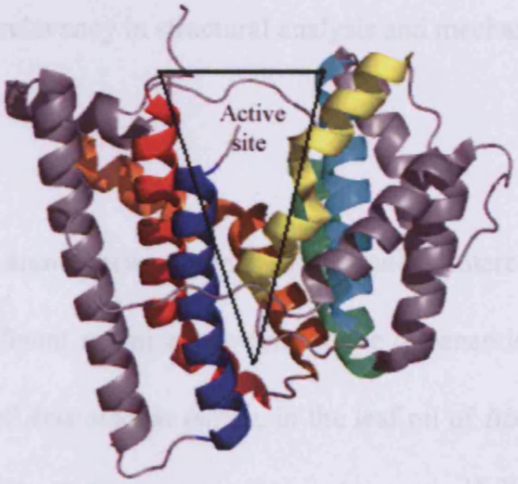


Figure 1.15: *Ribbon diagram representation of the crystal structure of aristolochene synthase from P. roqueforti (1DGP.pdb). The six α-helices with different colours (from gray) form the hydrophobic active site cavity.*

Aristolochene synthase from *Aspergillus terreus* also catalyses the cyclization of farnesyl diphosphate to form (+)-aristolochene as a single sesquiterpene product.[76] Its molecular weight is 36000, which is less than aristolochene synthase from *Penicillium roqueforti* (Mw = 38000). Apart from that, the amino acid sequence of the aristolochene synthase from *A. terreus* is 61% identical with the enzyme from *P. roqueforti*.[49] The recent work of Christianson *et al.*

demonstrated a tetrameric quaternary structure from the crystallized aristolochene synthase from *A. terreus*,[50] in which each subunit adopts the α-helical class I terpene synthase fold with the active site in a solvent-exposed conformation. However, from the 2.15 Å resolution crystal structure of its complex with inorganic pyrophosphate and three Mg^{2+} ions indicates the $Mg^{2+}{}_3$-PP_i cluster binds only to subunit D of the tetramer, which is stabilized in the "closed" conformation required for catalysis. There are two metal binding motifs in the enzyme - the DDLLE motif beginning with D90 and the NSE/DTE motif beginning with N219.[50] Because of the similar active site contours of aristolochene synthases from *A. terreus* and *P. roqueforti*, these two enzymes have directly relevancy in structural analysis and mechanistic implications.[49, 50, 56]

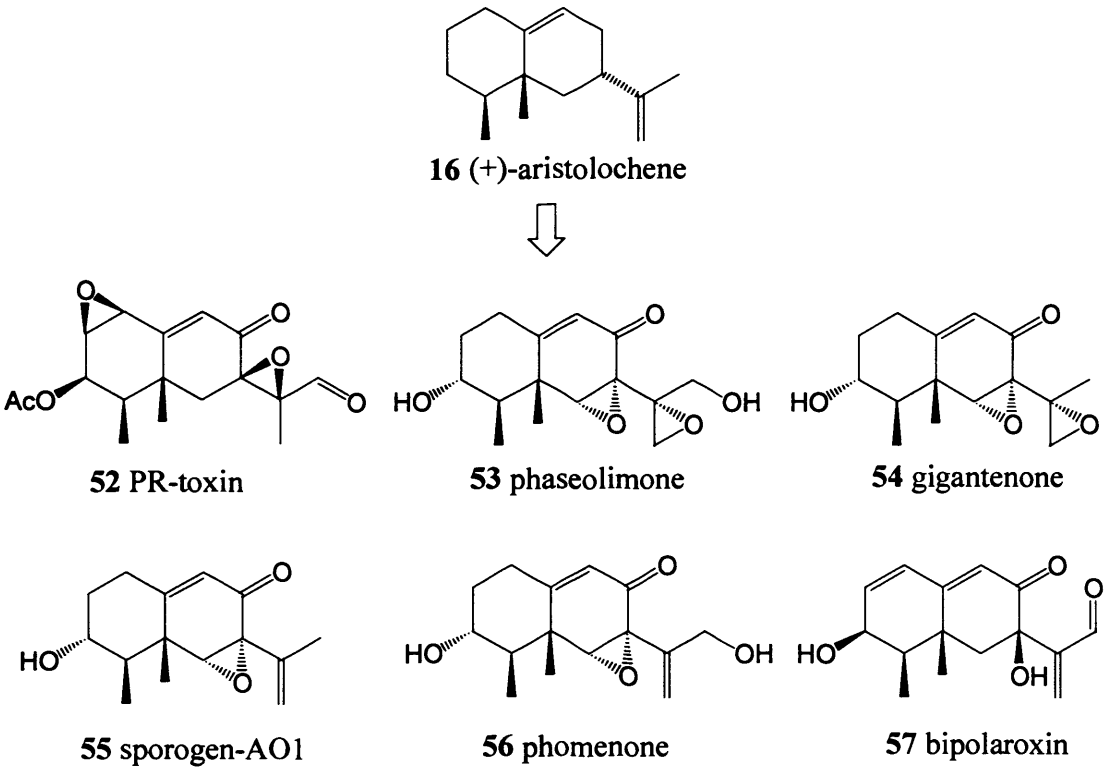
1.7.2 Aristolochene

Aristolochene is a bicyclic eremophilane-type sesquiterpene.[77] Interestingly, it has been isolated from several sources in different enantiomeric forms. The (–)-enantiomer has been found in the hexane extract of the root of *Aristolochia indica*, in the leaf oil of *Bixa orellana*, in the liverwort of *Dumortiera hirsute* and in defensive secretions of termites.[13, 78-80] Fungal sources such as *Penicillium roqueforti* and *Aspergillus terreus*, and the liverwort *Porella arborisvitae* give (+)-aristolochene.[41, 75, 81, 82] The diastereomeric sesquiterpene 5-epi-aristolochene (**51**) has been identified in tobacco (*Nicotiana tabacum*) and results from the cyclization of farnesyl pyrophosphate by 5-epi-aristolochene synthase by a similar mechanism to aristolochene synthase.[49]

16 51

Figure 1.16: *Structures of (+)-aristolochene* (**16**) *and its diastereomeric isomer 5- epi-aristolochene* (**51**).

(+)-Aristolochene formation is thought to be the first committed step in the biosynthesis of a large group of sesquiterpenoid fungal toxins, the most poisonous of which is the bis-epoxide PR-toxin **52** (Figure 1.17).[74]



52 PR-toxin **53** phaseolimone **54** gigantenone

55 sporogen-AO1 **56** phomenone **57** bipolaroxin

Figure 1.17: *Structures of fungal toxins thought to be derived from aristolochene (blue colour indicates the mother molecule - aristolochene).*

1.7.3 Description of the proposed catalytic mechanism of AS

The general cyclisation mechanism of sesquiterpene cyclases involves the ionization of FPP, followed by nucleophilic attack of one of the double bonds present in the FPP structure to yield a cyclic intermediate. The starting geometry of the substrate and its orientation within the active site is a critical determinant of the ultimate cyclisation product. The terpene cyclase active site plays a critical role as a template in binding the flexible polyisoprenoid substrate in the proper conformation for catalysis.[50] A cascade of reactions including hydride shifts, and Wagner-Meerwein rearrangements that relocate a positive charge over different atoms of the reaction intermediates will then take place. Final deprotonation or capture of an exogenous nucleophile such as water leads to product formation.[83]

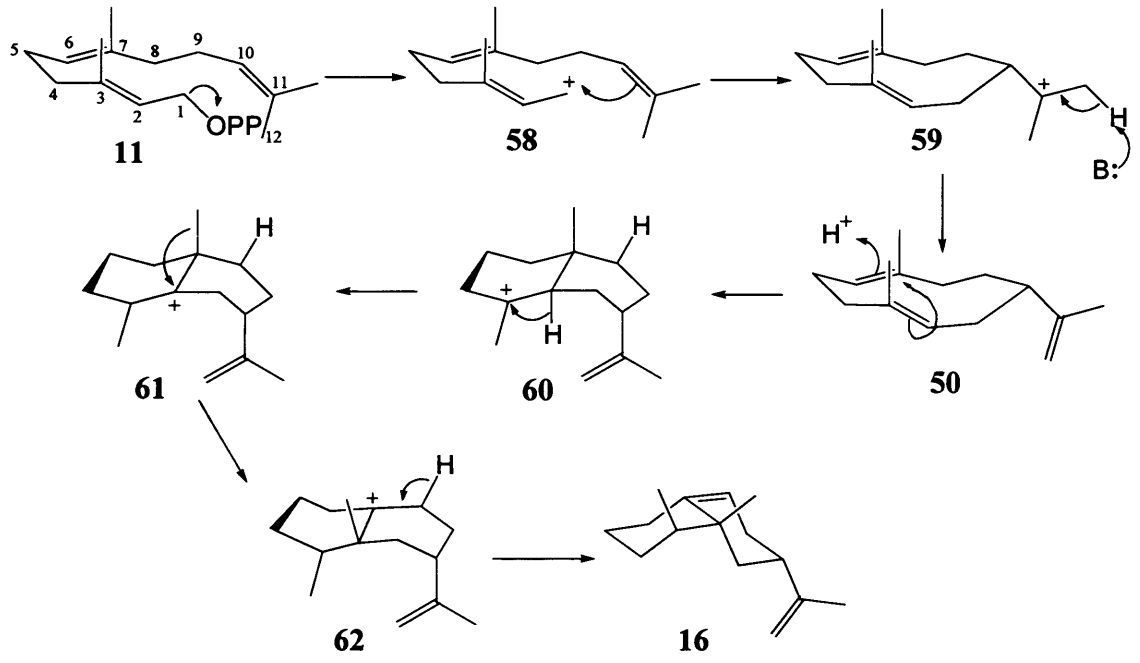
The most common mechanism for the initial ionization of the substrate in sesquiterpene cyclases is through the coordination of the diphosphate group to metal cations bound to the active site through specific residues, leaving behind a polyisoprenyl cation. In addition, the diphosphate moiety, that most likely remains bound throughout the cyclisation reaction, may play an important role in the stabilization of the positively charged intermediates.[52] However, it has been reported that other terpene cyclases achieve ionisation by the enzymatic protonation of a double bond of the substrate with an acidic residue or by epoxide opening.[51]

Wagner-Meerwein-like rearrangements are often thought to proceed through carbocations that would be considered less favourable.[83, 84] The amino acid residues in the vicinity of reacting parts of the substrate and intermediates stabilise intermediate species such as carbocations.[55] Many

aromatic residues can be found in the active site. The electrostatic component arises from interactions of the quadrupole moments of the aromatic rings, which are produced by the uneven distribution of charge. Greater electron density concentrates on the face of the ring, resulting in reduced electron density on the edge of the ring, giving rise to the quadrupole moment. Several geometries of interaction between aromatic rings have been proposed.[85, 86] Cation-π interactions involve the quadrupole moment of the aromatic ring, suggesting that aromatic residues within the active site of proteins may bind cationic substrates.[87] These aromatic residues are an ideal source for electrostatic stabilisation of carbocations in the reaction pathway.[47, 56-58, 87]

Based on mechanistic studies with labeled substrates and through the analysis of the reaction products obtained with mutant aristolochene synthase, a reaction mechanism was proposed, in which the initial cleavage of the alkyl diphosphate bond leads to farnesyl cation (**58**) prior to cyclization (Scheme 1.6).[64, 76, 77, 88] This cation is stabilised by residues F178 and F112.[57, 58] The PP_i leaving group is believed to be coordinated to metal ions bound to the highly conserved Mg^{2+} binding motifs, which are at the opposite side at the entrance of active site cavity. Attack by the C10-C11 double bond to produce germacryl cation (**59**) followed by proton loss from C12 has been proposed to lead to germacrene A (**50**).[89] Residue Y92 was initially suggested to act as an acid in the formation of the eudesmane cation (**60**).[59] However, after Felicetti & Cane removed the hydroxyl group, creating a Y92F mutant, this still produced aristolochene, but in lower quantity.[76] Alternatively, protonation of germacrene A has been proposed to be carried out by a water molecule trapped in the active site.[76] After the protonation of the C6-C7 double bond and a further cyclisation by intramolecular attack of the resulting carbocation by the C2-C3 π-bond, the

uncharged germacrene A is converted to the bicyclic eudesmane cation (**60**).[90, 49, 59, 64] The positive charge on the eudesmane cation is thought to be stabilised by the π-system of W334.[56] Successive 1,2 hydride shift and methyl migration followed by the deprotonation of H_{Si} on C8 results in the generation of the product (+)-aristolochene **16**.[89]



Scheme 1.6: *Proposed mechanism for AS catalysed cyclization of FPP.*

Site directed mutagenesis of key amino acid residues has generated a lot of useful evidence supporting the above mentioned stepwise reaction mechanism and identifying important amino acid residues. Residue F178 of AS from *P. roqueforti* was identified as a residue that is involved in the stabilisation of transition states preceding germacrene A and following eudesmane cation, because a mutant of AS, in which residue phenylalanine 178 (F178) was replaced by valine, produced significant amounts of α-farnesene (**63**) and β-farnesene (**64**) as well as α-selinene (**65**), β-selinene (**66**) and selina-4,11-diene (**67**).[57] The residue F178 is also postulated to be involved in

preventing erroneous deprotonation in the last step of synthesis of aristolochene, as the F178C mutant of AS produced increased proportions of valencene (**68**).[58]

When residue 112 was replaced by alanine, the presence of farnesenes **63** and **64** suggested that farnesyl cation accumulated during catalysis by F112A mutant of AS, leading to deprotonation from C4 and C15. Residue 112, therefore, most likely contributes significantly to the stabilisation of the transition state following farnesyl cation formation. Changing F112 to alanine, terminated the reaction at germacrene A (**50**), indicating that production of eudesmane cation can not be induced in the absence of the side chain of F112.[58]

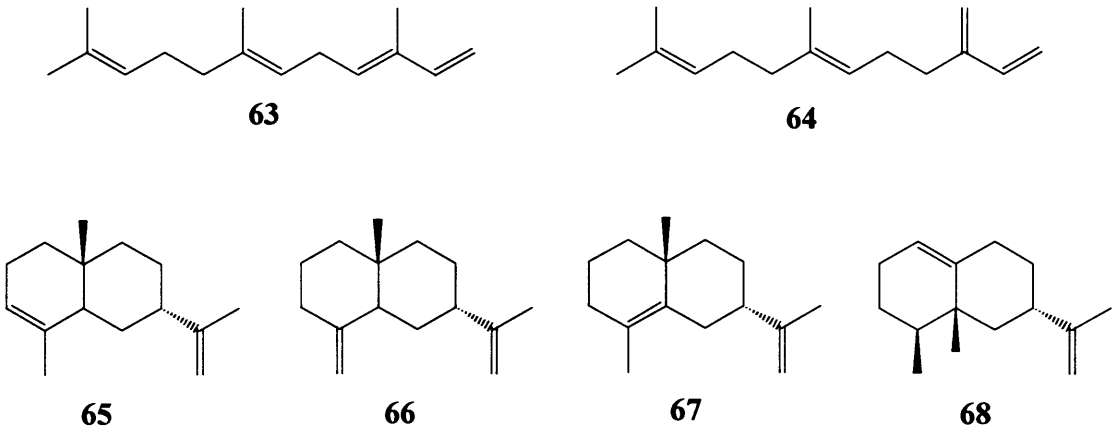


Figure 1.18: *Structures of (E,E)-α-farnesene* (**63**), *(E)-β-farnesene* (**64**), *α-selinene* (**65**), *β-selinene* (**66**), *selina-4,11-diene* (**67**), *valencene* (**68**).

The positive charge of the eudesmane cation (**60**) also seems to be stabilised by the π-system of W334, because only small amounts of aristolochene (**16**) were produced and the main reaction product was germacrene A (**50**) when this aromatic residue was replaced with an aliphatic amino acid.[56]

Even though there is not enough evidence to prove that residue Y92 acts as the proton donor in the protonation of germacrene A, its size and position (in the bottom of active site) have been shown to enforce a quasi-cyclic conformation on the substrate, thus helping to form of the 10-membered ring. When this amino acid was replaced by alanine, cysteine, valine and phenylalanine, it was shown that a decrease in the size of residue 92 produces an increase in the production of linear compounds.[59, 91] Perturbation of the active site by site directed mutagenesis of these residues has led to mutant AS enzymes that are considerably more promiscuous than the wild type enzyme. As mentioned above, the finding that the Y92F mutant of AS from *P. roqueforti* produced significant amounts of (+)-aristolochene, disfavours the possibility that the hydroxyl group of Y92 was involved in proton donation to the double bond of germacrene A as the active site acid. Felicetti *et al.* therefore proposed that a water molecule in the active site might act as the acid although there was no evidence from the crystal structure.[76]

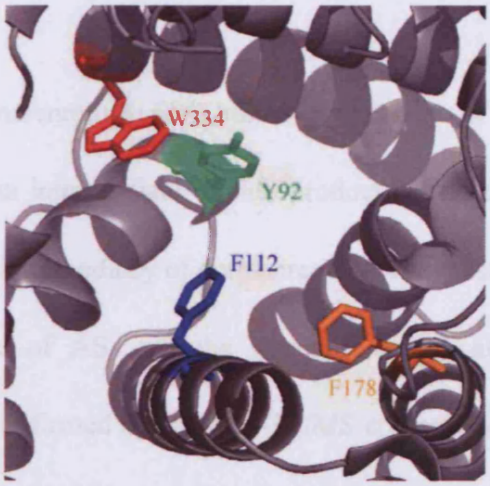


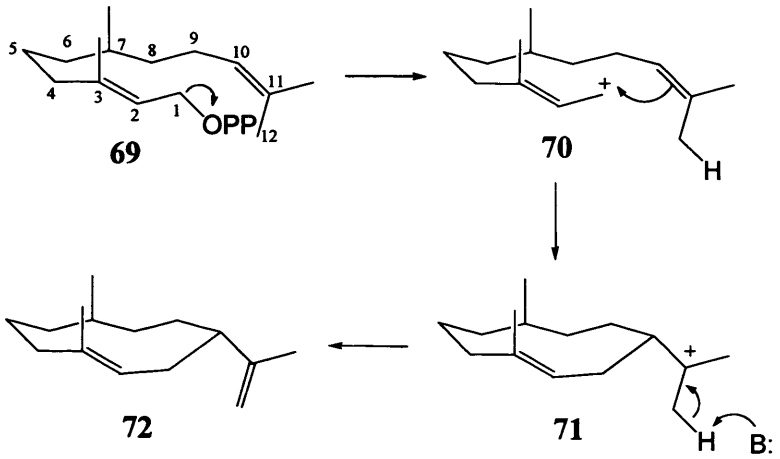
Figure 1.19: *Ribbon diagram representation of the crystal structure of aristolochene synthase from P. roqueforti (1DGP.pdb). Aromatic residues in the active site are shown.*

However, Shishova *et al.* modelled the product - aristolochene into the active site of *A. terreus* AS, and found the aristolochene was precisely and tightly packed into the active site cavity in the "closed" conformation, so it is unlikely that a water molecule could trapped in the enclosed active site to perform a general acid/base function. The authors therefore propose that the pyrophosphate moiety is the most likely candidate for the general acid/base in the active site.[50]

FPP analogues have also been employed to study the catalytic mechanism of AS. In order to determine which methyl group of FPP undergoes deprotonation in the generation of germacrene A and which hydrogen atom is lost in the formation of the C7-C8 double bond of aristolochene, Cane and co-workers made four ^{2}H labelled FPP analogues at position C12, C13 and C8 and incubated them with aristolochene synthase. After analysis of the products by ^{2}H NMR, it was concluded that the C12 methyl and H-8_{si} were deprotonated during the synthesis of aristolochene.[89]

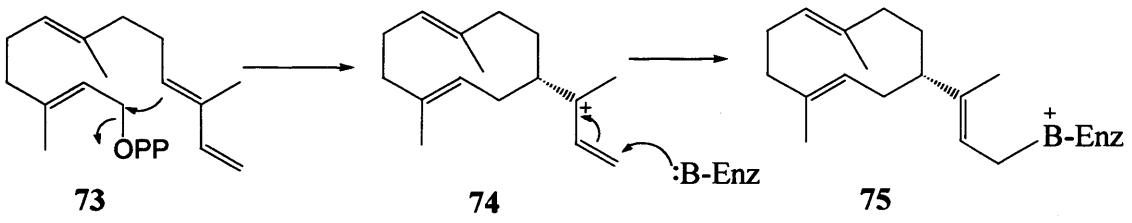
The observation of (–)-germacrene A as a minor by-product of the reaction indicates that (–)-germacrene A is either an intermediate or side product during the cyclisation.[64] Cane *et al.* found good evidence for the intermediacy of germacrene A. They tested the 6,7-dihydro-analogue of FPP (**69**) as a substrate of AS, and the structure of the abortive cyclisation product, dihydrogermacrene A, was confirmed by a direct GC/MS comparison with a synthetic sample of this compound.[90] According to the proposed catalytic mechanism of AS (Scheme 1.6), after the generation of germacrene A, this uncharged intermediate is then postulated to undergo protonation of the C6-C7 double bond and undergo a further cyclisation by intramolecular attack

of the resulting carbocation by the C2-C3 π-bond to form the bicyclic eudesmane cation (**60**). However this cyclisation would be prevented because of the lack of C6-C7 double bond in this 6,7-dihydro-analogue of FPP, and dihydro-germacrene A (**72**) was then generated (Scheme 1.7).[90]



Scheme 1.7: *Proposed sequence of reactions leading to 6,7-dihydrogermacrene A* (**72**) *from 6,7-dihydro FPP* (**69**) *catalysed by P. roqueforti AS.*

The vinyl analogue of FPP, 1,2-methylidenefarnesyl pyrophosphate, (VFPP) (**73**), was shown to act as a mechanism-based inhibitor to AS (Scheme 1.8). The initial ionisation and cyclization of the VFPP by aristolochene synthase possibly gives rise to the allylic cation species **74**. This cation could react with the active site base of a nearby nucleophilic amino acid side chain to form a covalent bond, which results in permanent inactivation of the enzyme.[92]



Scheme 1.8: *Proposed inactivation process of AS by 1,2-methylidenefarnesyl pyrophosphate* (**73**).

1.8 Approaches for studying the catalytic mechanism of sesquiterpene synthases

Sesquiterpenes are all formed from FPP alone yet they are a family of compounds comprising thousands of known members possessing more than 300 known carbon skeletal arrangements.[13] Understanding of how this one compound may be transformed by a family of structurally related enzymes into myriad derivatives would be a great step forward in modern enzymology. There are two main approaches to the study of this problem. One is site directed mutagenesis of key amino acid residues. Another approach is turnover of modified FPP analogues.

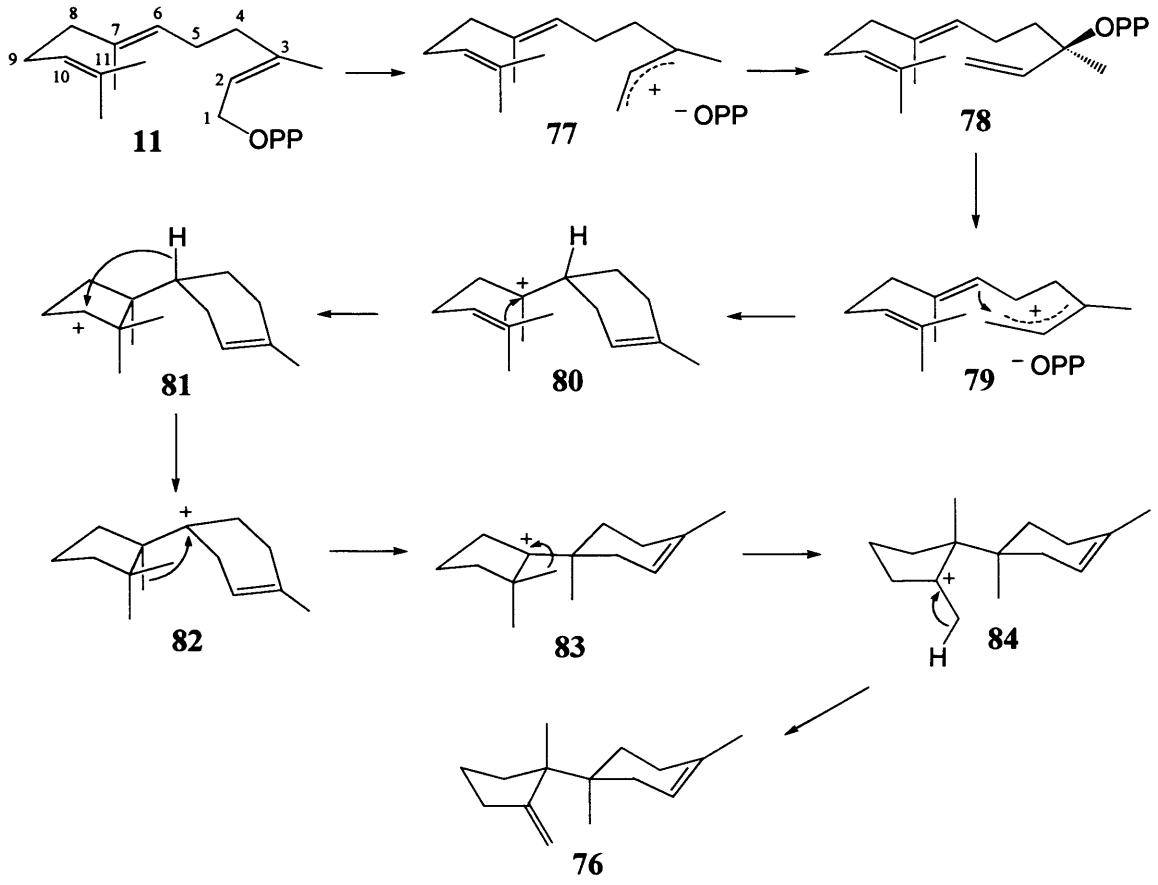
1.8.1 Site directed mutagensis

Site directed mutagenesis of a protein allows a residue to be replaced and the effects on function to be probed. A mutant sesquiterpene synthase may give a different distribution of products to the wild type enzyme. Structure activity relationships in sesquiterpene cyclases have been widely studied through site directed mutagenesis to identify residues in the active site which are involved in the catalytic mechanism.[46, 93]

Trichodiene synthase (TS) from *Fusarium sporotrichioides* catalyzes the cyclization of farnesyl diphosphate (**11**) to trichodiene (**76**) in the presence of Mg^{2+} (Scheme 1.9). It has a conserved aspartate rich domain, from residue 100 to 104 (DDSKD). In order to demonstrate the use the of aspartate residues in mediating substrate binding by chelation of the divalent metal ion, the three aspartate residues in the aspartate rich region were individually replaced by glutamate.[94-96] The D100E and D101E mutants of TS showed an increase in K_m and a reduction in k_{cat} compared to

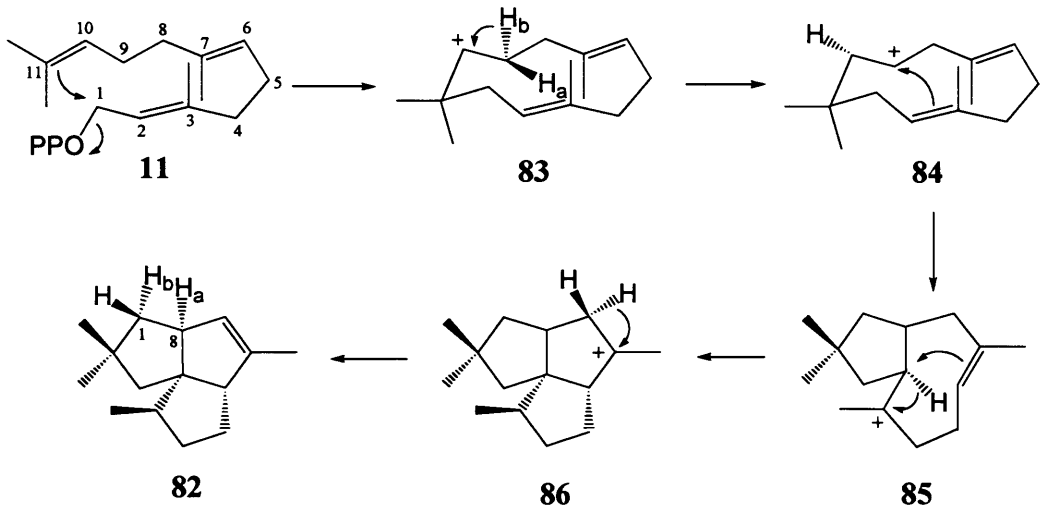
the wild type. At the same time, all three mutants produced anomalous sesquiterpene products in addition to trichodiene when incubated with farnesyl diphosphate. With regard to the arginine-rich region of TS, 302-306 (DRRYR), site-directed mutagenesis study also gave important information.[97] The R304K mutant showed an obvious increase in K_m, as well as a 200-fold reduction in k_{cat}; mutant Y305T exhibited a 80-fold increase in K_m and and 120 fold reduction in k_{cat}. Both of these two mutants converted FPP to a mixture of trichodiene and other sesquiterpene hydrocarbons.[97] The above evidence estabilished the importance of the aspartate-rich domain and arginine-rich domain in catalysis of the trichodiene synthase reaction.

Cane *et al.* also showed that two cysteine residues (C146 and C190) were important at the active site of TS by generation of C146A and C190A mutants. The CD spectra of each of the mutant proteins showed negligible differences compaired with that of native enzyme. However, the C190A mutant displayed significantly reduced activity, with a reduction in k_{cat}/K_m of 3000 compared to the wild-type TS, while the C146A mutant was essentially inactive. [98]



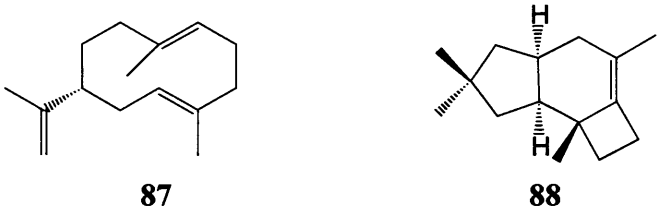
Scheme 1.9: *Proposed mechanism for TS catalysed cyclization of FPP.*

Another sesquiterpene synthase is pentalenene synthase (PS) from *Streptomyces* UC5319. This enzyme catalyzes the cyclization of farnesyl diphosphate (**11**) to the sesquiterpene hydrocarbon pentalenene (**83**).[53] In order to find out the functional active site base in the active site of pentalenene synthase that mediates the postulated series of deprotonations and reprotonations, incruding the final deprotonation that generates pentalenene (Scheme 1.10), the only basic residues in the active site cavity - histidine-309 and the possible Lewis base tryptophan (W308) were both tested by site directed mutagenesis.[53]



Scheme 1.10: *Proposed mechanism for PS catalysed cyclization of FPP.*

Mutants H309F, W308F and double mutant W308F/H309F all retained substantial pentalenene synthase activity. In addition, incubation of FPP with the W308F or W308F/H309F mutants of pentalenene synthase yielded pentalenene, accompanied by varying proportions of (+)-germacrene A. By contrast, single H309 mutants gave rise to both (+)-germacrene A (**87**) and protoilludene (**88**) in addition to pentalenene. Both anomalous cyclization products result from derailment of the normal cyclization cascade, presumably due to relaxed control over the conformation of the substrate FPP and derived intermediates in the active sites of H309 mutant. From this result, three possibilities also can be considered: the possible active site base could be enzyme-bound water(s), a carbonyl oxygen atom of the peptide backbone or the bound pyrophosphate ion.



87 **88**

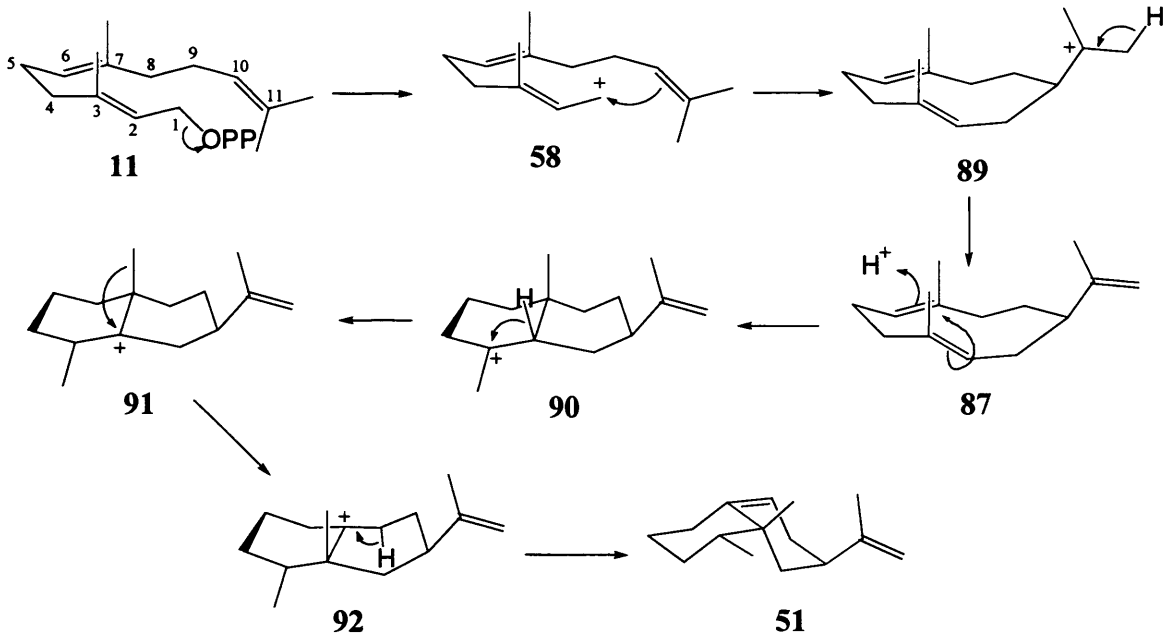
Figure 1.20: *Structures of (+)-germacrene A* (**87**) *and protoilludene* (**88**).

To explore the role of the aspartate-rich domain (^{80}DDLFD) of pentalenene synthase, each of the three aspartate residues was separately replaced by glutamate.[53] Both the D80E and D81E mutants of PS suffered a significant decrease in k_{cat} and a significant increase in K_m compared to wild type. By contrast, the D84E mutant displayed relative minor changes in the steady-state kinetic parameters (2.5-fold increase in measured k_{cat} offset by a 7-fold increase in K_m). Asparagine 219 of pentalenene synthase is part of the DXX(S/T)XXXE motif in the active site, and it is thought to be chelated to a Mg^{2+} ion. Consistent with the expected role of N219 in binding Mg^{2+}, the corresponding N219L mutant of pentalenene synthase is completely inactive despite the observation from its crystal structure that nominally isosteric amino acid substitution does not result in any significant conformational changes for neighboring residues in the enzyme active site.

Both theoretical and experimental studies have proved support for the importance of aromatic amino acid residues in stabilizing carbocations at enzyme active sites.[87] Among the many aromatic residues that can be seen within the active site of pentalenene synthase, F76 and F77 appear to be well placed and can interact with positive charge at C-1, C2 and C-3 of the farnesyl residue and derived intermediates. Replacement of either F76 or F77 by alanine led to a more than 10-fold reduction in catalytic activity. Mutant F77Y, in which the phenylalanine is replaced by a more electron-rich tyrosine residue, also produced pentalenene, with essentially no change in K_m compared to the value for the wild type but with k_{cat} reduced by a factor of 20, suggesting that the precise geometry of interaction of the aromatic residues with the intermediate carbocations is

critical to enhancement of the rate of the reaction.[53]

Based upon the three-dimensional structure of tobacco 5-epi-aristolochene synthase (TEAS), a detailed mechanism has been proposed for TEAS catalysis (Scheme 1.12).[13, 68] (+)-Germacrene A (**87**) is proposed as an intermediate during the cyclization. In order to demonstrate the formation of this postulated intermediate, site directed mutagenesis was undertaken to alter residue Y520 to Phe.[99] This residue is proposed to be responsible for the activation of germacrene A (**87**) to a eudesmane cation (**89**) with proton donation.[68] The identity of the hexane-extractable material produced from FPP by TEAS-Y520F was elucidated through GC-MS analysis. The results indicate the introduction of the Y520F mutation into the TEAS active site results in formation of germacrene A as the final product. This observation supports the intermediacy of germacrene A in TEAS catalysis and strongly supports the proposal that the hydroxyl group of Y520 is indeed the proton donor initiating transformation of germacrene A to the eudesmane cation.



Scheme 1.11: *Proposed mechanism for TEAS catalysed cyclization of FPP.*

The role that terpene synthases play as templates for their substrates and intermediates can be probed by the site-directed mutagenesis within the active site not only by insertions and/or deletions, but also by swapping entire domains between proteins to create chimeric cyclases that will generate a mixture of products.[47, 55, 100] To identify not just the role played by a single residue, but complete functional domains of sesquiterpene cyclases, a domain-swapping strategy has been carried out. For example, swapping exons between both enzymes identified functional domains of epi-aristolochene synthase from *Nicotiana tabacum* and vetispiradiene synthase from *Hyoscyamus muticus*. The resulting chimeric protein produced both epi-aristolochene and vetispiradiene.[101, 102] Above example indicated that domain swapping between structurally related terpene synthases is a useful tool to express functional terpene synthases without interrupting enzyme integrity. Combining two functional domains from different proteins resulted in a novel enzyme able to synthesise reaction products of both parent enzymes.

1.8.2 Substrate analogues

Another powerful technique for studying the catalytic mechanism of sesquiterpene synthases is the use of substrate analogues. The preparation of a series of FPP analogues and the analysis of how they bind to and are transformed by the chosen enzymes may lead to a deeper understanding of the processes at work. In other words, modification of FPP at appropriate positions with functional groups capable of stabilising or destabilising cations in their vicinity will perturb the mechanism in a predictable and testable fashion. Several studies on aristolochene synthase with different FPP analogues have shown insight into its mechanism (See section 1.7.3). The method

of anomalous substrates is also widely used in other sesquiterpene synthase studies.

With regard to the mechanism of TS catalysed cyclization of FPP to trichodiene, Cane *et al.* proposed that the substrate FPP underwent initial syn isomerization to the tertiary allylic isomer, nerolidyl diphosphate (NPP) (**78**) (See Scheme 1.10).[13, 103] In order to support above postulation, two enantiomeric analogues of FPP, (7*S*)-6,7-dihydrofarnesyl diphosphate (**93**) and (7*R*)-6,7-dihydrofarnesyl diphosphate (**94**), were tested as anomalous substrates of trichodiene synthase.[103, 104]

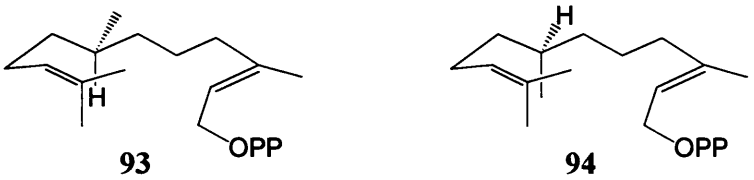
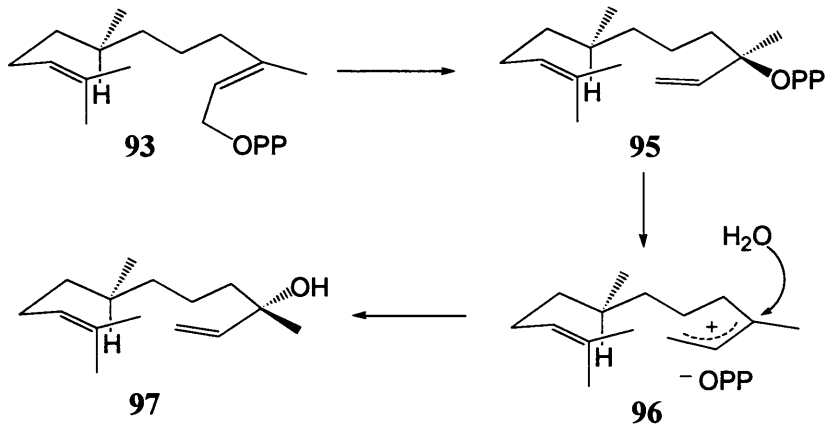


Figure 1.21: *Structures of (7S)-6,7-dihydrofarnesyl diphosphate* (**93**) *and (7R)-6,7-dihydrofarnesyl diphosphate* (**94**).

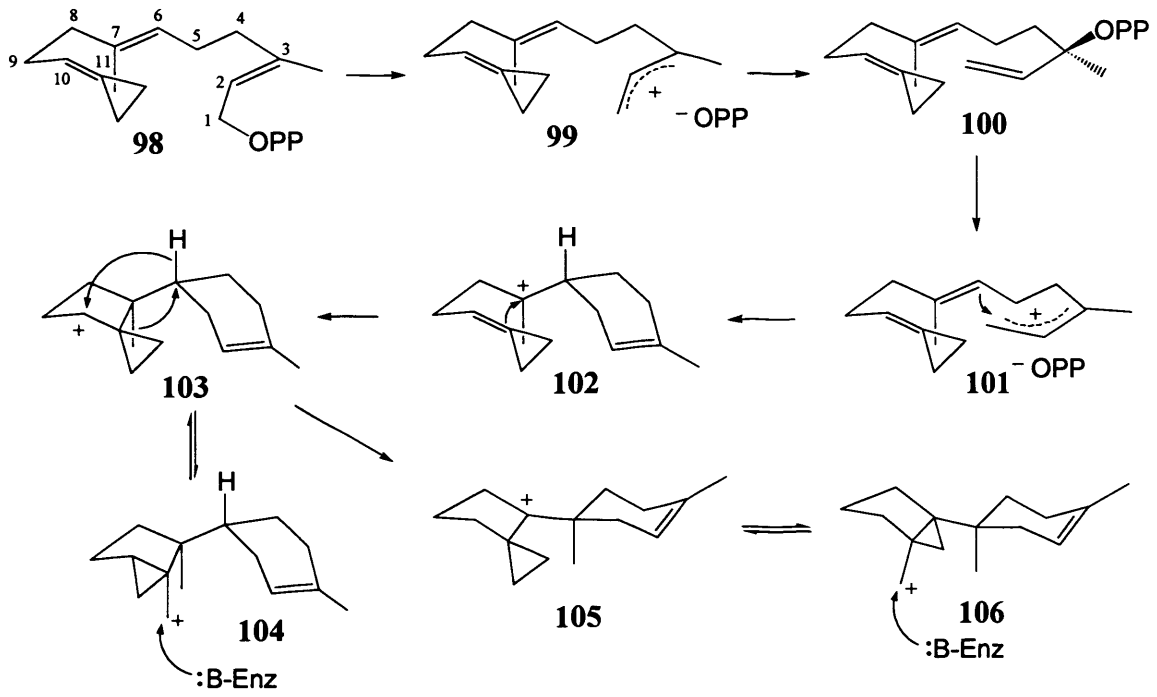
In principle, the enzyme should be able to isomerize each of these substrate analogues to the corresponding tertiary allylic diphosphate, 6,7-dihydronerolidyl diphosphate (**95**), but further cyclization would be blocked due to the absence of 6,7-double bond. Unfortunately, in neither case was any of the tertiary allylic pryophosphate **95** directly observed. However, the observed formation of 6,7-dihydronerolidol (**97**) can be accounted for by backside attack of water on an allylic cation-pyrophosphate anion pair generated by syn allylic rearrangement of dihydrofarnesyl diphosphate and ionization of the resulting 6,7-dihydronerolidyl diphosphate (**95**). So this analogue study provided further support for the proposed isomerization of FPP to (3R)-nerolidyl

diphosphate (**78**) in the enzymatic formation of trichodiene.[104]



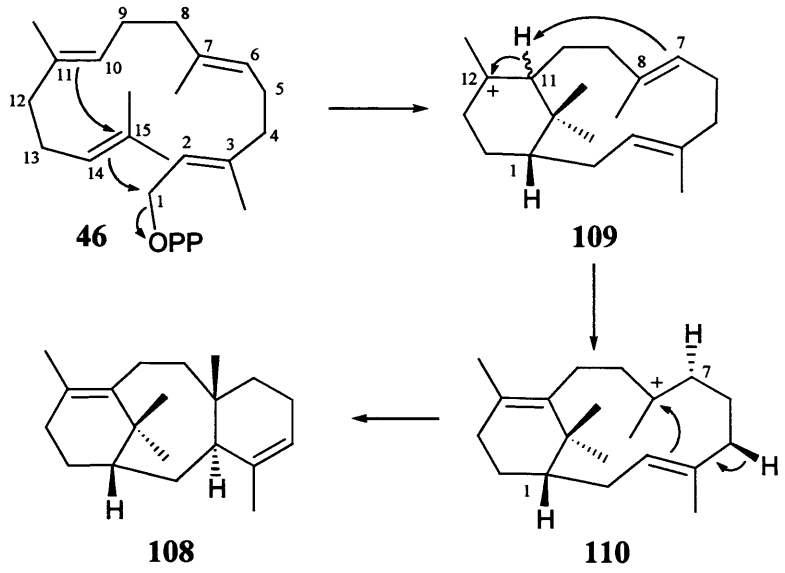
Scheme 1.12: *Proposed sequence of reactions leading to 6,7-dihydronerolidol* (**97**) *from 6,7-dihydrofarnesyl diphosphate* (**93**) *catalysed by recombinant TS.*

FPP analogues also can act as inhibitors of sesquiterpene cyclase enzymes. This also can help to probe the factors affecting substrate recognition and indicate the catalytic mechanism of enzymes. For example, Cane *et al.* designed the 10-cyclopropylidene farnesyl diphosphate (**98**) as a mechanism-based inhibitor of TS.[105] As an analogue of FPP, diphosphate **98** was expected to bind well to the active site and undergo the usual ionisation, isomerization and cyclization to yield the cation **102** (Scheme 1.13). Further cyclization of **102** would yield the cyclopropylcarbinyl intermediate **103**. This type of cation is known to undergo extensive rearrangement through a cyclobutyl cation intermediate. Alternatively, cation **103** could undergo the normal hydride shift and methyl migration to give species **105** that would be subject to a similar rearrangement. These rearrangements would put the positive charge to regions of the active site which do not normally encounter carbocations, and the derailment intermediates are intercepted by nearby nucleophilic amino acid residues, leading to alkylation of the enzyme.



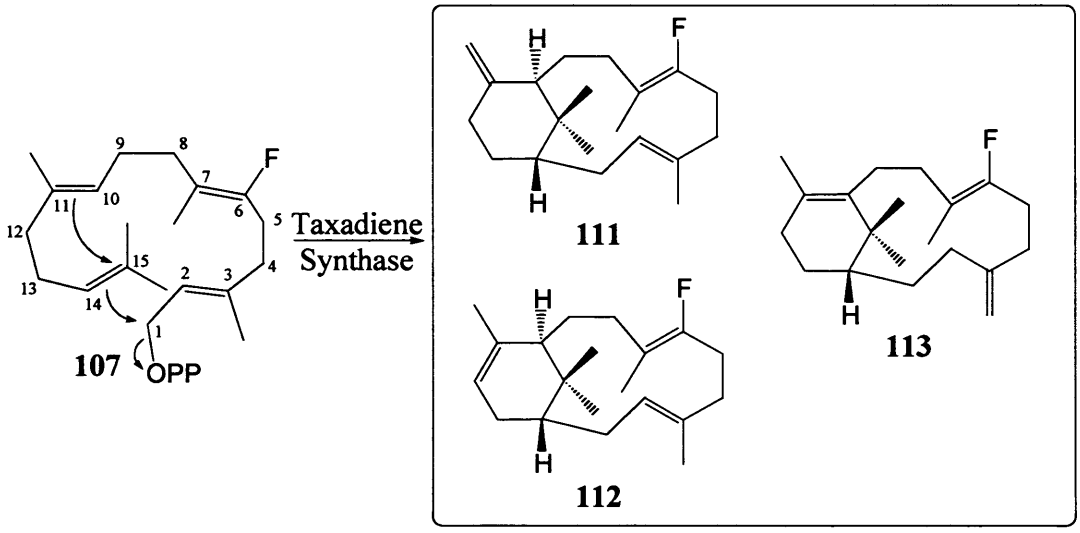
Scheme 1.13: *Proposed inactivation process of TS by 10-cyclopropylidenefarnesyl diphosphate* (**98**).

The inductive electron-withdrawing effect of the fluoro-substituent on 6-fluorogeranylgeranyl diphosphate (GGPP) (**107**) was also demonstrated in the study of taxadiene synthase, which converts GGPP (**46**) to taxadiene (**108**). A mechanism involving a transannular proton transfer that converts a verticillen-12-yl carbocation (**109**) to verticillen-8-yl carbocation (**110**) (Scheme 1.14).[106]



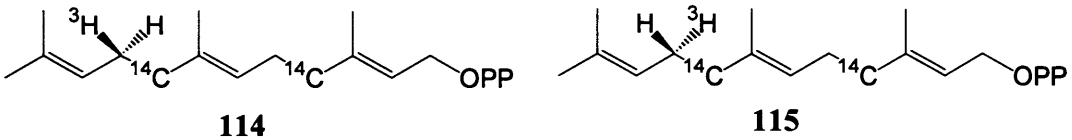
Scheme 1.14: *Proposed mechanism for taxadiene synthase catalysed cyclization of GGPP.*

The fluoro-substituent was expected to prevent this specific proton transfer and thereby derail the reaction at the bicyclic stage. Three fluorinated verticillene products (**111**, **112** and **113**) were found and analysed by GC-MS and NMR (Scheme 1.15). The products provided support for the contention that the reaction proceeds via an H-11 to H-7 proton transfer.[107]



Scheme 1.15: *Products from incubation of 6-fluoroGGPP and recombinant taxadiene synthase.*

Isotopically labeled FPP analogues have been useful in determining the regio and stereochemical selectivity during the FPP cyclization to sesquiterpenes. For example, in order to explore the stereochemistry of the deprotonation step in cyclization of FPP (**11**) to pentalenene (**82**) by pentalenene synthase from *Streptomyces* UC5319. Both (9*R*)-[9-^{3}H,4,8-^{14}C]FPP (**114**) and (9*S*)-[9-^{3}H,4,8-^{14}C]FPP (**115**) were incubated with PS separately. It was thereby established that H-9*re* (H_a) of FPP become H-8 of pentalenene while H-9*si* (H_b) of the substrate undergoes intramolecular transfer to C-1 of pentalenene (See Scheme 1.10).[108]



114 **115**

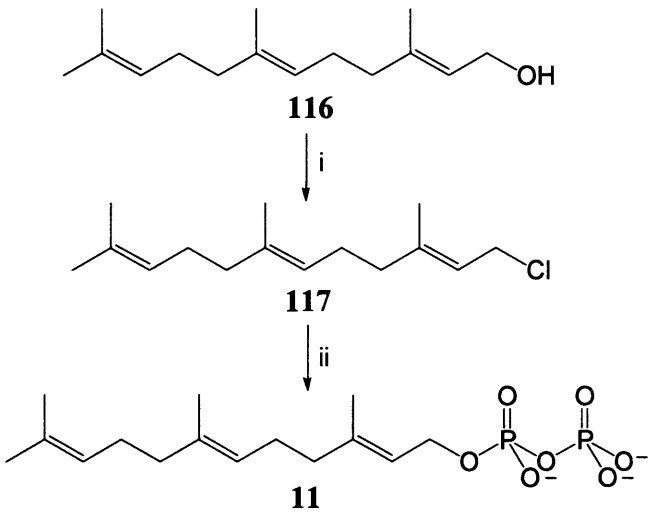
Figure 1.22: *Structures of (9R)-[9-^{3}H,4,8-^{14}C]FPP* (**114**) *and (9S)-[9-^{3}H,4,8-^{14}C]FPP* (**115**).

1.9 Synthetic preparation of FPP analogues

From the above discussion, it is clear that FPP analogues have been widely used for the mechanistic study of these enzymes. In the following sections, synthetic methods for the preparation of FPP analogues will be discussed.

1.9.1 Preparation of pyrophosphate salts

The first reaction used routinely to prepare allylic pyrophosphates was published by Cramer and Bohm in 1959.[109] This procedure involves treatment of an alcohol with inorganic phosphate and trichloroacetonitrile. Whereupon, the desired product is then isolated from a complex mixture of organic and inorganic mono-, di-, and triphosphates by ion-exchange chromatography. This method is difficult to manage on a big scale and the yield is poor. So far, the most successful method for diphosphate preparation is that described by Poulter *et al.* (Scheme 1.16).[110]



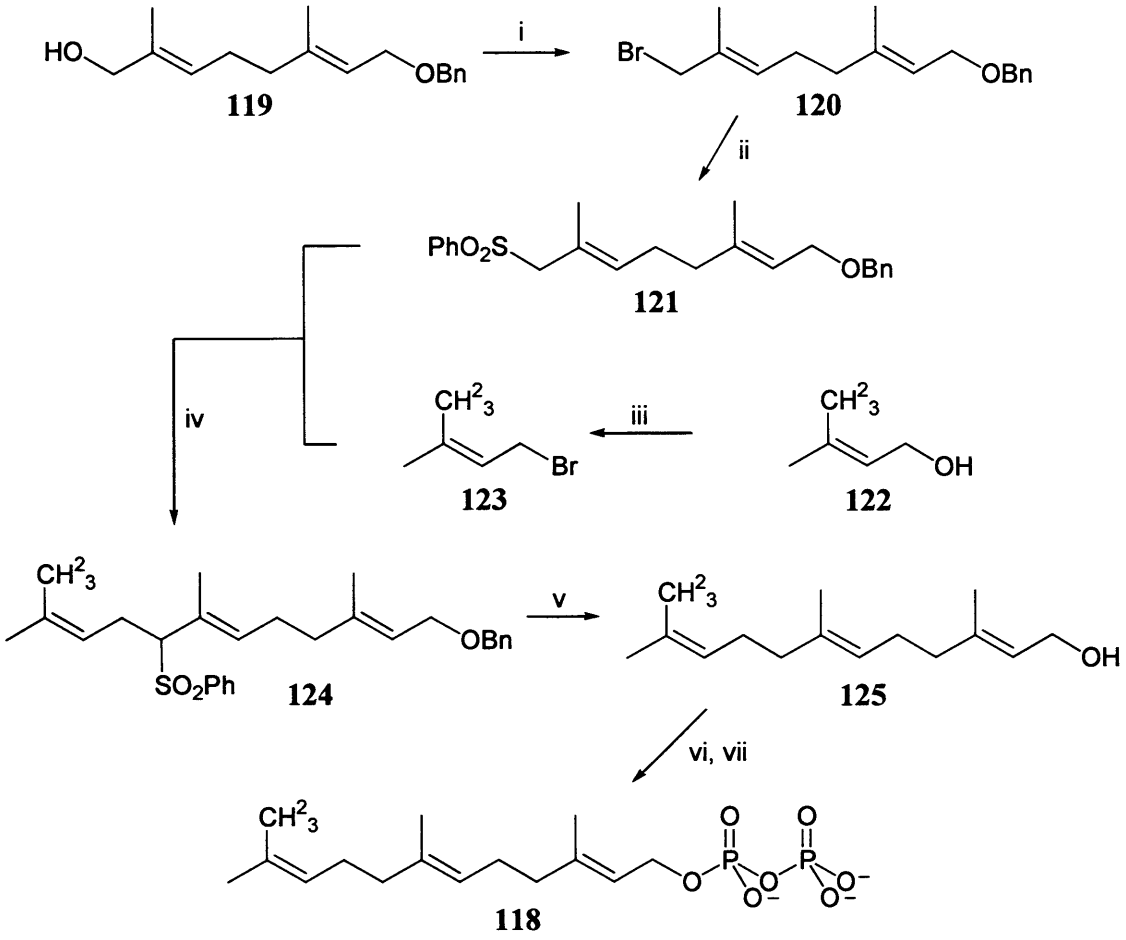
Scheme 1.16: *Synthesis of FPP* (**11**) *from farnesol* (**116**).

Reagents and conditions: i) NCS, DMS, -30 °C to RT, 2 h; ii) $(Bu_4N)_3HP_2O_7$, MeCN, RT, 2 h, 72%.

Generation of the pyrophosphate ester from the allylic alcohol, is a two steps process. The allylic alcohol **116** can be first transformed into an allylic chloride **117** by treatment with dimethyl sulfide (DMS) and N-chlorosuccinimide (NCS) at low temperature in anhydrous dichloromethane,[110] or into an allylic bromide by treatment with methane sulfonyl chloride and lithium bromide in anhydrous tetrahydrofuran,[111] or into a tosylate by treatment with *p*-toluenesulfonyl chloride and 4-(*N*,*N*-dimethylamino)pyridine in anhydrous dichloromethane.[110] Treatment with the tris-tetrabutylammonium salt of pyrophosphoric acid in anhydrous acetonitrile followed by ion exchange chromatography gives the tris-ammonium salt **11** of the target substance. Purification by reverse phase HPLC then yields the required pure product.[112]

1.9.2 Sulfonylation-alkylation-desulfonylation methodology

The sulfonylation-alkylation-desulfonylation methodology was first developed by Biellmann and Ducep in 1969.[113] This sequence of reactions is then widely used in the preparation of isoprenoid diphosphate analogues. For example, [12,12,12-$^2H_3$]FPP and [13,13,13-$^2H_3$]FPP have both been synthesized using this methodology (Scheme 1.17).[89]



Scheme 1.17: *Synthesis of diphosphate* **111**.

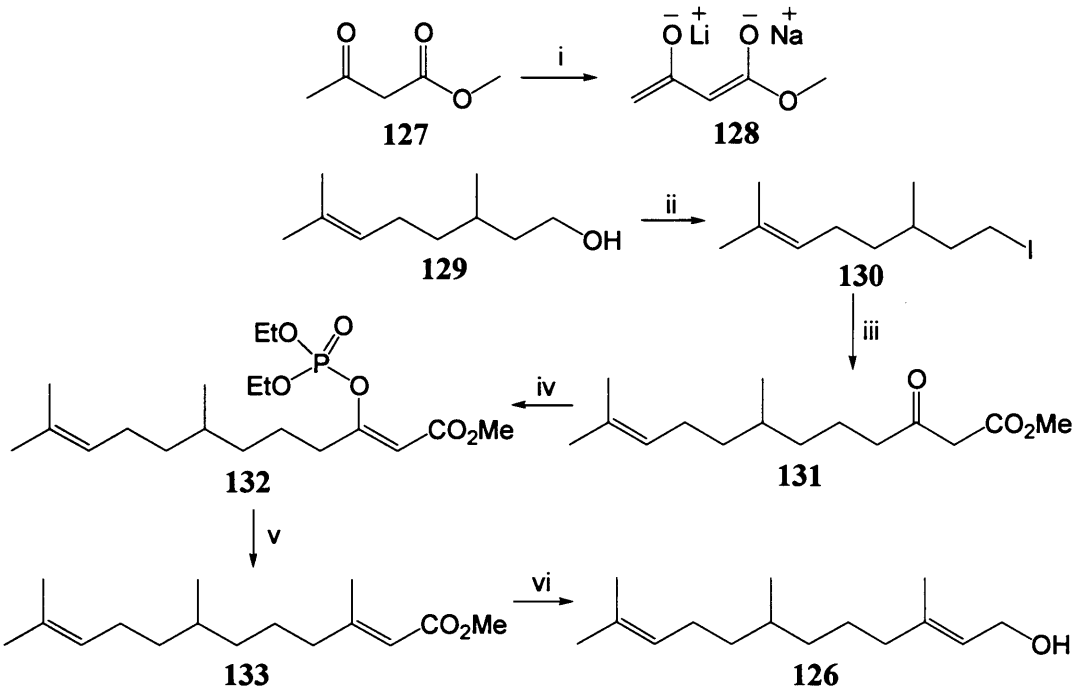
Reagents and conditions: i) PBr_3, DCM ii) $PhSO_2Na$, DMF, 3 h, 76%; iii) PBr_3, THF, 15 h, 81%; iv) n-BuLi, HMPA, THF, -78 °C, 6 h, 56%; v) Li, $EtNH_2$, THF; vi) CCl_4, PPh_3, vii) $(Bu_4N)_3HP_2O_7$, CH_3CN, 2 h.

It can be concluded that the forward synthetic steps of this methodology include synthesis of two major units. One unit is the bromide **123**, which is made by bromination of the corresponding alcohol **122** as described above.[114] The other unit is the sulfone **121**, which can be made by successive halogenation of the alcohol **119** and then treatment of the resulting halide with benzenesulfinic acid, sodium salt.[115] These two species are coupled together in the presence of an appropriate base, which is followed by a reductive desulfonylation process. The desulfonylation

process is the most critical step as it can potentially lead to double bond migration in the product, and there are many reagents available that can achieve the desired result. For example, lithium in $EtNH_2$,[116] lithium with *tert*-butyl alcohol,[116] $LiBHEt_3$ in the presence of palladium catalyst.[117]

1.9.3 Weiler and Sum chain extension methodology

The Weiler and Sum chain extension methodology was first introduced in 1981[118] and its utility has been much extended by Gibbs *et al.*[119] This elegant and robust chain extension methodology was extensively employed in the synthesis of isoprenoids. For example, this methodology was successfully used by Cane *et al.* for the synthesis of 6,7-dihydrofarnesol (**126**) (Scheme 1.18).[103]

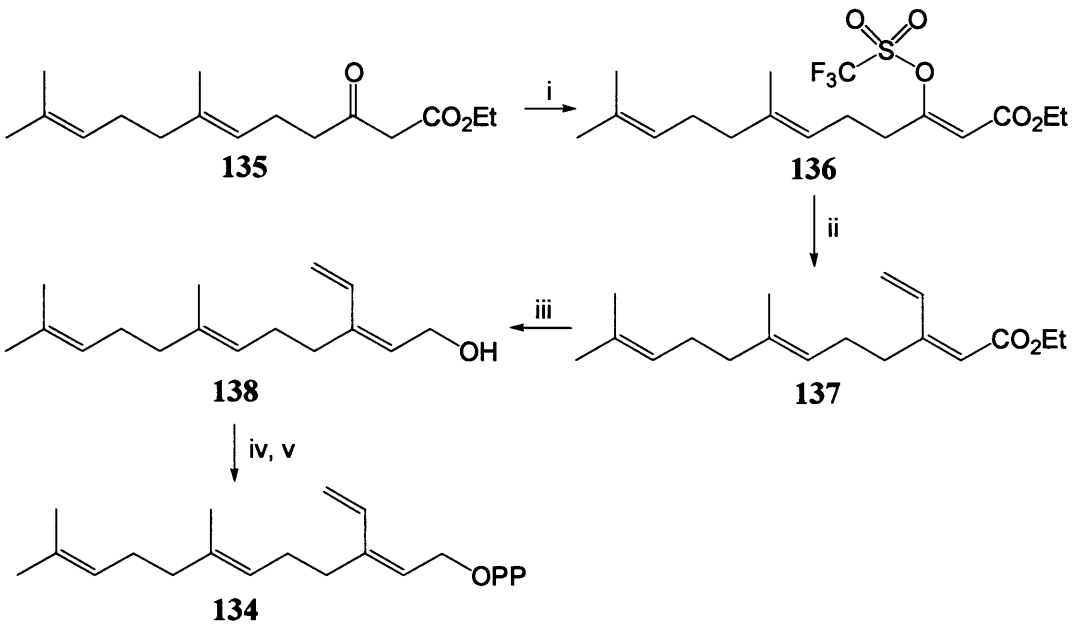


Scheme 1.18: *Synthesis of 6,7-dihydrofarnesol* (**126**).

Reagents and conditions: i) NaH, n-BuLi, THF, 0 °C, 30 min; ii) MsCl, TEA, NaI, DCM, 0 °C, 22 h, 79%; iii) **128**, THF, 0 °C, 55% for two steps; iv) Diethyl chlorophosphate, NaH, Et_2O, 0 °C, 12 h, 76%; v) CuI, MeLi, Et_2O, -78 °C, 3.5 h, 84%; vi) $LiAlH_4$, EtOH, Et_2O, 0 °C, 27 h, 63%.

The main synthetic steps of Weiler and Sum chain extension comprise the bromination of the starting alcohol **129**, alkylation of lithio-sodio acetoacetate dianion **128** (derived from methyl acetoacetate **127**) with the resulting bromide to give the β-keto ester **131**. This was then treated with diethyl chlorophosphate and sodium hydride to give the *Z*-enol phosphate **132**, which was followed by coupling with lithium dimethylcuprate to give ester **133**. Derivatisation of this compound to the farnesol analogues **126** was achieved by the reduction with $LiAlH_4$.

This method was modified by Poulter and Gibbs to allow the introduction of a wide range of modifications to FPP.[119] Their vinyl triflate-mediated route has proven to be suitable for the synthesis of a wide variety of FPP and GGPP analogues. It differs from the classic Weiler chain extension, by use of an enol triflate as a more reactive leaving group before the coupling instead of using phosphate. This improvement allowed the introduction of a different functional group into the 3 position of the farnesyl structure rather than the methyl group. For example, 13-methylidenefarnesyl diphosphate (**134**) was made through this improved methodology (Scheme 1.19).[119, 120]



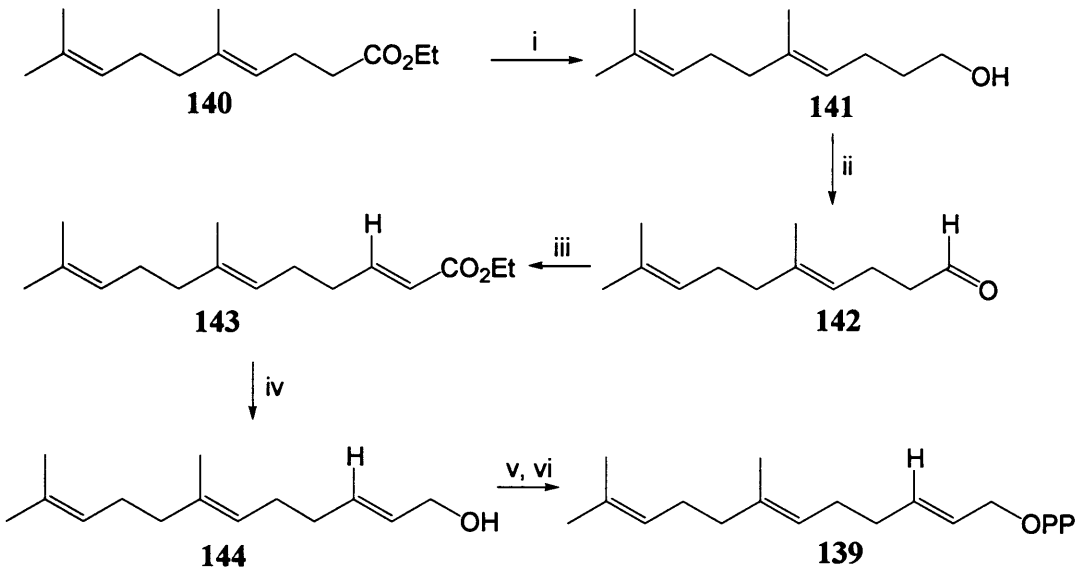
Scheme 1.19: *Synthesis of diphosphate* **134**.

Reagents and conditions: i) $(Me_3Si)_2NK$, THF, -78 °C; $(CF_3SO_2)_2NPh$, THF, -78 °C to RT, over night, 52%; ii) CH_2=$CHSnBu_3$, $Pd(AsPh_3)_2$, CuI, N-methylpyrrolidone, 15 h, 69%; DCM, 0 °C, 22 h, 79%; iii) DIBAL-H, PhMe, -78 °C, 1 h, 43%; iv) NCS, Me_2S, DCM, -40 °C to 0 °C, 2.5 h, 76%; v) $(Bu_4N)_3HP_2O_7$, CH_3CN, 2.5 h, 20%.

By using potassium bis(trimethylsilyl)amide as the base to react with the β-keto ester **135** to generate the potassium enolate, followed by quenching the reaction with $(CF_3SO_2)_2NPh$ or 2-(5-chloropyridyl)$N(SO_2CF_3)_2$,[121] the substitution is stereoselective and primarily the desired *Z* isomer of the vinyl triflate **136** was obtained. The coupling of the resulting triflate **136** with organostannane reagent with Pd(0)/CuI catalyst in *N*-methylpyrrolidone ensures the coupling proceeds with high ratio of the desired *Z* isomer **137**.[119] This stereoselective coupling also can be performed with Grignard reagent in the presence of CuCN in diethyl ether.[121, 122]

1.9.4 Wittig reaction and Horner-Emmons reaction

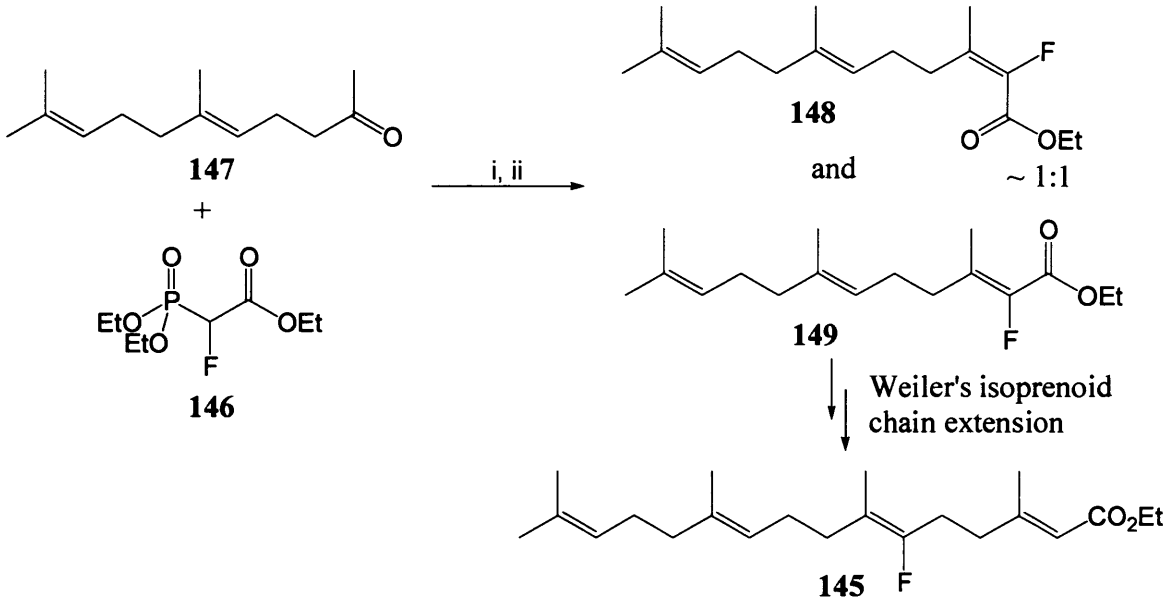
Witting and Horner-Emmons condensations are also useful for the construction of the isoprenoid chain and allow the addition of key functional groups. For instance, Poulter successfully made the 13-desmethylfarnesyl diphosphate **139** through a synthetic route based on a Horner-Emmons Wittig condensation (Scheme 1.20).[123] The ester **140** was reduced to alcohol **141** using lithium aluminium hydride, and the alcohol was oxidized to aldehyde **142** with tetrapropylammonium perruthenate (TPAP) and 4-methylmorpholine *N*-oxide (NMO). Treatment of aldehyde **142** with triethyl phosphonoacetate and sodium hydride gave ester **143** as a single isomer. Reduction of the conjugated ester provided alcohol **144**. Derivatisation of this alcohol to diphosphate analogue **139** was achieved by the diphosphorylation methodology of Poulter *et al.*[110]



Scheme 1.20: *Synthesis of diphosphate* **139**.

Reagents and conditions: i) $LiAlH_4$, Et_2O, -78 °C, 45 min, 94%; ii) NMO, TPAP, DCM, overnight, 91%; iii) triethyl phosphonoacetate, NaH, THF, overnight, 49%; iv) DIBAL-H, DCM, -78 °C to -50 °C, 1.5 h, 93%; v) CBr_4, PPh_3, DCM, 2 h; vi) $(Bu_4N)_3HP_2O_7$, CH_3CN, 2 h, 76% over two steps.

In addition to the simple condensation to make carbon-carbon double bonds, Wittig or Horner-Emmons reactions can also be used to put the functional groups onto the vinyl positions in the isoprenyl chain. For example, the Horner-Emmon condensation was successfully used by Coates *et al.* to produce 6-fluoro (*E,E,E*)-geranylgeranyl diphosphate **145** (Scheme 1.21). In addition to the overall Weiler's isoprenoid chain extension, they used the triethyl fluorophosphonoacetate **146** as a Horner-Emmons reagent to put the fluorine in the C6 vinyl position.[107]



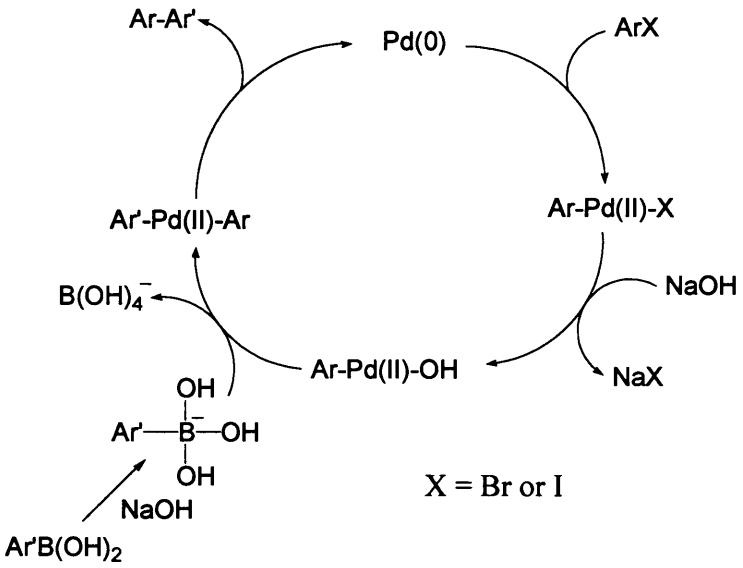
Scheme 1.21: *Synthesis of 6-fluoro (E,E,E)-geranylgeranyl diphosphate* (**145**).

Reagents and conditions: i) NaH, THF, 16 h, 98%; ii) $LiAlH_4$, THF, overnight, 51%.

1.10 Development of new Suzuki coupling methodology

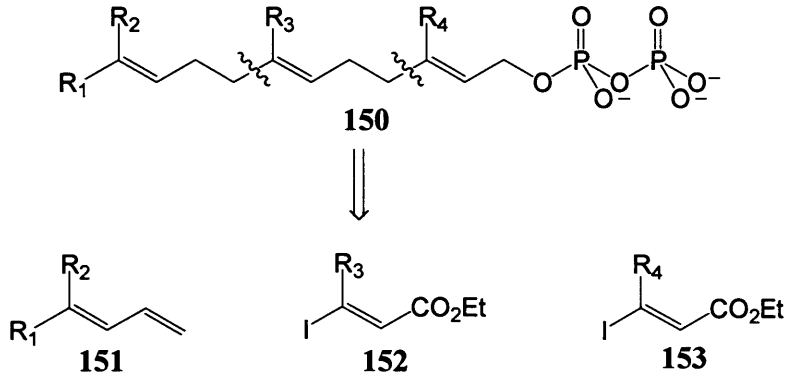
It was one of the intentions of this project to develop a novel Suzuki-Miyaura coupling methodology to perform the synthesis of the compounds with changes to the methyl groups of FPP. It seemed an appropriate method for constructing isoprenoid chains and would ensure no

problems with double bond geometry were possible. The Suzuki coupling is a palladium-catalysed cross coupling between an organoboronic acid and a halide. Recent catalyst and method developments have broadened the possible applications of this reaction enormously, so that the scope of the reaction partners is not restricted to aryls, but includes alkyls, alkenyls and alkynyls.[124, 125] The reaction mechanism is illustrated in Scheme 1.22.



Scheme 1.22: *The catalytic cycle of Suzuki coupling.*

From the retrosynthesis Scheme 1.23, it seemed that a disconnection that exploited the oligomeric nature of FPP would be both appropriate and efficient. Hence a series of monomers was envisaged with one group being 1,3-dienes **151** representing the C-terminal end of the farnesyl group and the other set being various *E*-crotonyl iodides **152** and **153**. The key forward reaction to connect these two monomers would involve transformation of the dienyl group with a hindered borane such as 9-BBN followed by Suzuki-Miyaura coupling with the appropriate iodide.[124, 126-129]



$R_{1\text{-}4}$= CH_3, Ph or CH_2F

Scheme 1.23: *Retrosynthesis of FPP analogues using the planned Suzuki-Miyaura coupling.*

1.11 Aim of the project

The aim of this project was to design and synthesize farnesyl diphosphate analogues for modulating the chemistry of aristolochene synthase. In other words, one part of this project was to produce libraries of FPP analogues with different methodologies as those indicated in Scheme 1.18, Scheme 1.20 and Scheme 1.23. It was anticipated that the methodologies developed in this project would be feasible (rapid, efficient and low cost) and extendable to the preparation of precursor-analogues of other terpene classes.

Another part of this project was to test the developed FPP analogues as substrates or inhibitors of aristolochene synthase. The study of their transformation by aristolochene synthase should further our understanding of the mechanism of action of this enzyme and provide generic insights applicable to other enzyme systems.

The following chapters will describe the successful synthesis of some of the required FPP derivatives using such methodology and their testing as mechanistic probes of aristolochene synthase.

CHAPTER 2

SYNTHESIS OF FPP ANALOGUES

2.1 Target molecules

From the previous introduction, it was concluded there were two important approaches for studying the catalytic mechanism of sesquiterpene synthase, site directed mutagenesis of key amino acid residues and turnover of modified FPP analogues. However, the interpretation of results from site-specific mutants of terpene cyclases requires some caution, since there is a possibility that the change of only a single amino acid in their active site can alter their templating potential. Such local geometrical changes may be inherently difficult to detect since they may not affect the global fold of the proteins but nevertheless lead to the synthesis of novel products through subtle alterations of the reaction pathway. So in this case, the method of turn over of modified FPP analogues has its advantage. It is one of the intentions of this project to synthesize a wide range of analogues of FPP, which can be used to explore more deeply the mechanism of the reaction catalysed by aristolochene synthase.

As stated in Chapter one (see section 1.7.4), the cationic intermediates generated during the enzyme catalysed reaction can be stabilised or destabilised by functional groups in their vicinity. So different functional groups in different positions have predictable and testable effects on the cations, which might be helpful for understanding the reaction mechanisms. Nine FPP analogues with phenyl and fluoro substituents have been made through different methodologies (Figure 2.1).

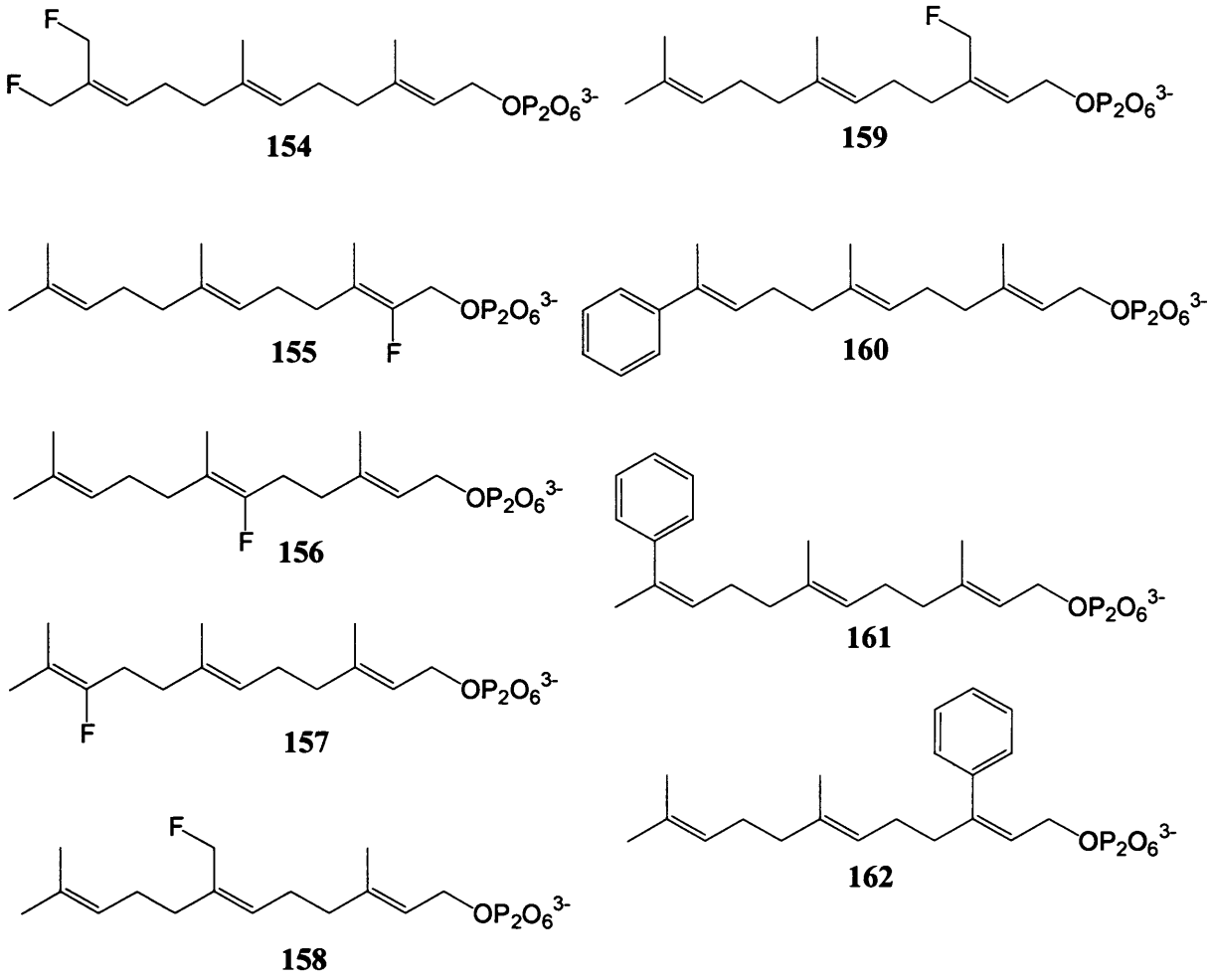


Figure 2.1 *Structures of target FPP analogues.*

The phenyl group is a bulky aromatic group capable of stabilising cationic intermediates, both its size and the affinity to cation will be the interesting points that require to be investigated. Fluoro substituents do not greatly affect the binding affinities to the enzyme as a consequence of size and shape but at the same time exert a strong influence on the electronic environment at the site of replacement[107, 130] in that they stabilize cations on the α-carbon by π-donation but exert a destabilizing inductive effect on cations located on the β-carbon (Figure 2.2). The strong influence on the reactivity exerted by fluoro substituents without significantly affecting size and shape may make these reagents more suitable than mutagenesis experiments to decipher the

intricate details of terpene synthase chemistry. Fluoro-substituent analogues of prenyldiphosphates recently were successfully used as powerful reagents to study the mechanism of the enzyme-catalysed cyclisations to terpenoids products.[107, 123, 130]

Stabilised cation Destabilised cation

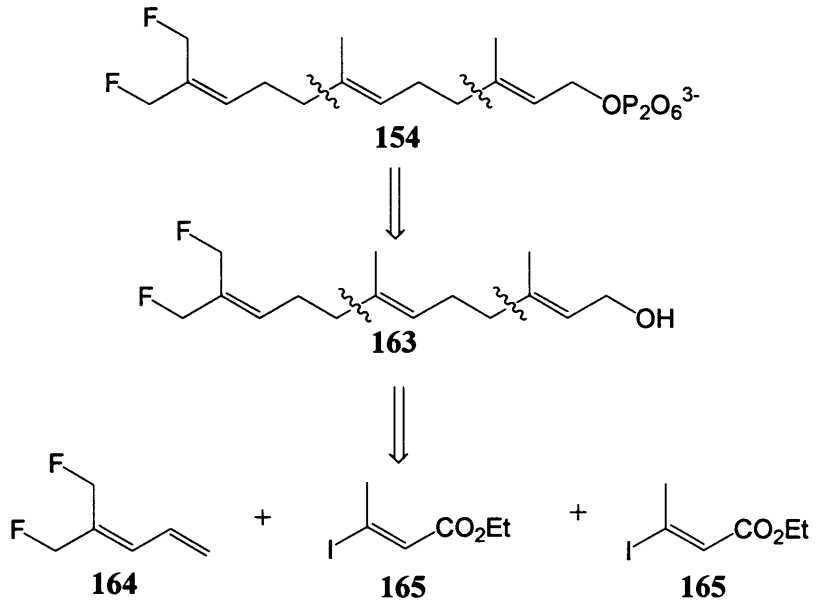
Figure 2.2: *Fluoro substituent's influence on the carbon cation in its vicinity.*

2.2 Synthesis of 12,13-difluoro farnesyl diphosphate (154)

The first trial synthesis of an FPP analogue was 12,13-difluoro farnesyl pyrophosphate **154**. Several different synthetic methodologies were tried to produce this analogue; the key methodologies used included Suzuki-Miyaura coupling, Wittig reaction, Weiler chain extension and the use of iron tricarbonyl protecting groups.

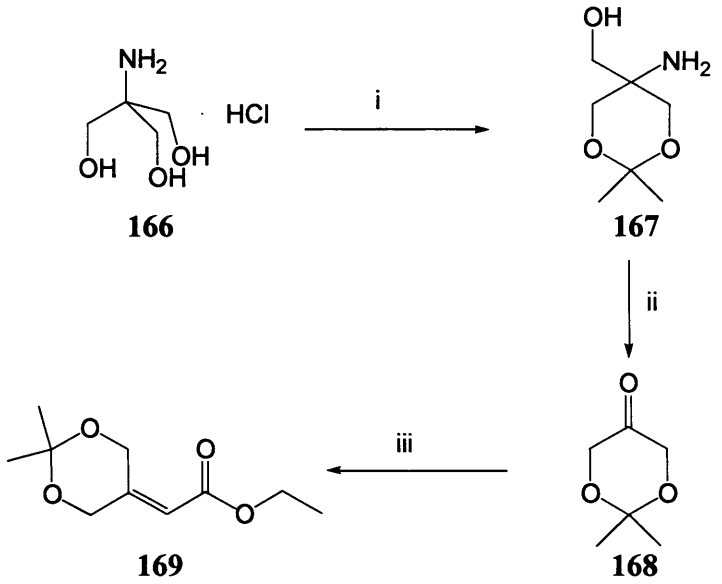
2.2.1 Suzuki coupling methodology

As introduced in Chapter 1 (see section 1.10), our newly developed Suzuki coupling methodology for the synthesis of FPP analogues starts from two groups of monomers; one group was dienes representing the C-terminal end of the farnesyl group and the other set was various *E*-crotonyl iodides (Scheme 2.1). In this case, the diene **164** possesses two fluorine substituents and the *E*-crotonyl iodide is a simple iodide **165** with a methyl group. The retrosynthesis leading to diene **164** is shown in Scheme 2.1.



Scheme 2.1. *Retrosynthesis of 12,13-difluoro FPP* **154**.

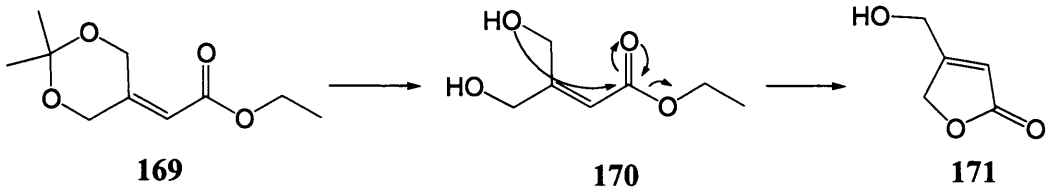
Reaction of the tris(hydroxymethyl)aminomethane hydrochloride (**166**) with 2,2-dimethoxypropane in the presence of acid catalyst *p*-toluenesulfonic acid in anhydrous DMF gave 5-amino-5-hydroxymethyl-2,2-dimethyl-1,3-dioxane (**167**).[131, 132] As purification after vacuum distillation gave only a 23% yield, the crude product **167** was directly treated with a solution of sodium periodate at *p*H 6 to give 2,2-dimethyl-5-oxo-1,3-dioxane (**168**) as a colorless oil in 68% overall yield.[132] Wittig reaction of ketone **168** with (carbethoxymethyl)triphenyl-phosphonium bromide in dry ethanol afforded the ester **169** in 77% yield after purification by silica column chromatography.[133]



Scheme 2.2: *Synthesis of Ester* **169**.

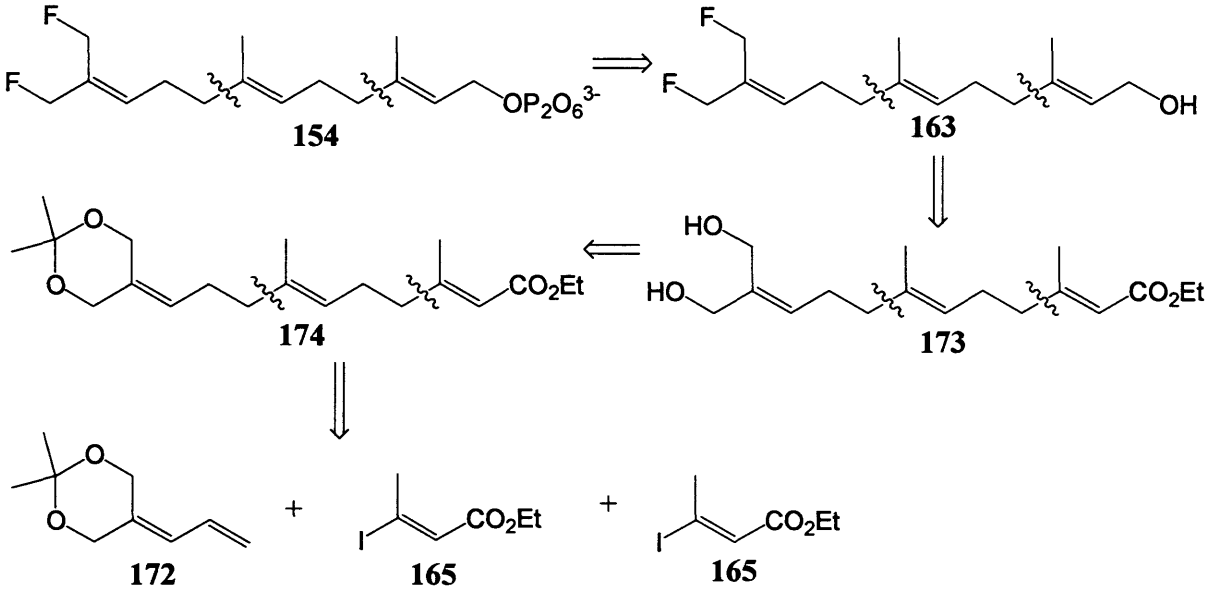
Reagents and conditions: i) 2,2-dimethoxypropane, PTSA, DMF, RT, 15 h, 28%; ii) $NaIO_4$, KH_2PO_4, 0 °C to RT, 15 h, 68% for two steps; iii) (carbethoxymethyl)-triphenyl phosphonium bromide, Na, EtOH, RT, 15 h, 77%.

Attempts to convert the ester **169** to the diol **170** with TFA in ethanol [134] failed probably due to lactone formation (Scheme 2.3).



Scheme 2.3: *Proposed mechanism of generation of the lactone* **171**.

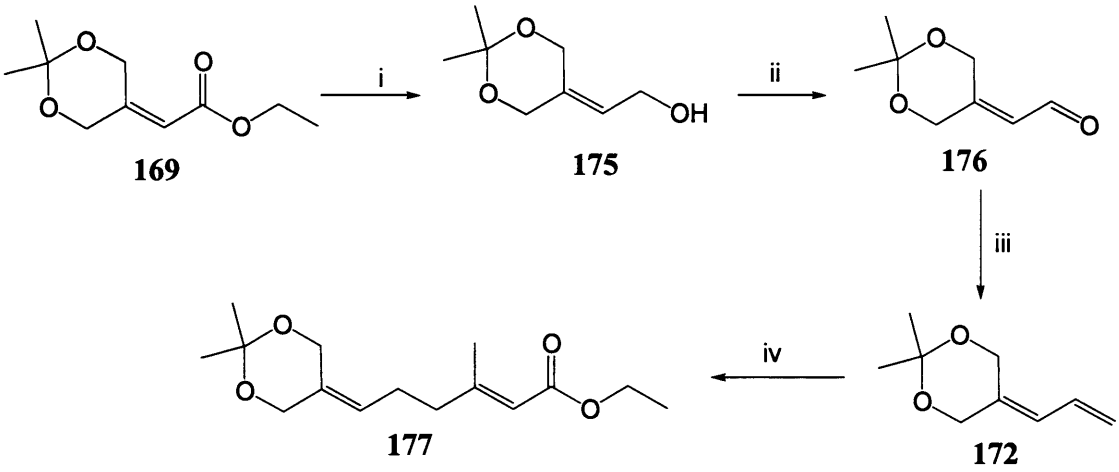
Consequently, a new retrosynthetic plan was formulated leading to diene **172** (Scheme 2.4).



Scheme 2.4: *Alternative retrosynthesis of 12,13-difluoro FPP* **154**.

In this case, diene **172** would be coupled with an appropriate iodide using a Suzuki-Miyaura coupling reaction. The protective acetal group could be removed and the resulting diol then fluorinated with diethylaminosulfur trifluoride (DAST) before transformation to the target pyrophosphate.

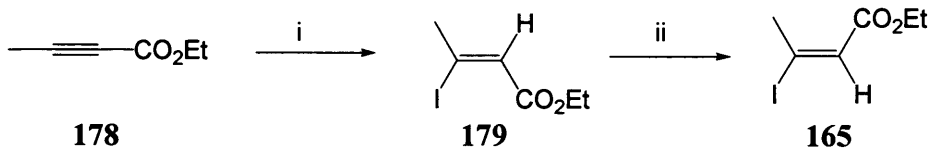
Reduction of ester **169** with DIBAL-H gave alcohol **175** in 97% yield. Pyridinium chlorochromate oxidation[135, 136] in anhydrous DCM then gave aldehyde **176** in 55% yield (Scheme 2.5).[137] Wittig reaction of aldehyde **176** with the ylid of methyl phosphonium bromide then afforded the diene **172** in 62% yield after purification by silica flash column chromatography.[133]



Scheme 2.5: *Synthesis of ester* **177**.

Reagents and conditions: i) DIBAL-H, THF, 0 °C, 2 h, 97%; ii) PCC, DCM, RT, 4 h, 55%; iii) methyl triphenyl phosphonium bromide, BuLi, THF, RT, 18 h, 62%; iv) 9-BBN, $PdCl_2dppf$, $AsPh_3$, **165**, NaOH, THF, 50 °C, 15 h, 62%.

The other compound of the Suzuki-Miyaura coupling, *E*-iodide **165** was synthesised in two steps from ethyl 2-butynoate (**178**) (Scheme 2.6). Reaction of ester **178** with sodium iodide and glacial acetic acid at 115 °C gave the *Z*-iodide **179** in 98% yield.[138-140] This was then converted to the more stable *E*-isomer **165** by heating the *Z*-isomer in a sealed tube for 4 hours under argon. Separation of the resulting isomeric mixture by flash column chromatography then gave the pure *E*-isomer in 47% yield.[139]

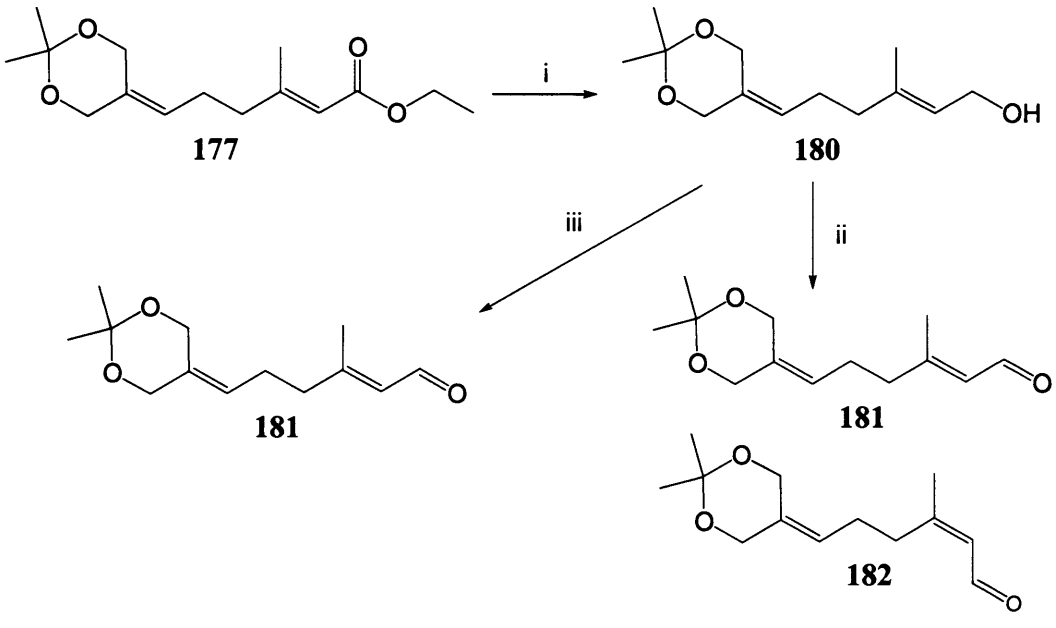


Scheme 2.6: *Synthesis of iodide* **165**.

Reagents and conditions: i) NaI, CH_3COOH, 115 °C, 1.5 h, 98%; ii) sealed tube under argon, 220 °C, 2 h, 47%.

Suzuki-Miyaura coupling of diene **172** with iodide **165** using triphenyl arsine, $PdCl_2$dppf and 6 M NaOH afforded ester **177** in 62% yield (Scheme 2.5).[124, 126]

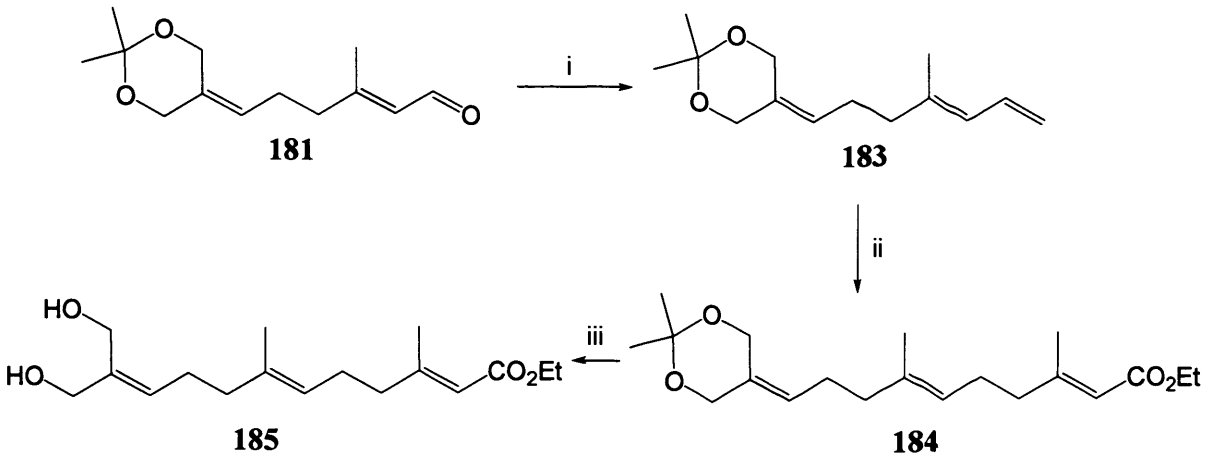
Reduction of ester **177** with DIBAL-H in anhydrous THF gave alcohol **180** in 88% yield (Scheme 2.7).[141, 142] Oxidation of alcohol **180** with pyridinium chlorochromate (PCC) led isomerisation, probably due to the slightly acidic nature of the reagent. The ^{1}H NMR spectrum showed two doublet resonances around 10 ppm indicating two distinct aldehyde protons, i.e. the *E* and *Z* isomers **181** and **182**. However, when the oxidation was carried out using tetra-n-propylammonium perruthenate (TPAP), N-methylmorpholine-N-oxide (NMO) and ground 4 Å molecular sieves, the desired *E*-aldehyde **181** was obtained exclusively in 78% yield.[143]



Scheme 2.7: *Synthesis of aldehyde* **181**.

Reagents and conditions: i) DIBAL-H, THF, -78 °C, 2 h, 88%; ii) PCC, DCM, RT, 4 h; iii) NMO, TPAP, CH_3CN, RT, 15h, 78%; .

The *E*-aldehyde was then subjected to a Wittig reaction with methyl triphenyl phosphonium bromide to give 1,3-diene **183** in 82% yield (Scheme 2.8). Suzuki coupling of this with iodide **165** in the same manner as for the preparation of **177** (see above) afforded ester **184** in 35% yield. Removal of the diol protecting group using TFA in ethanol then gave diol **185** in 79% yield.[144]



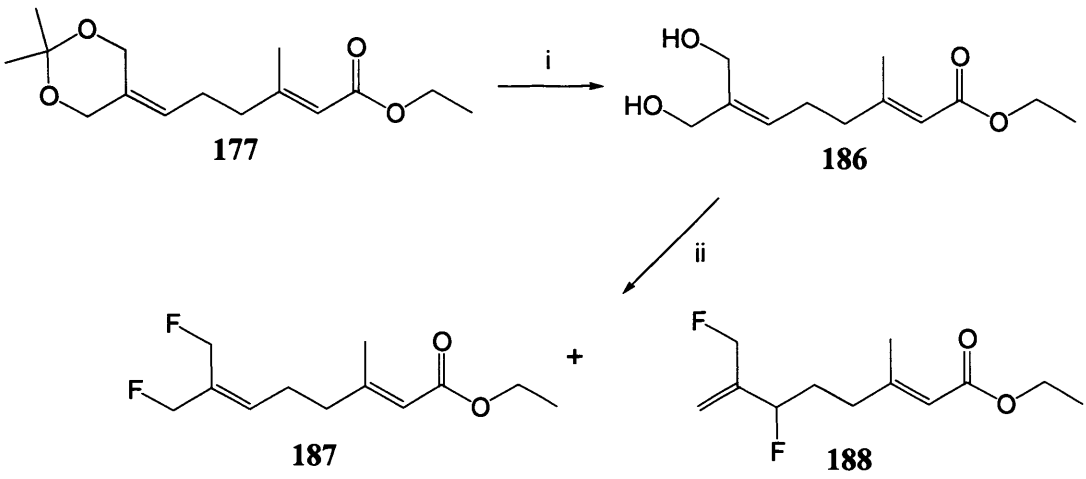
181 **183** **185** **184**

Scheme 2.8: *Synthesis of diol* **185**.

Reagents and conditions: i) $CH_3P^+Ph_3Br^-$, BuLi, THF, RT, 18 h, 82%; ii) 9-BBN, $PdCl_2$dppf, $AsPh_3$, **165**, NaOH, THF, 50 °C, 15 h, 35%; iii) TFA, EtOH, RT, 3 h, 79%.

2.2.2 Introduction of fluoro groups

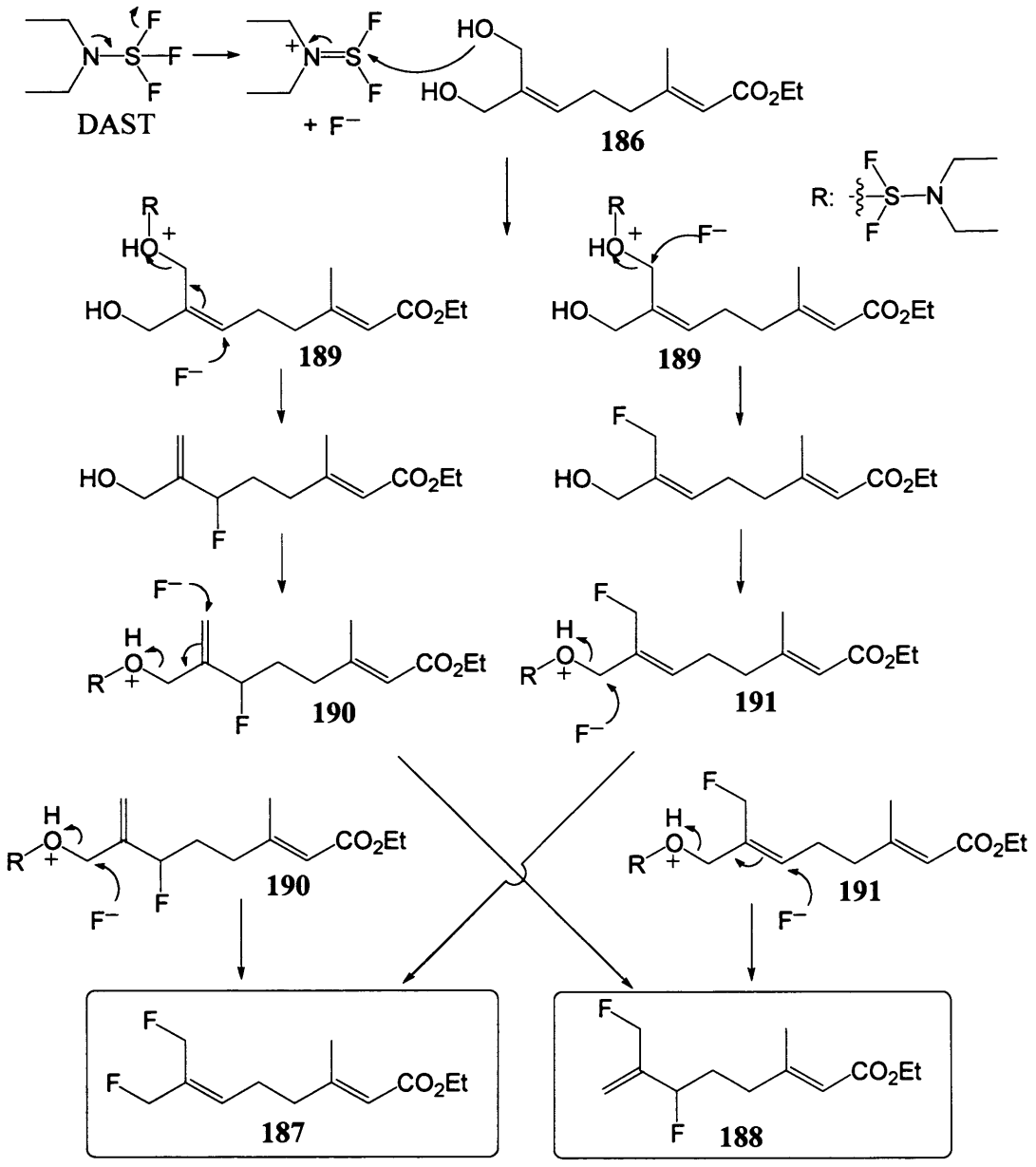
In order to test the methodology of fluorination with DAST, ester **177** was used as it was available in larger quantities than **184** and simpler to prepare. The ester **177** was converted to the diol **186** by treatment with TFA in ethanol.[144] Diol **186** was recovered in 90% yield after purification by flash chromatography on silica gel. Treatment of the diol **186** with DAST in dry DCM at –78 °C gave a mixture of products, difluoride **187** and the undesired product **188** in 19% and 48% yield respectively (Scheme 2.9).[145, 146]



Scheme 2.9: *Synthesis of difluoride* **187**.

Reagents and conditions: i) TFA, EtOH, RT, 2 h, 98%; ii) DAST, DCM, -78 °C to RT, 5 h.

The ^{19}F NMR spectrum of difluoride **187** had two doublets with similar chemical shifts (δ_F = –212.84 and –217.05 ppm), suggesting that the two fluorine atoms in this molecule are in similar magnetic environments. However, the ^{19}F NMR spectrum of difluoride **188** had two doublet at very different chemical shifts (δ_F = –182.64 and –216.76 ppm) due to them being in different molecular environments. A possible mechanism leading to the formation of **187** and **188** is shown in Scheme 2.10.

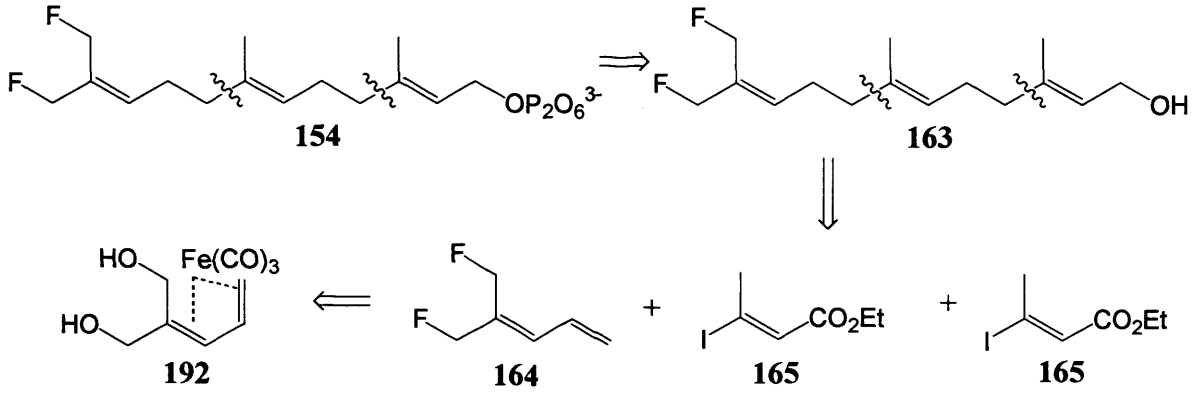


Scheme 2.10 Possible mechanism for the formation of **187** and **188**.

2.2.3 Investigation of diene protection with iron tricarbonyl group

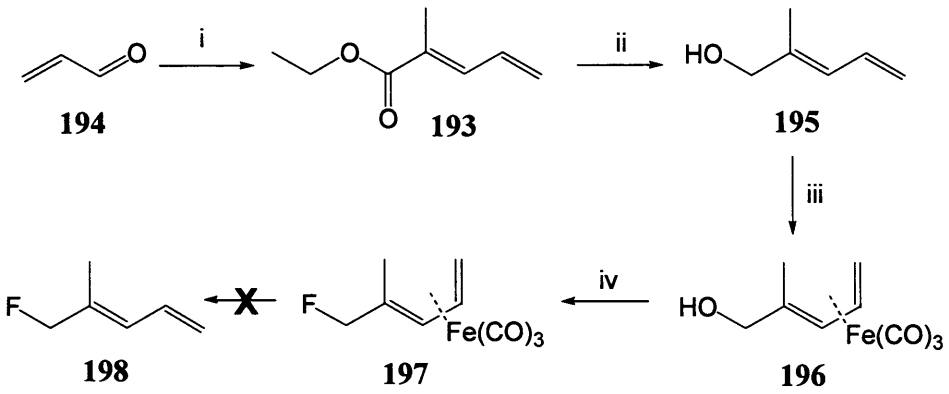
Although the initial approach towards difluoride **187** looked positive, low yields due to double bond migration were observed when the fluorination was carried out on the allylic alcohol. This was not ideal and therefore an alternative method was sought. As it was noticed that the allylic double bond was involved in the fluorinations, how to 'lock' the double bond was the key issue.

When the allylic double bond is a part of 1,3-diene, allylic fluorinations have been shown to be regiospecific when iron tricarbonyl is used to coordinate the diene group and therefore this was investigated (Scheme 2.11).[147]



Scheme 2.11. *Methology with using of iron tribcarbonyl coordination.*

To test the fesibility of the iron tricarbonyl methodology, diene **193** was used as it was available in larger quantities than **172** and simpler to prepare. Acrolein **194** was treated with carbethoxyethylidene triphenyl phosphorane in DCM to afford ester **193** in 79% yield (Scheme 2.12);[148] This ester then was reduced to the alcohol using DIBAL-H. A mixture of diiron nonacarbonyl and diene **195** was heated to 50 °C for 15 h under argon atmosphere to afford complex **196** in 81% yield.[149] Fluorination of complex **196** using DAST[145, 146] in DCM at –78 °C yielded the expected fluoride **197** in 56% yield. Unfortunately, the cleavage of the iron tricarbonyl group proved difficult. After several attempts (e.g. $FeCl_3$ in CH_3CN, 4-methylmorpholine N-oxide in DCM),[150] there were no separable products from the reaction mixture, presumably because the product was highly volatile (estimate of its boiling point is below 80 °C) and therefore lost during reaction work up.

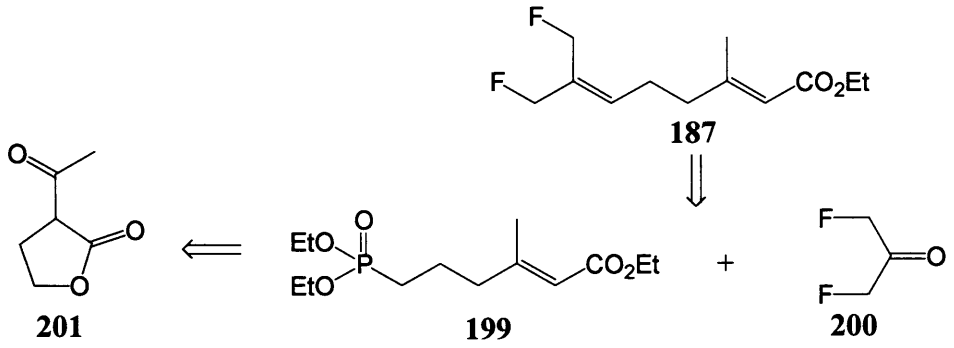


Scheme 1.12: *Investigation of diene protection with iron tricarbonyl.*

Reagents and conditions: i) carbethoxyethylidiene triphenyl phosphorane, DCM, RT, 5 h, 79%; ii) DIBAL-H, THF, -78 °C to 0 °C, 3 h, 76%; iii) $Fe_2(CO)_9$, THF, reflux, 15 h, 81%; iv) DAST, DCM, -78 °C, 6 h, 56%.

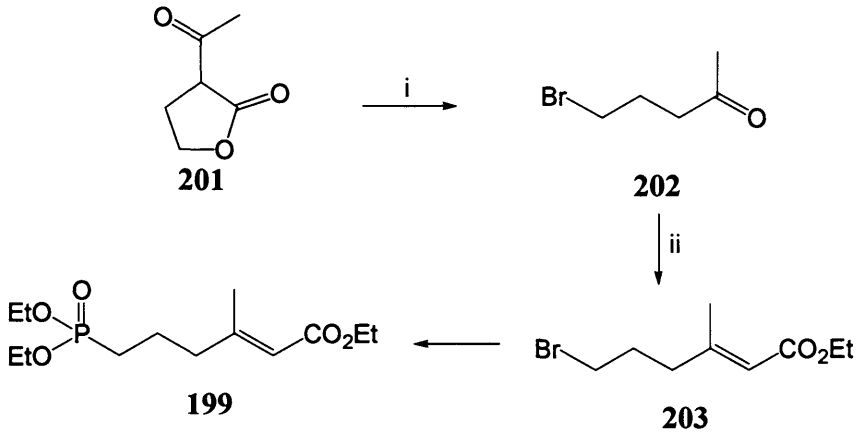
2.2.4 Use of Horner-Emmons chemistry

As the previous approach to synthesise 12,13-difluoro FPP was unsuccessful, a new methodology had to be developed. Because of the difficulty of converting the allylic diols to difluorides, it was decided to introduce this functionality using Horner-Emmons chemistry. The retrosynthesis scheme is shown below (Scheme 2.13).



Scheme 1.13: *Retrosynthesis of difluoride* **187**.

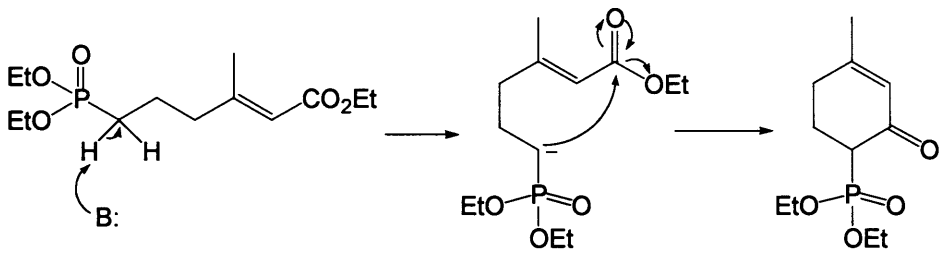
The forward reaction starts with α-acetyl butyrolactone (**201**), which was treated with hydrobromic acid in the presence of sulfuric acid to give bromoketone **202** in 92% yield (Scheme 2.14).[151, 152] This was then initially subjected to a Horner-Emmons reaction with triethyl phosphonoacetate to generate the bromoester **203** with good stereoselectivity (*E*:*Z* = 11:1).[153] An Arbuzov reaction using triethyl phosphite then gave phosphonoacetate **199** in 81% yield after purification by silica flash column chromatography.[154, 155]



Scheme 2.14: *Synthesis of phosphonoacetate* **199**.

Reagents and conditions: i) HBr, H_2SO_4, toluene, 70 °C, 18 h, 92%; ii) Triethyl -phosphonoacetate, NaH, THF, 0 °C, 14 h, 68%; iii) Triethyl phosphite, 130 °C, 3.5 h, 81%.

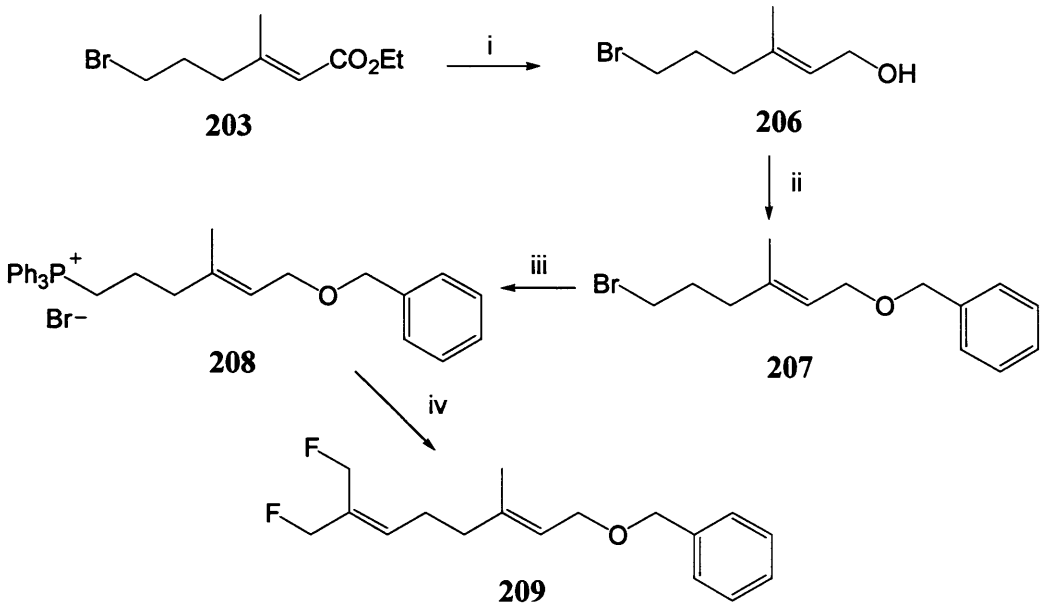
A Horner-Emmons reaction on this phosphonoacetate with 1,3-difluoroacetone **200**[156] using different bases (t-BuOK, KHMDS, NaH, LDA) proved unsuccessful due to possible six membered ring formed in a Dieckmann-like condensation (Scheme 2.15). In order to prevent this problem, the ester functionality was removed, and the use of alternative protecting groups was explored.



199 **204** **205**

Scheme 2.15: *Proposed mechanism of ring formation from phosphonoacetate* **199.**

At the same time, we decided to use Wittig reaction rather than the Horner-Emmons reaction, because the reagent 1,3-difluoroacetone is a symmetrical molecule and the Horner-Emmons reagent is less reactive than the Wittig reagent despite its *E*-selectivity in stereochemistry. The benzyl-protecting group was tried as the first alternative group (Scheme 2.16). Reduction of the ester **203** with DIBAL-H in THF gave alcohol **206** in 98% yield. Treatment with benzyl bromide and NaH in THF then gave bromo benzyl ether **207** in 73% yield.[157] This was then initially converted to the triphenyl phosphonium bromide **208** by heating with triphenyl phosphine overnight in acetonitrile,[158] and then treatment with 1,3-difluoro acetone in a Wittig reaction to give the desired difluoro benzyl ether **209** in 93% yield.[156]



Scheme 2.16: *Synthesis of difluoro benzyl ether* **209**.

Reagents and conditions: i) DIBAL-H, THF, -78 °C to 0 °C, 2 h, 98%; ii) Benzyl bromide, NaH, THF, 0 °C to RT, 3 h, 73%; iii) Ph_3P, CH_3CN, reflux, 14 h, 87%; iv) 1,3-difluoro acetone, LiHMDS, THF, -78 °C to RT, 1 h, 93%.

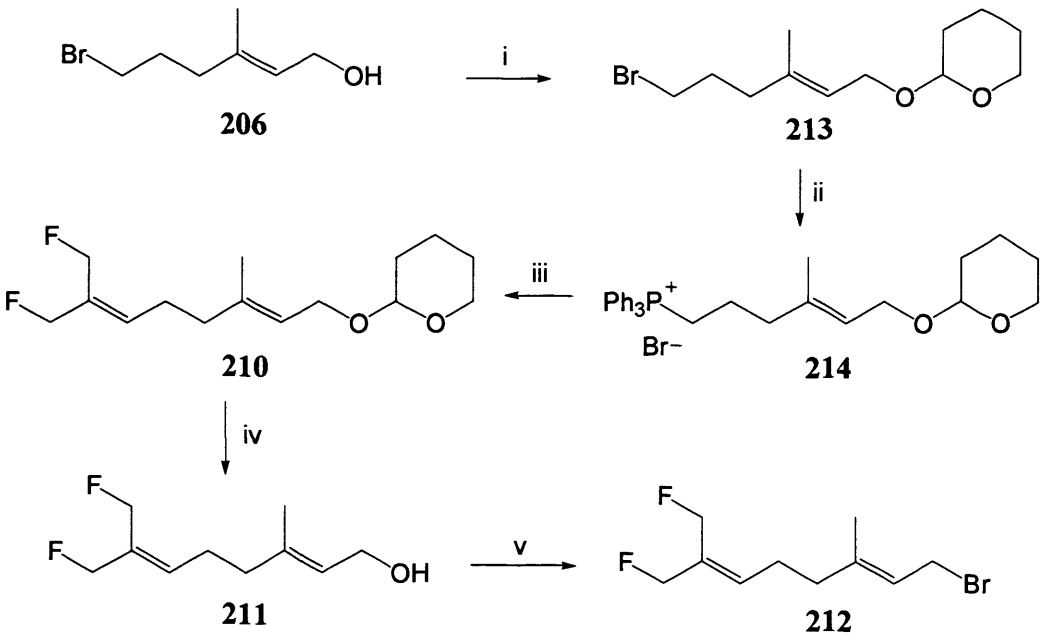
Several attempts were made to remove the benzyl protecting group. Dissolving metal reduction using sodium liquid ammonia was unsuccessful because the fluorine group was removed. The resultant compound did not show any peaks in the ^{19}F NMR spectrum. The difluoro benzyl ether was inert to treatment with methanesulfonic acid. Attempted hydrogenolysis with H_2 on Pd was successful but the double bonds were also reduced.[157] So another, more readily removable protecting group was required.

2.2.5 Weiler and Sum chain extension methodology

The THP protecting group was then selected as it could be introduced in high yield and removed readily.[159] The THP protected difluoride **210** was made in the same manner as difluoro benzyl

ether **209** in 60% overall yield for the four steps following reduction of the ester **203** with DIBAL-H (Scheme 2.17).[159, 160]

After successful preparation of difluoride **210**, the THP protecting group was easily removed by heating the compound with pyridinium *p*-toluenesulfonate in ethanol,[160, 161] and the alcohol **156** was isolated in 97% yield (Scheme 2.17). To extend the geraniol analogue to farnesol analogue, the chain extension methodology first published by Weiler and Sum (see section 1.8.3) was attempted.[118, 162] Alcohol **211** was converted to a bromide **212** by reaction with methanesulfonyl chloride and lithium bromide.[163]

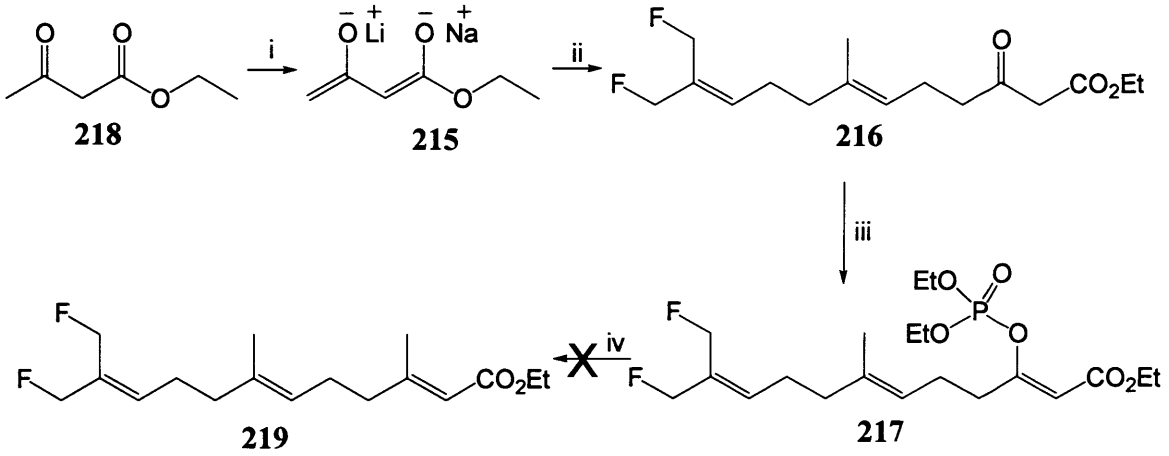


Scheme 2.17: *Synthesis of difluoro bromide* **212**.

Reagents and conditions: i) 3,4-DHP, p-TsOH, DCM, 0 °C to RT, 16 h, 93%; ii) Ph_3P, CH_3CN, reflux, 14 h; iii) 1,3-difluoro acetone, LiHMDS, THF, -78 °C to -20 °C, 2 h, 66% for 2 steps; iv) PPTS, EtOH, 55 °C, 2 h, 97%; v) MsCl, Et_3N, LiBr, THF, -55 °C to 0 °C, 2 h.

Alkylation of bromide **212** with three equivalents of lithio-sodio acetoacetate dianion (215) gave

the β-keto ester **216** in 88% yield after purification by silica flash column chromatography.[118] Reaction with diethyl chlorophosphate then gave the enol phosphate **217** as a single isomer in 88% yield (Scheme 2.18).[162] The proposed final step, a Michael addition with lithium dimethylcuprate,[162] however, resulted in loss of fluorine atoms, as indicated by the absence of resonance in the ^{19}F spectrum, most likely due to elimination of hydrogen fluoride.



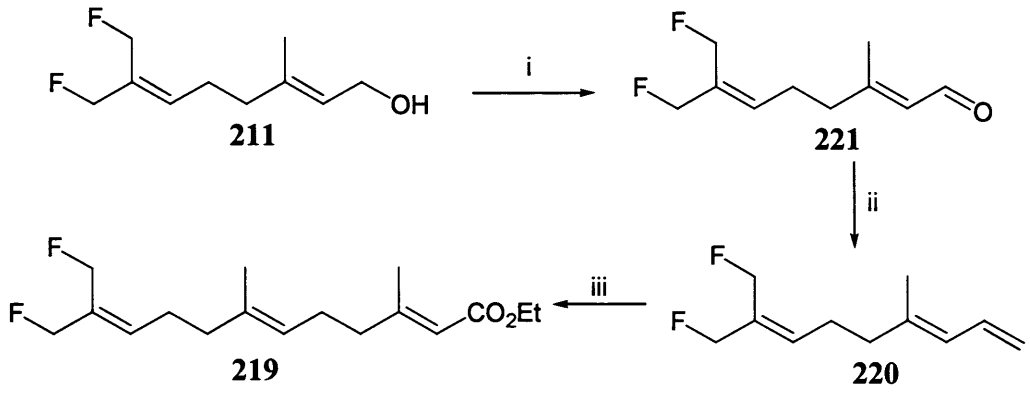
Scheme 2.18: *Attempted synthesis of difluoro ester* **219**.

Reagents and conditions: i) NaH, n-BuLi, THF, 0 °C, 20 min; ii) **212**, THF, 0 °C, 89% for two steps; iii) Diethyl chlorophosphate, NaH, Et_2O, 0 °C, 1 h, 88%; iv) CuI, MeLi, Et_2O, -78 °C, 3h.

2.2.6 Reuse of Suzuki coupling methodology

As the chain extension methodology of Weiler and Sum was unsuccessful for our purpose, the Suzuki coupling methodology was revived since this route had been abandoned due to problems of regioselective fluorination, a problem now solved. The alcohol **211** was converted to the 1,3-diene **220** by successive TPAP oxidation[143] and Wittig reaction in 83% overall yield (Scheme 2.19).[133] A Suzuki-Miyaura coupling[124, 126] with the crotonyl iodide **165** resulted in the production

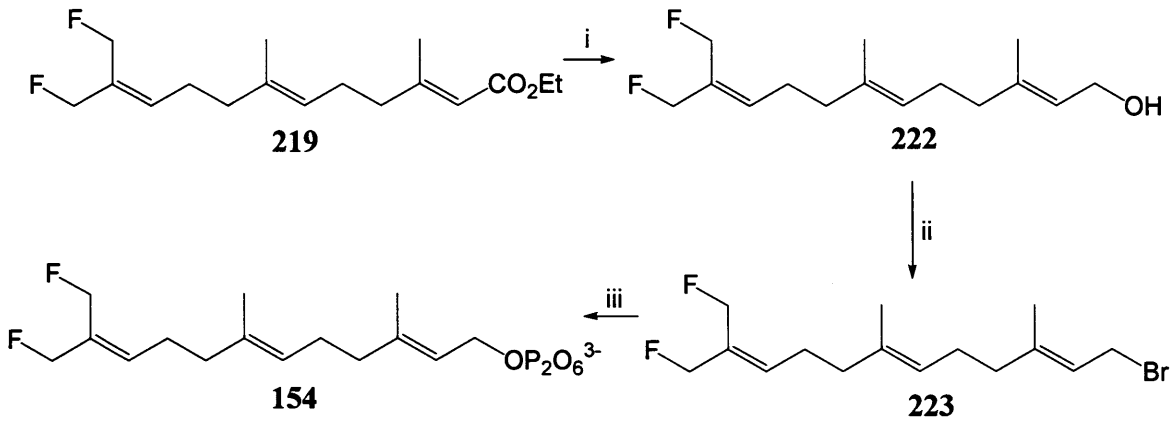
of ester **219** in 29% yield in the same manner as the described procedure for ester **177** (see Scheme 2.5).



Scheme 2.19: *Synthesis of difluoro ester* **219**.

Reagents and conditions: i) TPAP, NMO, CH_3CN, RT, 16 h, 82%; ii) CH_3PPh_3Br, n-BuLi, THF, RT, 16 h, 92%; iii) 9-BBN, $PdCl_2dppf$, $AsPh_3$, **165**, NaOH, THF, 50 °C, 15 h, 29%.

Reduction of ester **219** with DIBAL-H gave the alcohol **222** in 68% yield.[142] This was then converted to bromide **223** by treating with methanesulfonyl chloride and lithium bromide.[163] Treatment of the bromide with freshly recrystallized tris(tetra-n-butylammonium) hydrogenpyrophosphate in anhydrous acetonitrile generated the desired difluoro farnesyl pyrophosphate analogue **154** in 10% yield for the last two steps.[110] This tetra-butyl ammonium salt had to be converted to the ammonium salt via cation exchange chromatography prior to purification by reverse phase HPLC, as the original tetra-butylammonium salt caused serious separation problem in the HPLC step because of its long tail.

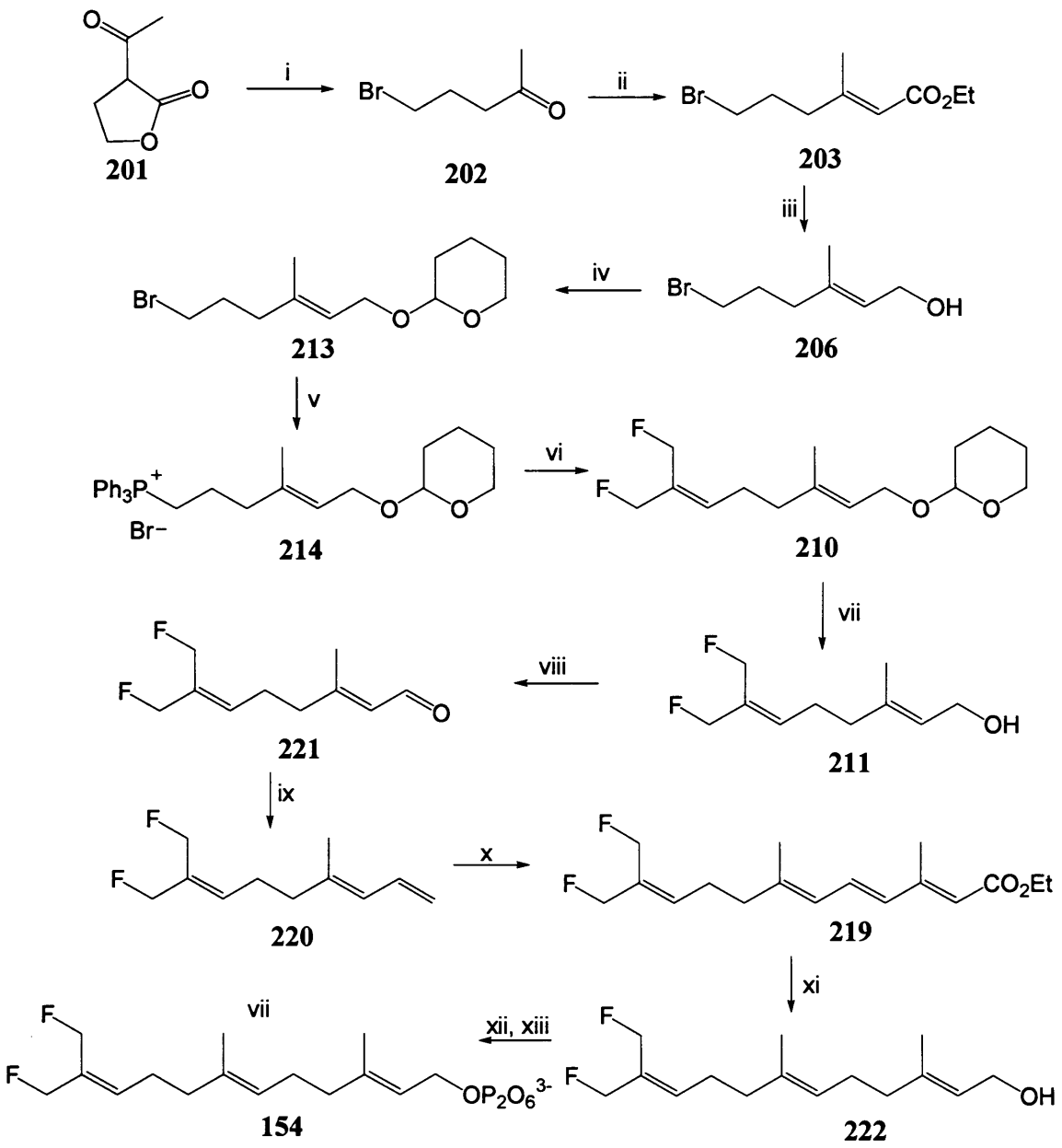


Scheme 2.20: *Synthesis of diphosphate* **154**.

Reagents and conditions: i) DIBAL-H, THF, -78 °C, 2 h, 68%; ii) MsCl, NEt_3, LiBr, THF, -45 °C, 2 h; iii) $(Bu_4N)_3HP_2O_7$, CH_3CN, 2 h, then DOWEX ion exchange Bu_4N^+/NH_4^+, 10% for two steps.

2.2.7 Summary

After trying different methodologies, the synthesis of 12,13-difluoro farnesyl pyrophosphate was finally achieved in 13 steps using Wittig reaction and Suzuki-Miyaura coupling as key steps. The overall yield is 0.8%. The summary scheme is shown below (Scheme 2.21).



Scheme 2.21: *Synthesis of diphosphate* **154**. *(Summary)*

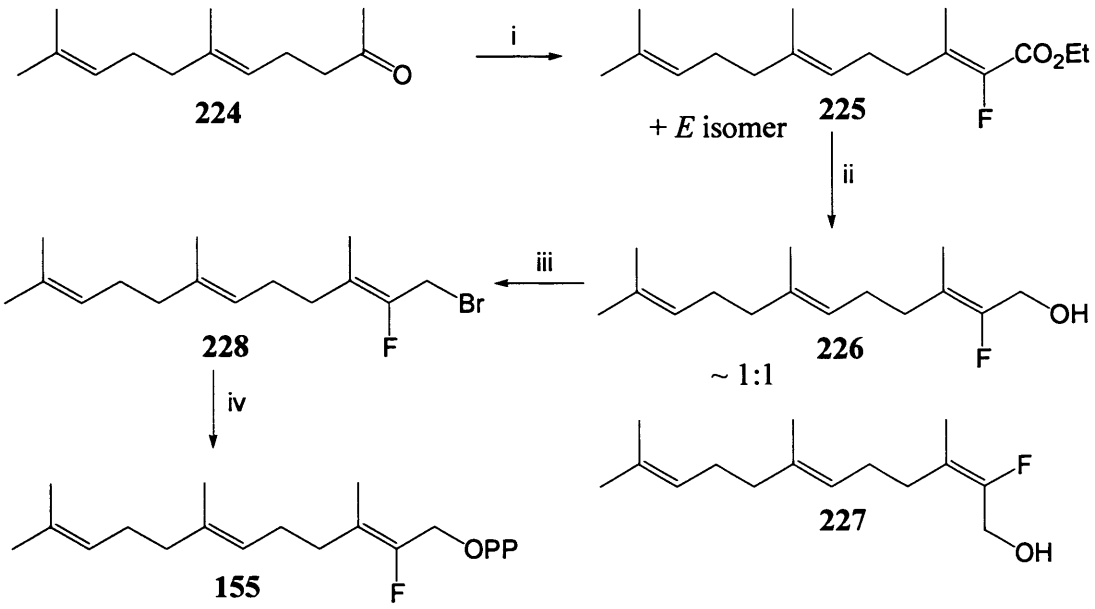
Reagents and conditions: i) HBr, H_2SO_4, toluene, 70 °C, 18 h, 92%; ii) Triethyl -phosphonoacetate, NaH, THF, 0 °C, 14 h, 68%; iii) i) DIBAL-H, THF, -78 °C to 0 °C, 2 h, 98%; iv) 3,4-DHP, p-TsOH, DCM, 0 °C to RT, 16 h, 93%; v) Ph_3P, CH_3CN, reflux, 14 h; vi) 1,3-difluoro acetone, LiHMDS, THF, -78 °C to -20 °C, 2 h, 66% for 2 steps; vii) PPTS, EtOH, 55 °C, 2 h, 97%; viii) TPAP, NMO, CH_3CN, RT, 16 h, 82%; ix) CH_3PPh_3Br, n-BuLi, THF, RT, 16 h, 92%; x) 9-BBN, $PdCl_2dppf$, $AsPh_3$, **165**, NaOH, THF, 50 °C, 15 h, 29%; xi) DIBAL-H, THF, -78 °C, 2 h, 68%; xii) MsCl, NEt_3, LiBr, THF, -45 °C, 2 h; xiii) $(Bu_4N)_3HP_2O_7$, CH_3CN, 2 h, then DOWEX ion exchange Bu_4N^+/NH_4^+, 10% for two steps.

2.3 Vinyl fluoro analogues of farnesyl pyrophosphate

The double bonds in FPP play a very important role in the formation of aristolochene according to the proposed mechanism shown in Scheme 1.7. For example, in the formation of germacryl cation and subsequent eudesmane cation[90] all three double bonds of FPP are involved. Vinyl fluoro analogues of FPP were therefore considered to be powerful tools to study the mechanism of the enzyme-catalysed cyclisations of sesquiterpenoids, reactions that are inherently difficult to study spectrometrically. We therefore decided to synthesize vinyl fluoro FPP analogues using the Weiler and Sum chain extension and sulfonylation-alkylation-desulfonylation methodologies (see section 1.8).

2.3.1 Synthesis of 2-fluoro farnesyl pyrophosphate (155)

The synthesis of 2-fluoro farnesyl pyrophosphate was achieved using a modification of a published procedure.[164] Geranyl acetone (**224**) was treated with triethyl 2-fluoro-2-phosphonoacetate and sodium hydride to give the fluoro ester **225** and its *E*-isomer in 96% yield after purification by silica flash column chromatography (Scheme 2.22). DIBAL-H reduction of the resulting ester mixture gave *E* and *Z* fluoro alcohols **226** and **227** in a ratio of approximately 1:1. Purification by silica column chromatography afforded the pure *Z*-isomer in 46% yield. Bromination followed by diphosphorylation[110] and purification by reverse phase HPLC in the same manner as for the difluorinated analogue **154** (see page 65) gave 2-fluoro FPP **155** in 36% yield over the two steps.

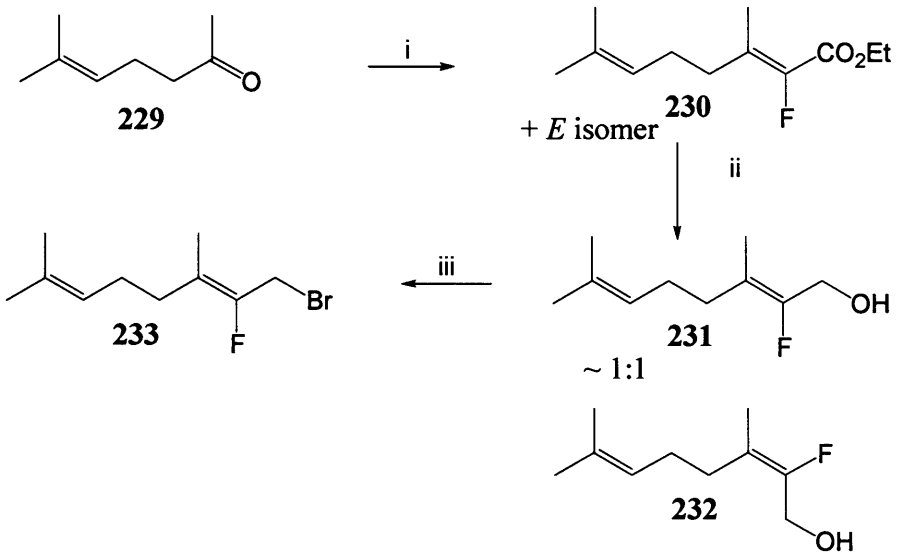


Scheme 2.22: *Synthesis of diphosphate* **155**.

Reagents and conditions: i) Triethyl 2-fluoro-2-phosphonoacetate, NaH, THF, 0 °C, 14 h, 96% including *E* isomer; ii) DIBAL-H, THF, -78 °C, 2 h, 46%; iii) MsCl, TEA, LiBr, THF, -45 °C to 0 °C, 2 h; iv) $(Bu_4N)_3HP_2O_7$, CH_3CN, 2 h, then DOWEX ion exchange Bu_4N^+/NH_4^+, 36% for two steps.

2.3.2 Synthesis of 6-fluoro farnesyl pyrophosphate (156)

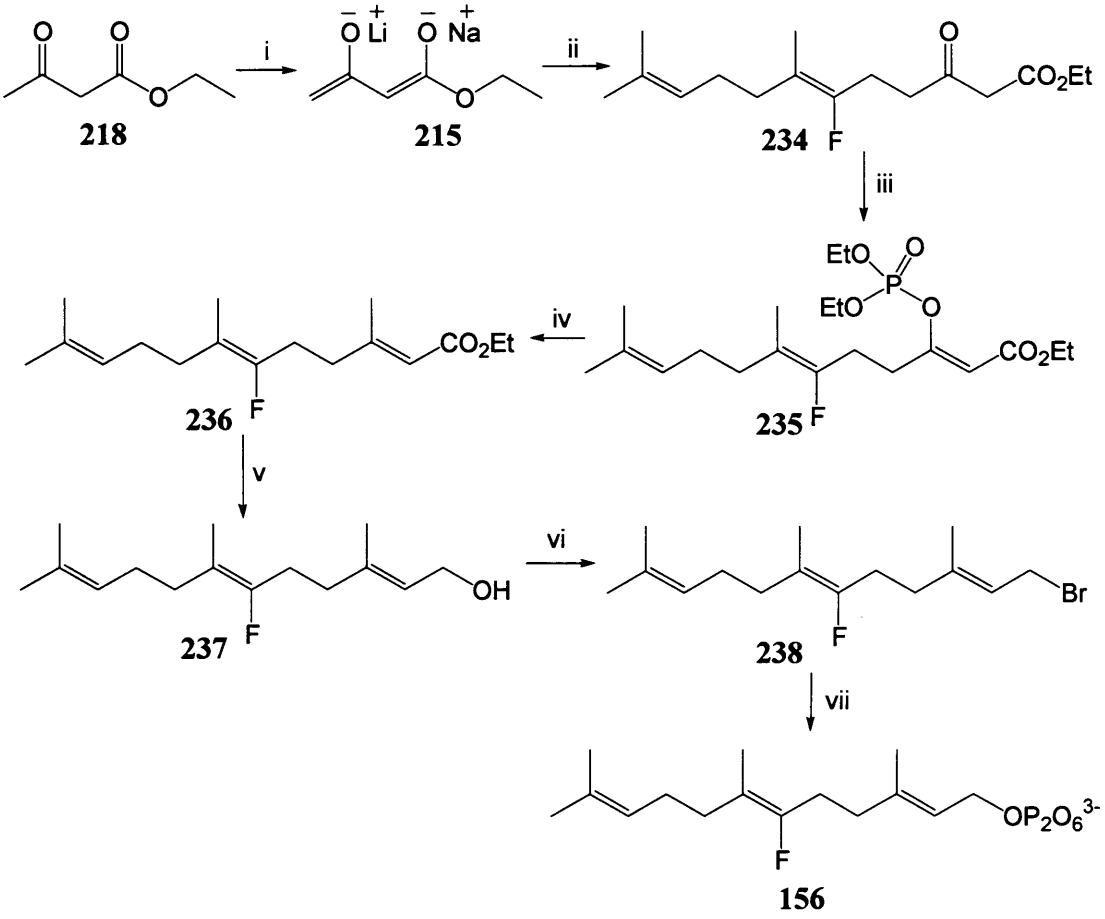
Synthesis of 6-fluoro farnesyl pyrophosphate was also achieved using a modification of a published procedure.[107] Condensation of 6-methyl-5-hepten-2-one (**229**) with triethyl fluorophosphonacetate gave fluoro ester **230** as a mixture of isomers (Scheme 2.23).[165] As separation of these by silica column chromatography proved difficult, the mixture was reduced directly using DIBAL-H to afford chromatographically separable 2-fluorogeraniol isomers, *E*-isomer **231** and *Z*-isomer **232** in approximately 1:1 ratio. The *Z* isomer was separated and then transformed into bromide **233** using methanesulfonyl chloride and lithium bromide.[163]



Scheme 2.23: *Synthesis of fluoro bromide* **233**.

Reagents and conditions: i) Triethyl 2-fluoro-2-phosphonoacetate, NaH, THF, 0 °C, 14 h; ii) DIBAL-H, THF, -78 °C, 2 h, 46%; iii) MsCl, TEA, LiBr, THF, -45 °C to 0 °C, 2 h, used directly in next step.

Alkylation of lithio-sodio acetoacetate dianion **215** with bromide **233** provided fluoro β-keto ester **234** in 98% yield. This was then converted to the *E* enol phosphate **235** using diethyl chlorophosphate and sodium hydride in 95% yield.[162] Treatment with lithium dimethyl cuprate at –78°C gave fluoro ester **236** in 95% yield.[162] Reduction with DIBAL-H then gave fluoro farnesol **237** in 91% yield. Activation of the alcohol as the allylic bromide **238**, followed by reaction with tris tetrabutyl ammonium hydrogen pyrophosphate gave 6-fluoro farnesyl pyrophosphate tetra-n-butylammonium salt. Cation-exchange chromatography followed by reverse phase HPLC purification finally gave the 6-fluoro farnesyl pyrophosphate ammonium salt **156** in 44% yield for the last two steps.[110]



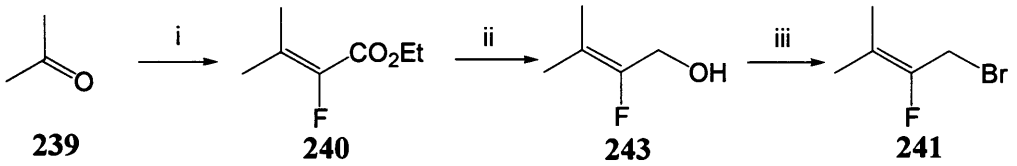
Scheme 2.24: *Synthesis of diphosphate* **156**.

Reagents and conditions: i) NaH, n-BuLi, THF, 0 °C, 20 min; ii) **233**, THF, 0 °C, 98% for two steps; iii) Diethyl chlorophosphate, NaH, Et_2O, 0 °C, 1 h, 97%; iv) CuI, MeLi, Et_2O, -78 °C, 3.5 h, 95%; v) DIBAL-H, THF, -78 °C, 91%; vi) MsCl, Et_3N, then LiBr, THF, -45 °C to 0 °C, 2 h; vii) $(Bu_4N)_3HP_2O_7$,CH_3CN, 2 h, then DOWEX ion exchange Bu_4N^+/NH_4^+, 48% for two steps.

2.3.3 Synthesis of 10-fluoro farnesyl pyrophosphate (157)

10-Fluoro farnesyl pyrophosphate was synthesized using a modification of the procedure of Cane *et al.*,[116] who tested it as an effective competitive inhibitor for trichodiene synthase. The key steps in this synthetic route were a coupling reaction between a sulfone and a bromide followed by a reductive desulfonylation.

Acetone (**239**) was treated with triethyl 2-fluoro-2-phosphonoacetate and sodium hydride to give ethyl 2-fluoro-3-methylbut-2-enoate (**240**) as a colorless oil in 47% yield (Scheme 2.25).[116] DIBAL-H reduction followed by treatment with methane sulfonyl chloride and lithium bromide gave bromide **241**. The bromide was not purified and was coupled directly with sulfone **242** (see Scheme 2.26).



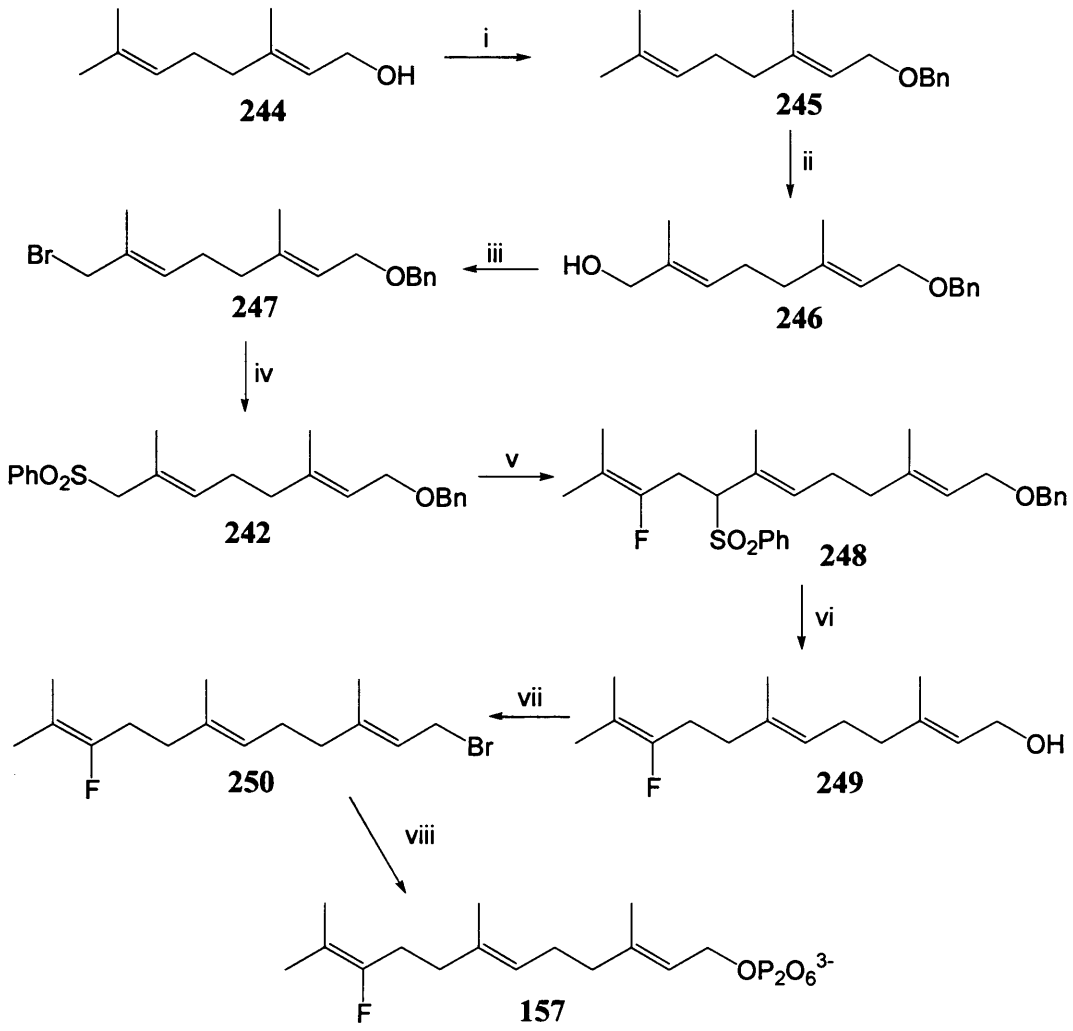
239 **240** **243** **241**

Scheme 2.25: *Synthesis of bromide* **241**.

Reagents and conditions: i) Triethyl 2-fluoro-2-phosphonoacetate, NaH, THF, RT, 14 h, 47%; ii) DIBAL-H, THF, -78 °C, 2 h; iii) MsCl, TEA, LiBr, THF, -45 °C to 0 °C, 2 h;

Geraniol (**244**) was treated with benzyl bromide and sodium hydride to afford the benzyl ether **245** in 94% yield.[157] Oxidation of the terminal methyl group in the presence of selenium dioxide, tert-butyl hydroperoxide and acetic acid gave alcohol **246** in 32% yield after purification by silica column chromatography. The poor yield was attributed to over oxidation with the aldehyde being isolated in 30% yield.[166, 167] The alcohol **246** was then treated with methanesulfonyl chloride and lithium bromide and the resulting crude bromide **247** was directly reacted with benzenesulfinic acid sodium salt to afford the sulfone **242** in 82% yield over the two steps.[168] Sulfone **242** was coupled to bromide **241** using *n*-BuLi at –78 °C to afford the fluoro sulfone **245** in 76% yield. Reductive desulfonylation in the presence of lithium and *tert*-butyl alcohol also lead to

debenzylation to afford the alcohol **249** in 28% yield.[116] Bromination and then diphosphorylation in the same manner as described previous gave diphosphate 157 in 56% yield.



Scheme 2.26: *Synthesis of diphosphate* **157**.

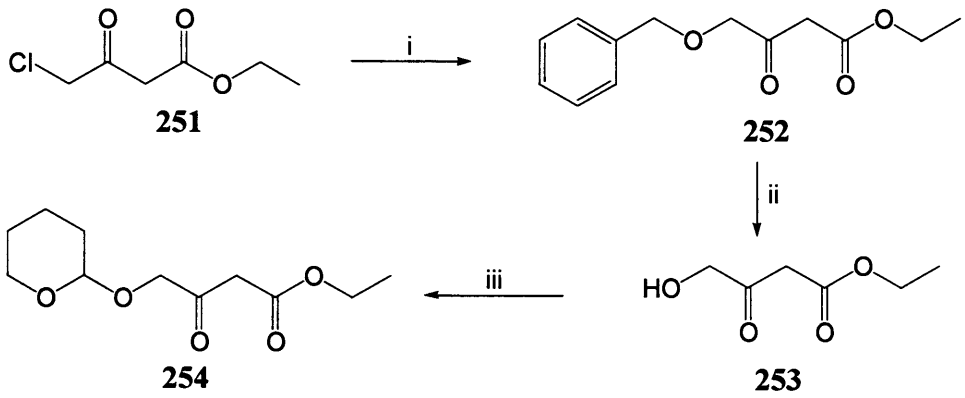
Reagents and conditions: i) Benzyl bromide, NaH, THF, 0 °C to RT, 3 h, 94%; ii) t-BuOOH, SeO_2, CH_3COOH, DCM, RT, 16 h, 32%; iii) MsCl, TEA, LiBr, THF, -45 °C to 0 °C, 2 h; iv) Benzensulfinic acid sodium salt, DMF, RT, 20 h, 82% for two steps; v) **241**, n-BuLi, THF, -78 °C to 0 °C, 3 h, 76%; vi) t-BuOH, Li, THF, RT, 20 h, 28%; vii) MsCl, TEA, LiBr, THF, -45 °C to 0 °C, 2 h; viii) $(Bu_4N)_3HP_2O_7$, CH_3CN, 2 h, then DOWEX ion exchange Bu_4N^+/NH_4^+, 56% for two steps.

2.4 14-Fluoro farnesyl diphosphate (158) and 15-fluoro farnesyl diphosphate (159)

The 14-F and 15-F FPP analogues were synthesized using similar metholodgy. The key steps involved the introduction of fluorine with TBAF and a Wittig or Horner-Wadsworth-Emmons reaction with the resulting fluorinated ketone.

2.4.1 Synthesis of 14-fluoro farnesyl pyrophosphate (158)

14-Fluoro FPP was synthesized using a modification of the method of Poulter *et al.*[123] Treatment of ethyl 4-chloroacetoacetate (**251**) with benzyl alcohol in the presence of sodium hydride gave the benzyl protected compound **252** in 82% yield.[169] Cleavage of the benzyl group was achieved by reductive hydrogenolysis using H_2 and 10% Pd/C catalyst gave the pure alcohol **253** in 95% yield after purification by silica column chromatography.[157] Treatment with 3,4-dihydro-2*H*-pyran and catalytic amount of *p*-toluenesulfonic acid then gave the THP ether **254**.[159]



251 **252** **253** **254**

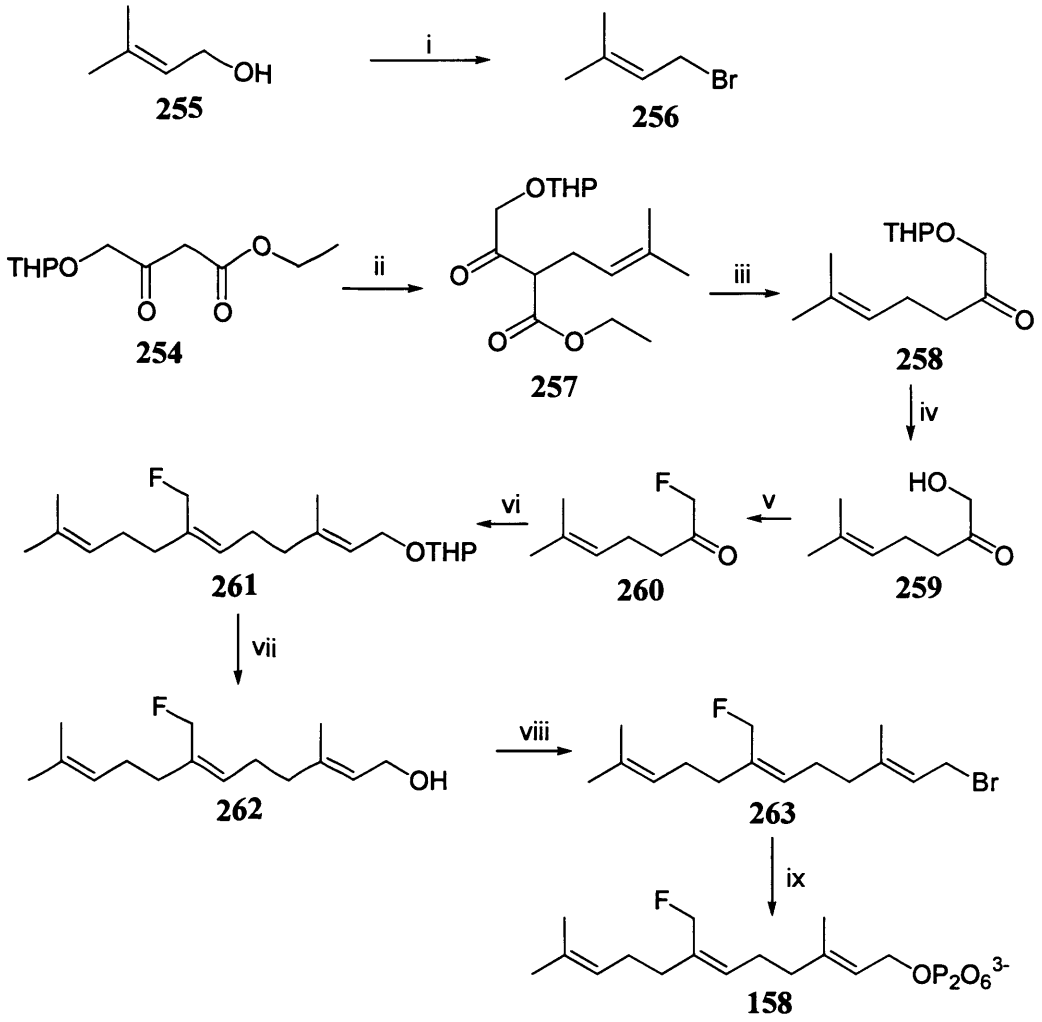
Scheme 2.27: *Synthesis of THP ether* **254**.

Reagents and conditions: i) Benzyl alcohol, NaH, toluene, 0 °C to RT, 18 h, 82%; ii)H_2, Pd/C (10% w/w), EtOH, 16 h, 95%; iii) 3,4-DHP, p-TsOH, DCM, 0 °C to RT, 16 h.

Alkylation of the THP ether **254** with 1-bromo-3-methylbut-2-ene (**256**) (derived from the reaction of 3-methyl-2-butene-1-ol (**255**) with methane sulfonyl chloride and lithium bromide) in the presence of sodium hydride gave ester **257** in 53% yield (Scheme 2.28). Base-mediated decarboxylation using KOH in ethanol afforded THP protected ketone **258** in 52% yield.[159]

The THP protecting group was then removed using pyridinium p-toluenesulfonate (PPTS) to afford keto alcohol **259** in 95% yield.[161] Fluorination was achieved using TBAF in the presence of triflic anhydride and 2,6-lutidine.[170] Due to the highly volatility of the resulting fluoride **260**, eluents from silica column chromatography were not concentrated completely under reduced pressure.

Fluoro ketone **260** (with residual solvent) was then subjected to a Wittig reaction with phosphonium salt **214** (see Scheme 2.17) to afford the THP ether **261** as major product in 30% yield over the three steps. Removal of the THP protecting group using PPTS gave alcohol **262** in 91% yield after purification by silica column chromatography.[161] Bromination and then diphosphorylation were carried out using the same method described previously. 14-Fluoro FPP (**158**) was eventually obtained in 45% yield (from **262**) after HPLC purification.



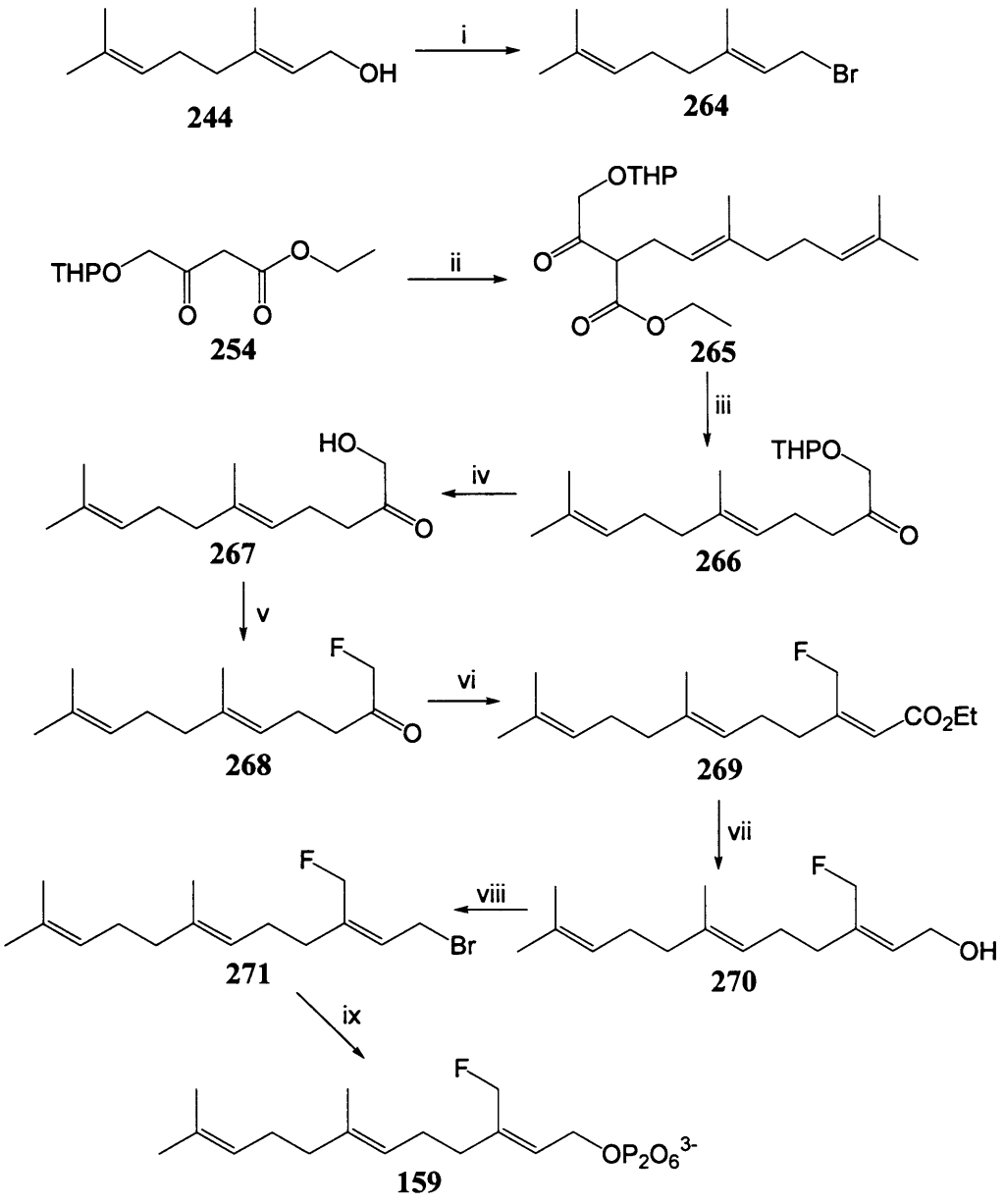
Scheme 2.28: *Synthesis of diphosphate* **158**.

Reagents and conditions: i) MsCl, TEA, LiBr, THF, -45 °C to 0 °C, 2 h; ii) NaH, **256**, THF, 0 °C to RT, 16 h, 53%; iii) KOH, EtOH, RT, 24 h, 52%; i) PPTS, EtOH, RT to 50 °C, 18 h, 95%; ii) 2,6-Lutidine, triflic anhydride, then TBAF, THF, 0 °C to RT, 2.5 h; iii) LiHMDS, **214**, THF, -78 °C to -20 °C, 3 h, 30% for 3 steps; iv) PPTS, EtOH, 55 °C, 2 h, 91%; v) MsCl, TEA, LiBr, THF, -45 °C to 0 °C, 2 h; vi) $(Bu_4N)_3HP_2O_7$, CH_3CN, 2 h, then DOWEX ion exchange Bu_4N^+/NH_4^+, 45% for two steps.

2.4.2 Synthesis of 15-fluoro farnesyl pyrophosphate (159)

15-Fluoro farnesyl pyrophosphate was also synthesized using a modification of a literature procedure.[123] The main steps similar to those described above for 14-fluoro FPP (**158**). Bromide **264**, required for alkylation with THP ether **254**, was synthesized from geraniol by treatment with methanesulfonyl chloride and lithium bromide (Scheme 2.29). Alkylation of THP ether **254** with bromide 264 using sodium hydride afforded keto ether **265** in 46% yield after purification by silica column chromatography.[123] Decarboxylation with KOH followed by treatment with PPTS gave the keto alcohol **267** in 60% yield over the two steps.[123]

Conversion of alcohol **267** to fluoride **268** was carried in the same manner as described for the preparation of fluoride **260**. However in this case, due to the larger molecular weight of fluoride **268** (compaired with fluoride **260**), this compound was less volatile and therefore isolated for full characterization. The fluoride **268** was then treated with triethyl phosphonoacetate and sodium hydride to give fluoro ester **269** as a mixture of *E* and *Z* isomers in a ratio of 1 : 10.[130] The two isomers had very similar chromatographic properties; the R_f values for *Z* isomer and *E* isomer were 0.18 and 0.17 for 60:1 hexane-ethylacetate, so medium performance liquid chromatography (MPLC) was used to perform the separation. After four rounds of MPLC, the *Z* isomer of **269** was isolated in 64% yield. The ester was finally converted to 15-fluoro FPP **159** using the usual sequence of reduction, bromination and diphosphorylation steps. Cation exchange followed by HPLC purification gave the desired compound in 34% yield over the last three steps.



Scheme 2.29: *Synthesis of diphosphate* **159**.

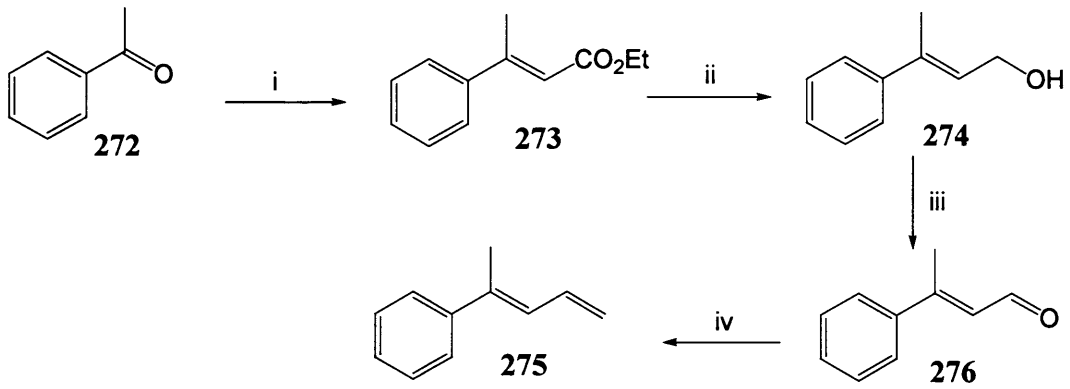
Reagents and conditions: i) MsCl, TEA, LiBr, THF, -45 °C to 0 °C, 2 h; ii) NaH, **264**, THF, 0 °C to RT, 16 h, 46%; iii) KOH, EtOH, 75%; iv) PPTS, EtOH, RT to 50 °C, 18 h, 81%; v) 2,6-lutidine, triflic anhydride, then TBAF, THF, 0 °C to RT, 2.5 h, 69%; vi) NaH, triethyl- phosphonoacetate, THF, 0 °C, 16 h, 64%; vii) DIBAL-H, THF, -78 °C, 2 h, 80%; viii) MsCl, TEA, LiBr, THF, -45 °C to 0 °C, 2 h; ix) $(Bu_4N)_3HP_2O_7$, CH_3CN, 2 h, then DOWEX ion exchange Bu_4N^+/NH_4^+, 43% for two steps.

2.5 Phenyl substituted FPP analogues

Three phenyl substituted FPP analogues were prepared using the novel Suzuki-Miyaura cross-coupling methodology in the key step (see section 1.10).

2.5.1 Synthesis of *E*- and *Z*-11-phenyl farnesyl diphosphate analogues 160 and 161

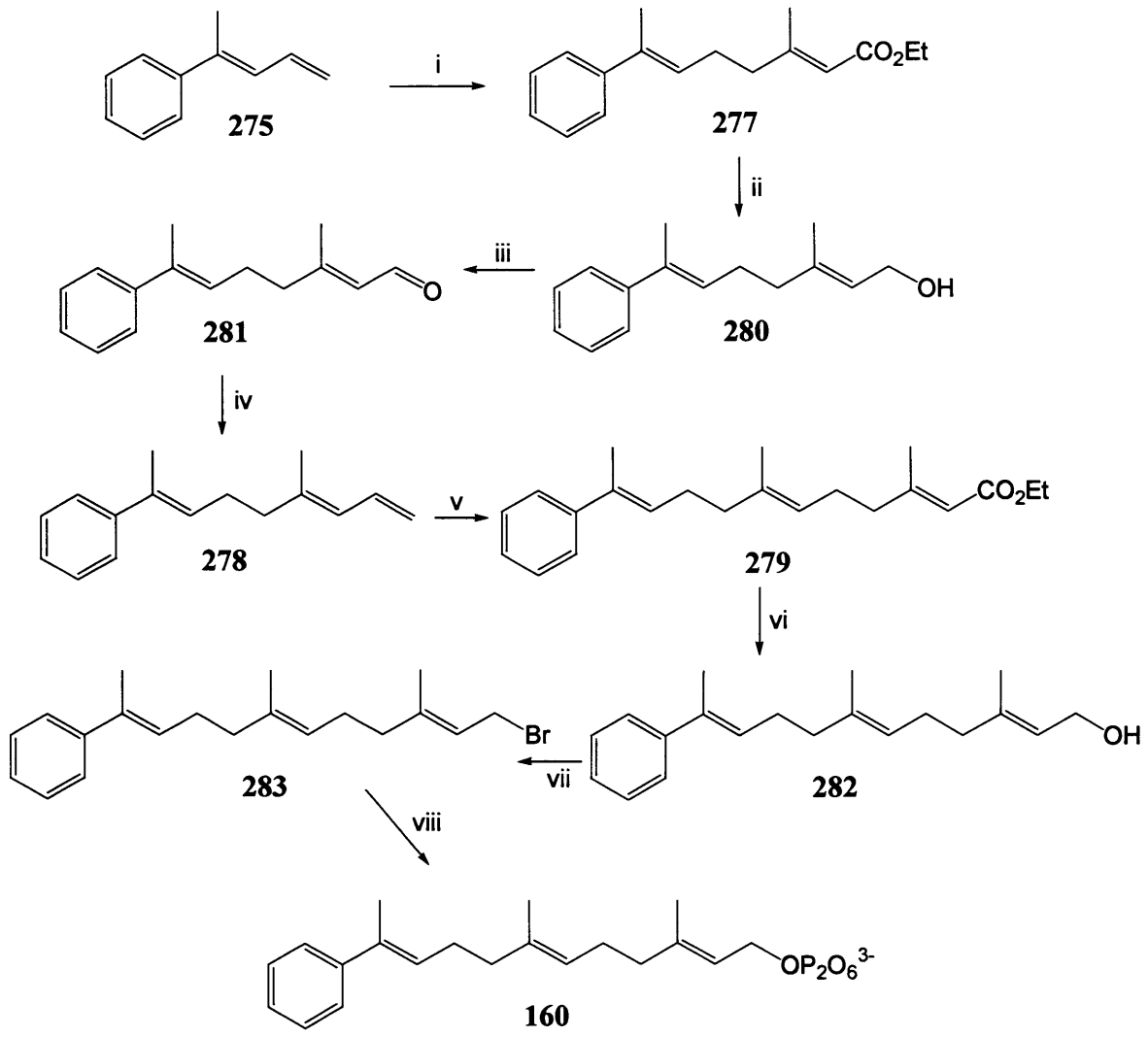
The synthesis of the *E*-11-phenyl FPP analogue **160** is shown in Schemes 2.30 and 2.31. Reaction of acetophenone (**272**) with triethyl phosphonoacetate under Horner-Emmons condensation lead to ester **273** in good stereoselectivity (*E* : *Z* = 12 : 1) (Scheme 2.30).[171] DIBAL-H reduction followed by oxidation of the resulting alcohol **274** using PCC[172, 173] and then a Wittig reaction with methyltriphenylphosphonium bromide gave the required *E*-phenyl diene **275** in 34% overall yield over the four steps.



Scheme 2.30: *Synthesis of diene* **275**.

Reagents and conditions: i) NaH, triethylphosphonoacetate, DME, 3 h, 61%; ii) DIBAL-H, THF, -78 °C, 2 h, 96%; iii) PCC, DCM, 4 h, 57%; iv) CH_3PPh_3Br, n-BuLi, THF, 78%.

E-Phenyl diene **275** was then hydroborated using crystalline 9-BBN as described before (see section 2.2.1) and immediately coupled to iodide **165** using $PdCl_2$dppf as catalyst, triphenylarsine as co-ligand and aqueous sodium hydroxide as base[124, 126-129] to give *E*-phenyl geranyl ester product **277** in 59% yield (Scheme 2.31). Homologation to the 1,3-diene derivative **278** was carried in a manner similar to that described above (Scheme 2.30). A second Suzuki coupling with iodide **165** then gave the farnesyl ester analogue **279** in 28% yield over the four steps. Ester **279** was transformed into the desired *E*-11 phenyl FPP analogue **160** in 27% yield following the normal sequence of reduction, bromination and diphosphorylation (Scheme 2.31).

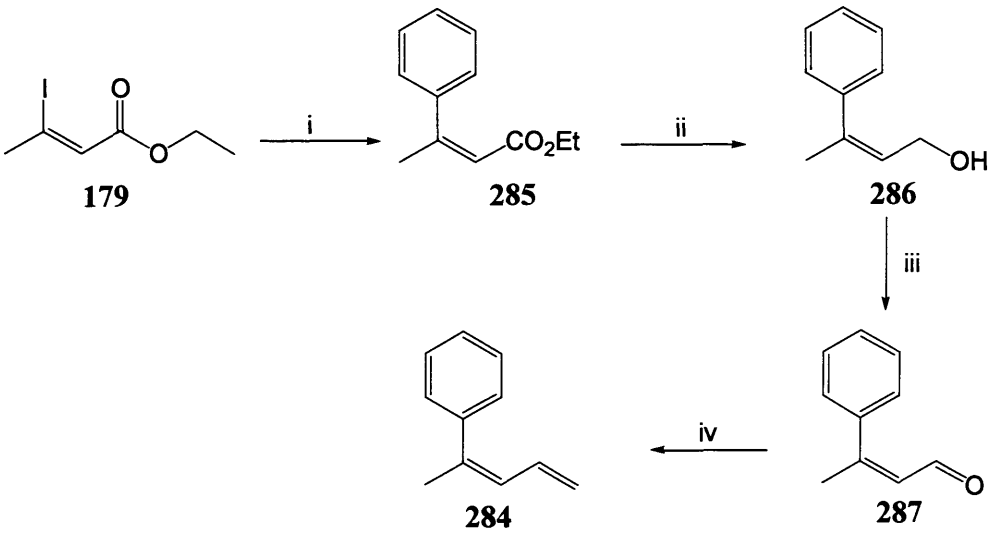


Scheme 2.31: *Synthesis of diphosphate* **160**.

Reagents and conditions: i) 9-BBN, THF, then iodide **165**, $PdCl_2$dppf, NaOH, $AsPh_3$, THF, 50 °C, 16 h, 59%; ii) DIBAL-H, THF, -78 °C, 2 h, 96%; iii) TPAP, NMO, CH_3CN, RT, 16 h, 89%; iv) CH_3PPh_3Br, n-BuLi, THF, 16 h, 81%; v) 9-BBN, THF, then **165**, $PdCl_2$dppf, NaOH, $AsPh_3$, THF, 50 °C, 16 h, 54%; vi) DIBAL-H, THF, -78 °C, 2 h, 86%; vii) MsCl, NEt_3, then LiBr, THF, -45 °C to 0 °C, 2 h; viii) $(Bu_4N)_3HP_2O_7$, CH_3CN, 2 h, then DOWEX ion exchange Bu_4N^+/NH_4^+, 31% for two steps.

Preparation of the *Z*-11-phenyl FPP analogue **161** was achieved in much the same fashion as shown in Schemes 2.32 and Scheme 2.33. Preparation of the initial *Z*-1,3-diene **284** was a little more complex because Horner-Emmons or Wittig modification of acetophenone (**272**) only

yielded a small amount of the *Z*-ester **285**. A Suzuki-Miyaura coupling of phenylboronic acid to *Z*-crotonyl iodide **179** (an intermediate in the preparation of **165**)[140] however, gave ester **285** in 85% yield after purification by silica column chromatography (Scheme 2.32).[174]

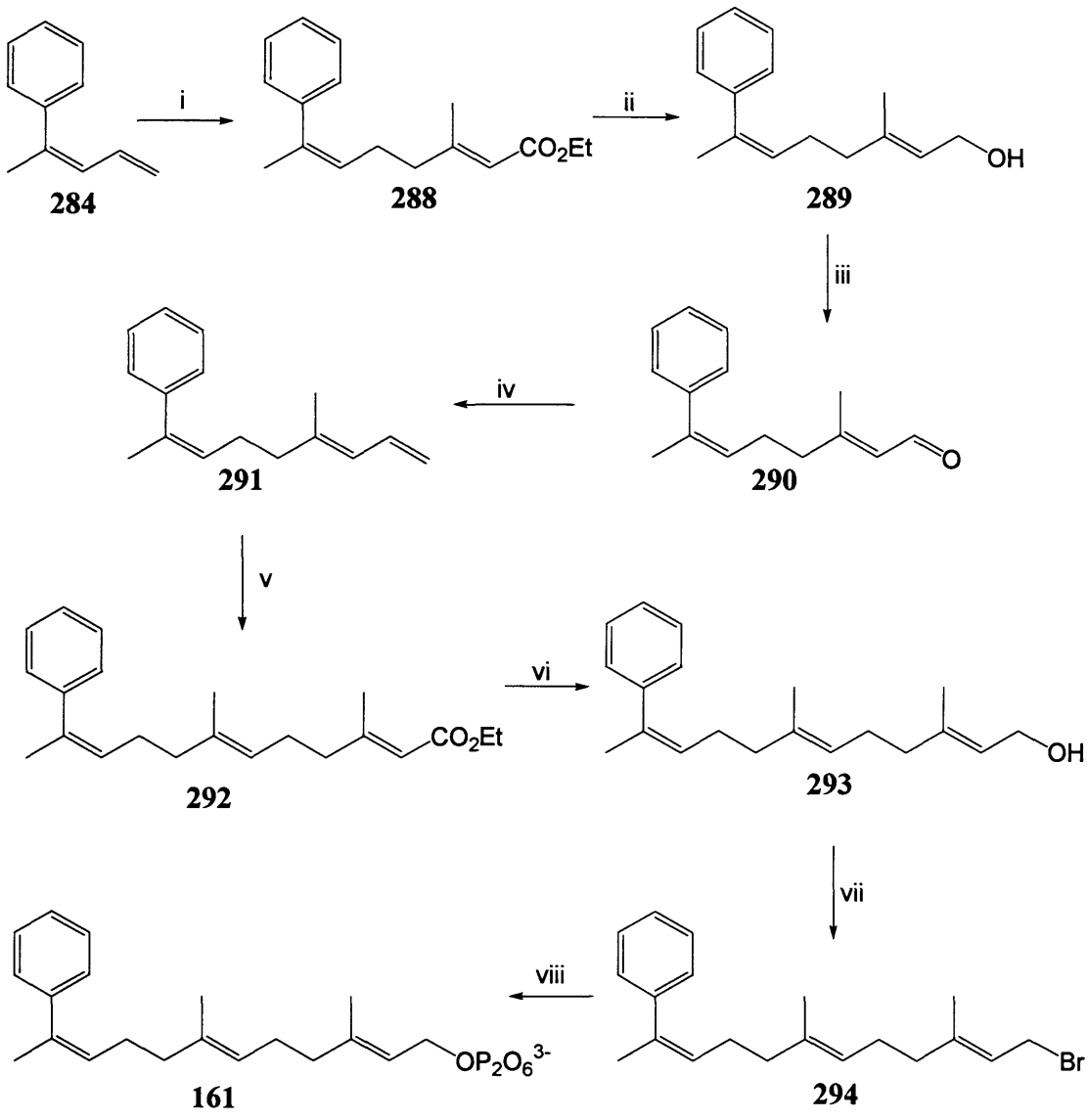


179 **285** **286** **287** **284**

Scheme 2.32: *Synthesis of diene* **284**.

Reagents and conditions: i) $PhB(OH)_2$, $Pd(OAc)_2$, $AsPh_3$, K_3PO_4, toluene, 90 °C, 6 h, 85%; ii) DIBAL-H, THF, -78 °C, 2 h, 99%; iii) oxalyl chloride, DMSO, DCM, -78 °C, then NEt_3, 30 min, 88%; iv) CH_3PPh_3Br, n-BuLi, THF, 16 h, 76%.

Using DIBAL-H ester **285** was transformed into alcohol **286** and then to aldehyde **287** by Swern oxidation[175] and followed by a Wittig reaction to generate 1,3-diene **284**. Diene **2284** was then converted to the *Z*-11-phenyl FPP analogue **161** using almost identical chemistry described above for the preparation of the *E*-11-phenyl FPP analogue **160** (Scheme 2.30 and 2.31). The two crucial Suzuki-Miyaura coupling steps took place in approximately 40% yield.

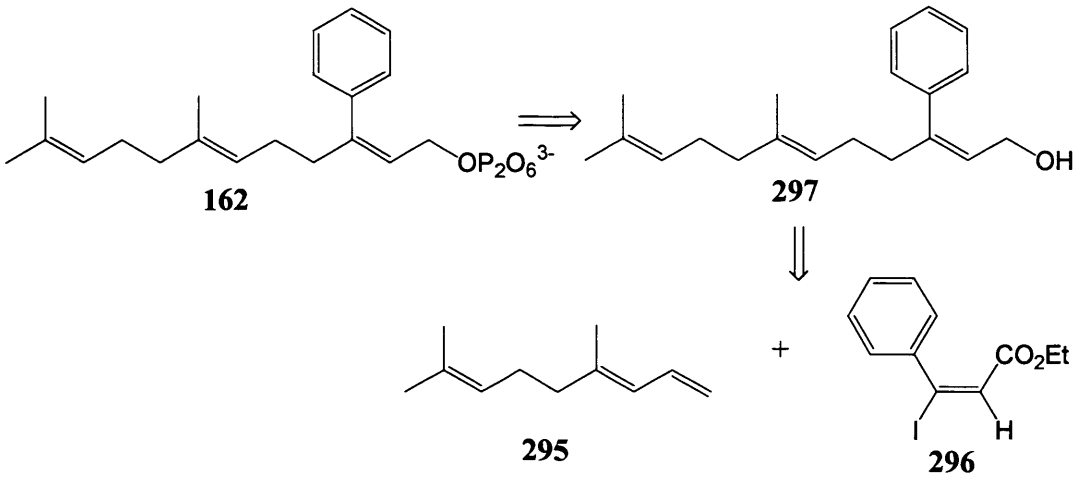


Scheme 2.33: *Synthesis of diphosphate* **161**.

Reagents and conditions: i) 9-BBN, THF, then **165**, $PdCl_2dppf$, NaOH, $AsPh_3$, THF, 50 °C, 16 h, 41%; ii) DIBAL-H, THF, -78 °C, 2 h, 91%; iii) TPAP, NMO, CH_3CN, 16 h, 87%; iv) CH_3PPh_3Br, n-BuLi, THF, 16 h, 86%; v) 9-BBN, THF, then **165**, $PdCl_2dppf$, NaOH, $AsPh_3$, THF, 50 °C, 16 h, 40%, vi) DIBAL-H, THF, -78 °C, 2 h, 77%; vii) MsCl, NEt_3, then LiBr, THF, -45 °C to 0 °C, 2 h; viii) $(Bu_4N)_3HP_2O_7$, CH_3CN, 2 h, then DOWEX ion exchange Bu_4N^+/NH_4^+, 36% for two steps.

2.5.2 Synthesis of 3-phenyl farnesyl diphosphate (162)

We envisaged synthesis of the 3-phenyl farnesyl diphosphate **162** starting from *Z*-crotonyl triene **295** and iodide **296** (Scheme 2.34).

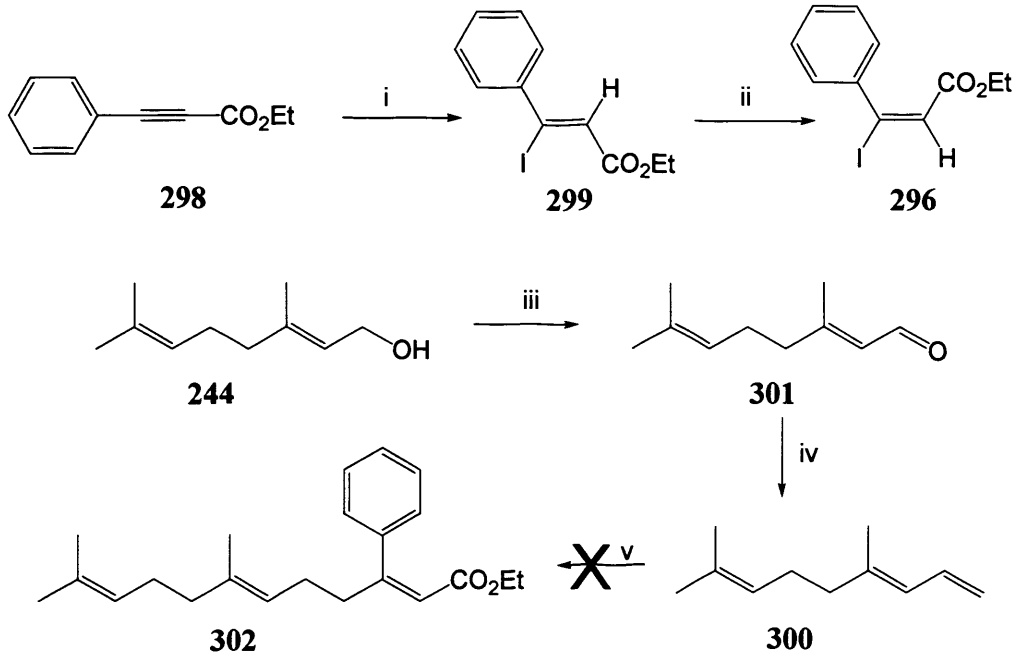


Scheme 2.34: *Retrosynthesis of diphosphate* **162**.

E-Crotonyl iodide (**296**) was prepared in two steps from ethyl phenylpropiolate (**298**) as described previously for iodide **165** (Scheme 2.35).[138, 139] However, as the separation of the isomeric mixture of iodides **296** and **299** by flash silica column chromatography proved difficult (due to very similar R_f values),[139] the mixture was used directly in the next step.

To synthesize the other component of the Suzuki coupling, geraniol (**244**) was subjected to TPAP oxidation[143] followed by a Wittig reaction with methylphosphonium bromide (Scheme 2.35). The resulting 1,3-diene **300** which was obtained in 64% yield, was subjected to our new Suzuki coupling methodology with iodide **296**, as described previously (*vide supra*). However, when the reaction was carried out even at a higher temperature (65 °C), no coupling occurred, presumably

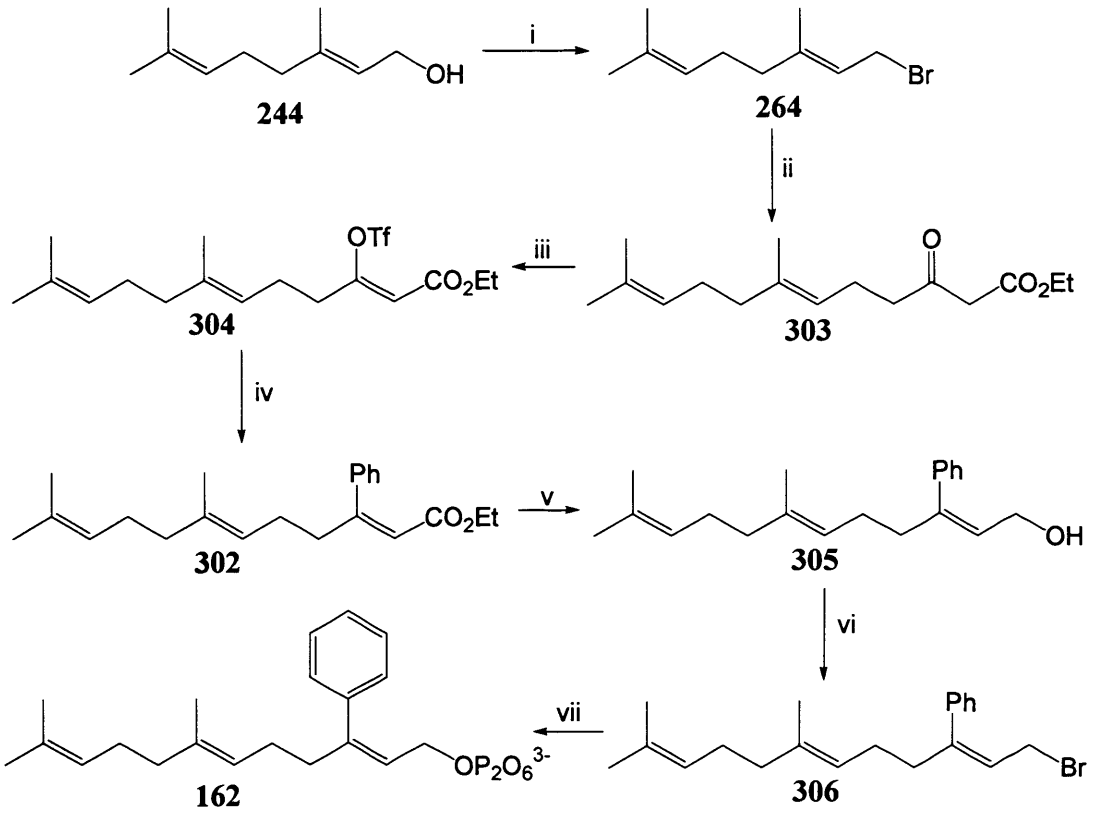
because the bulky phenyl ring hindered attack of the iodide.



Scheme 2.35: *Attempted synthesis of ester* **302**.

Reagents and conditions: i) NaI, CH_3COOH, 115 °C, 1.5 h, 87%; ii) sealed tube under argon, 220 °C, 3 h; iii) TPAP, NMO, CH_3CN, RT, 16 h, 82%; iv) CH_3PPh_3Br, n-BuLi, THF, 16 h, 80%; v) 9-BBN, THF, then **296**, $PdCl_2dppf$, NaOH, $AsPh_3$, THF, 65 °C, 16 h.

In order to resolve the above problem, we used the chain extension methodology of Weiler and Sum.[118] Geraniol (**244**) was converted into geranyl bromide (**264**) and then treated with dienolate **215** (see Section 2.2.5) to give β-ketoester **303** in 92% yield.[118, 162] Treatment with triflic anhydride and KHMDS gave the *Z*-enol triflate **304** in 51% yield.[176, 177] Suzuki-Miyaura cross-coupling with phenylboronic acid resulted in the formation of 3-phenyl ester **302** in 66% yield.[178, 179] Reduction followed by derivatisation in the usual manner gave 3-phenyl FPP analogue **162** in 26% yield over three steps.



Scheme 2.36: *Synthesis of diphosphate* **162**.

Reagents and conditions: i) MsCl, TEA, LiBr, THF, -45 °C to 0 °C, 2 h; ii) **215**, THF, 0 °C to RT, 2 h, 92%; iii) KHMDS, $(CF_3SO_2)_2O$, THF, -78 °C to RT, 16 h, 51%; iv) $PhB(OH)_2$, $Pd(OAc)_2$, $AsPh_3$, Ag_2O, THF, reflux, 15 h, 66%; v) DIBAL-H, THF, -78 °C, 2 h, 85%; vi) MsCl, NEt_3, then LiBr, THF, -45 °C to 0 °C, 2 h; vii) $(Bu_4N)_3HP_2O_7$, CH_3CN, 2 h, then DOWEX ion exchange Bu_4N^+/NH_4^+, 31% for two steps.

CHAPTER 3

INCUBATION OF FPP ANALOGUES WITH AS

3.1 Introduction

After obtaining the novel FPP analogues, the following task of this project was testing them with sesquiterpene synthase. As an analogue of FPP, they were expected to bind well to the active site, and there are two likely results from the incubation of FPP analogues and sesquiterpene synthases.

First, the FPP analogues are accepted as substrates by enzymes. This is an effective strategy for the investigation of sesquiterpene synthases catalysing multistep transformations in which the intermediates are sequestered by the enzyme. Substrate analogues are suitable designed so as to prevent completion of the normal catalytic cycle, ether by stabilization of an otherwise reactive intermediate or by the absence of a critical reactive centre.[90, 103, 104] The abortive cyclization products thus generated are released from the enzyme and are diagnostic of the structure and stereochemistry of the normal enzyme bound intermediates.[107, 121]

Second, the FPP analogues can act as an inhibitor to the enzymes. The development of mechanism-based inhibitors for sesquiterpene synthases is particularly challenging since these enzymes have evolved to handle highly reactive electrophilic species-cationic intermediates.[92] However, one strategy to overcome this problem is to use substrate analogues, which upon cyclization, could undergo rearrangement or delocalisation to place positive charge in a region of the protein that does not normally encounter reactive electrophilic centers and that therefore would be susceptible to alkylation.[92, 105, 116, 119, 120]

As introduced in the first chapter, it is known that the mechanistic details of the cyclisation of FPP by aristolochene synthase from *P. roqueforti* have only recently begun to emerge, mainly through the analysis of the reaction products generated by AS-mutants.[58] However, there is still some details that need further investigation, for example, little is known about the reaction mechanism of the formation of the decalin ring system. This could be formed by several mechanistically distinct pathways (see Scheme 3.1).[50, 57, 58, 77, 89] In order to explore more deeply the mechanism of the reaction catalysed by AS, the nine FPP analogues were incubated with AS and the results were analysed using a combination of mass spectrometry and NMR.

3.2 Preparation of wild-type aristolochene synthase

In order to understand how aristolochene synthase catalyses the cyclisation of FPP analogues, and also the effects of the substituent functional groups, the preparation of AS is necessary. Therefore, the cDNA that codes for aristolochene synthase, *Ari1*, was expressed, and the protein purified according to a the published method.[49, 59] The whole procedure is described in Experimental.

3.3 Incubation results for 12,13-difluoro FPP (154)

Incubation of 12,13-difluoro FPP (**154**) with purified recombinant aristolochene synthase from *Penicillium roqueforti* in *p*H 7.5 Tris buffer in the presence of Mg^{2+} at room temperature[58] did not lead to any hexane extractable products as judged by GC-MS analysis even after prolonged incubation up to seven days. Since 12,13-difluoro FPP clearly did not act as a substrate for AS, it was tested as an inhibitor of AS. The steady-state kinetic parameters of purified recombinant AS were measured in the presence of varying amounts of 12,13-difluoro FPP by incubation with

[1-^{3}H]-FPP and monitoring the formation of tritiated, hexane extractable products by Dr. David J. Miller, a co-worker on the project. A double reciprocal plot indicated that 12,13-difluoro FPP was a reversible competitive inhibitor of AS (Figure 3.1). The measured K_I of 0.8 ± 0.2 mM was comparable to the Michaelis constant for AS catalysis (K_M = 2.3 mM) reported previously indicating that the fluoro substituents do not have a negative effect on the affinity of the enzyme for this compound.[180]

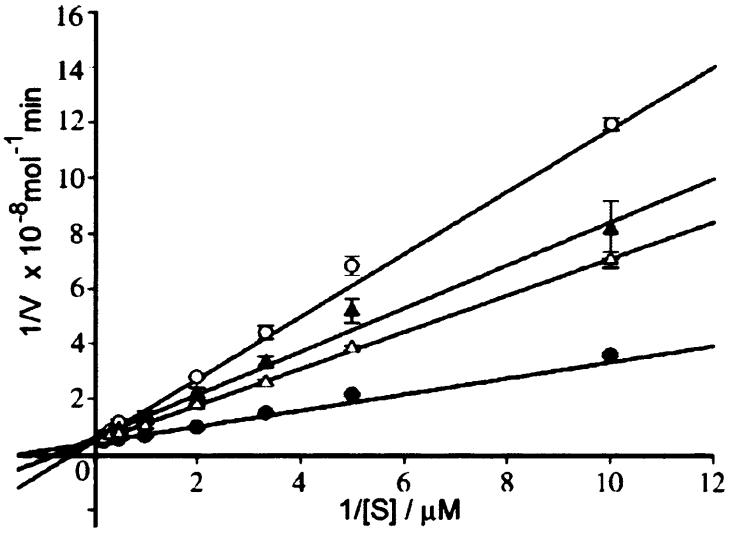
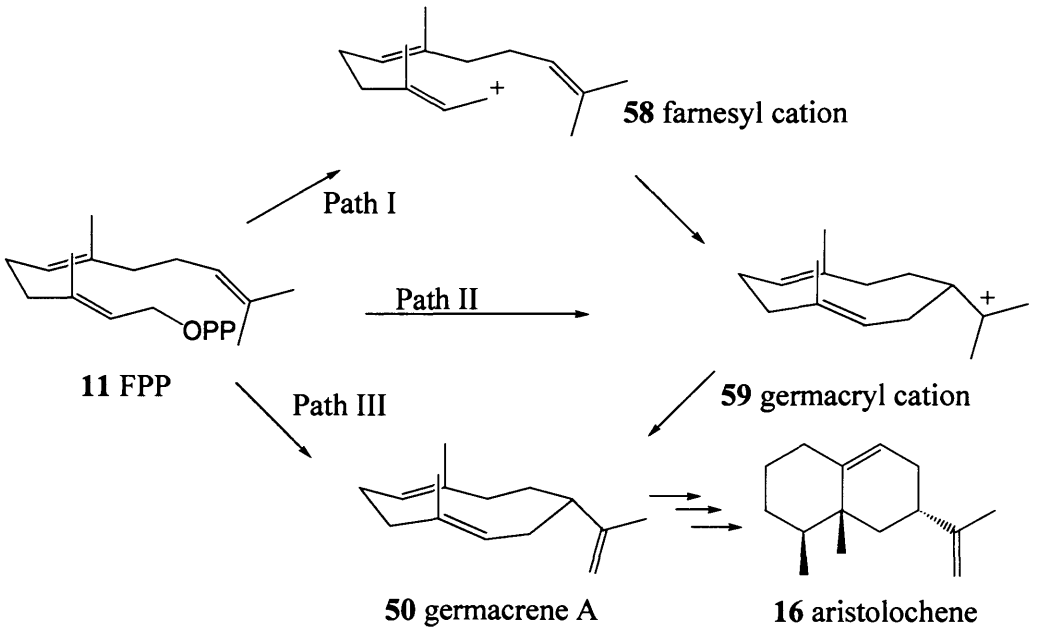


Figure 3.1: *Double reciprocal plot for inhibition of AS catalysed turnover of FPP* (**11**) *by 12,13-difluoro FPP* (**154**) *at 0 (•), 0.4 µM (Δ), 0.6 µM (▲)and 1 µM (○) of* **154**.

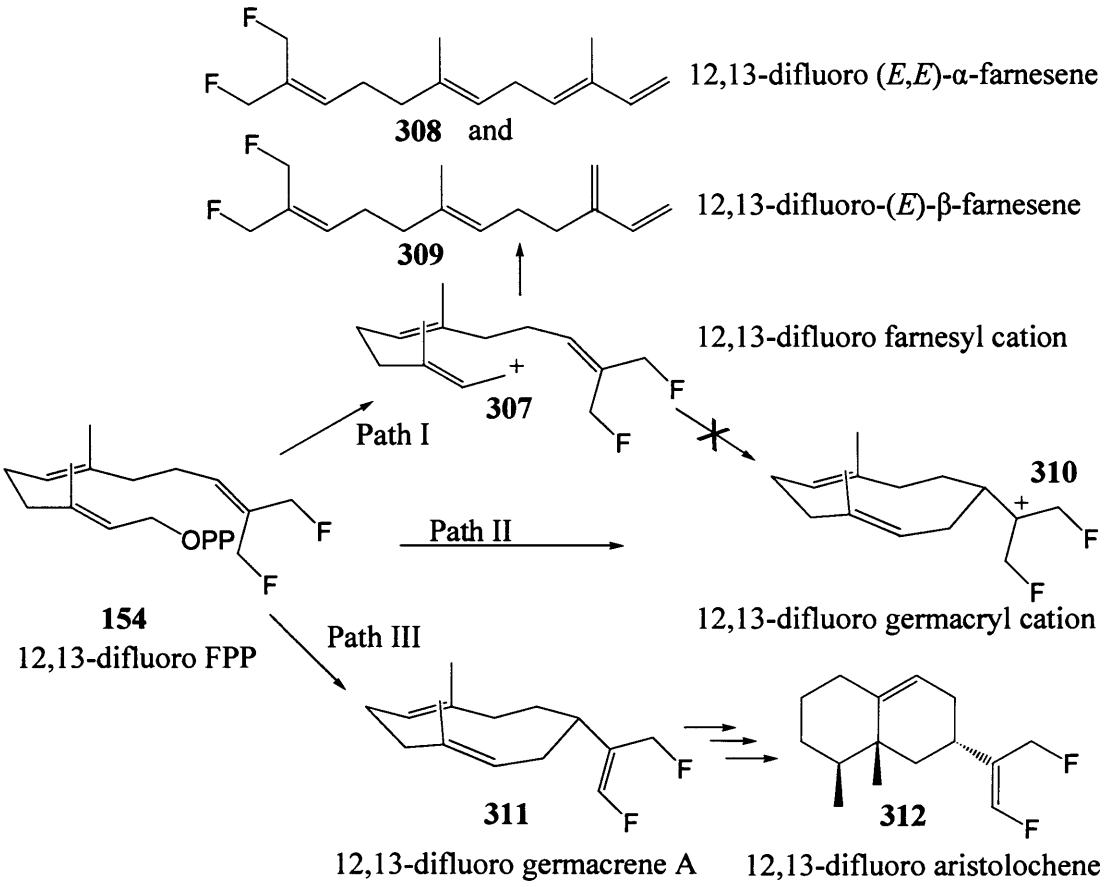
From the results of incubation of 12,13-difluoro FPP (**154**) with purified recombinant AS, one can gain deeper insight into the mechanism of action of this enzyme. The results obtained with site-specific mutants of AS, in which Phe 178 was replaced by valine and which gave relatively large amount of (*E*)-β-farnesene (9.2%) and (*E*,*E*)-α-farnesene (2.7%) suggested that diphosphate ionisation led to the formation of farnesyl cation which was then attacked by the C10-C11 π-bond to generate germacryl cation (path I, Scheme 3.1).[57] Alternatively, the formation of germacryl

cation could take place in a concerted reaction in which farnesyl-diphosphate ionisation is accompanied by electrophilic attack of C1 of FPP by the C10, C11 π-system (path II).[50] This mechanism is in agreement with the observation that the reaction of stereospecifically deuterated FPP occurred with inversion of configuration at C1.[77] In path III, cyclisation is initiated by deprotonation from C12 and concurrent bond formation between C10 and C1.[89]



Scheme 3.1: *Possible pathways for initial cyclisation during catalysis by aristolochene synthase.*

Due to the electronic effects of fluoro substituents on carbocations described above, incubation of 12,13-difluorofarnesyl diphosphate with AS would be expected to generate different outcomes for the three cyclisation pathways shown in Scheme 3.2.



Scheme 3.2: *Possible outcomes for 12,13-difluoro FPP during catalysis by aristolochene synthase.*

For path I, the destabilizing effect of the two fluoro substituents on the putative carbocation on C11 should lead to the accumulation of the 12,13-difluorofarnesyl cation (**307**) and the formation of 12,13-difluoro (*E*,*E*)-α-farnesene (**308**) and 12,13-difluoro-(*E*)-β-farnesene (**309**) through deprotonation from C4 or C15. For a reaction where pyrophosphate departure occurs simultaneously with electron flow from the C10, C11 double bond, 12,13-difluoro farnesyl diphosphate (path II) would be expected to act as a competitive inhibitor. In pathway III, the acidifying effect of the fluoro substituents on the protons on C12 and C13 should allow the reaction to proceed, ultimately generating 12,13-difluoro aristolochene (**312**).

The observation that 12,13-difluoro FPP (**154**) acts as an inhibitor of AS suggests that the cyclisation of FPP occurs along path II in which farnesyl diphosphate ionisation is accompanied by electrophilic attack of C1 by the C10, C11 π-bond with inversion of configuration at C1 resulting in the formation of germacryl cation. However, in previous publication, it was indicated that the farnesyl cation was an intermediate during the cyclization of FPP to aristolochene.[58] This latter conclusion was reached from experiments with a site-specific AS mutant, in which Phe 112 was replaced with alanine, leading to the production of 34.3% (*E*)-β-farnesene (**64**) and 53.1% (*E,E*)-α-farnesene (**63**) in addition to a small amount of germacrene A.[58] It was postulated that the presence of these linear sesquiterpenoids occurred because of the accumulation of farnesyl cation during catalysis in the mutant enzyme due to the lack of stabilization of germacryl cation through interaction with the aromatic ring of Phe 112. It was concluded that the aromatic ring of residue Phe 112 in addition to stablizing the germacryl carbocation might also play an important role in aligning the π-orbital of the C10, C11 double bond with the breaking C1-O bond of the diphosphate group due to its steric bulk. When the size of this side chain is reduced by replacing the benzyl group with the smaller methyl group this alignment may no longer be optimal thereby preventing the immediate quenching of the developing positive charge on C1 and leading to the accumulation of farnesyl cation and the production of the linear farnesenes (Figure 3.3).

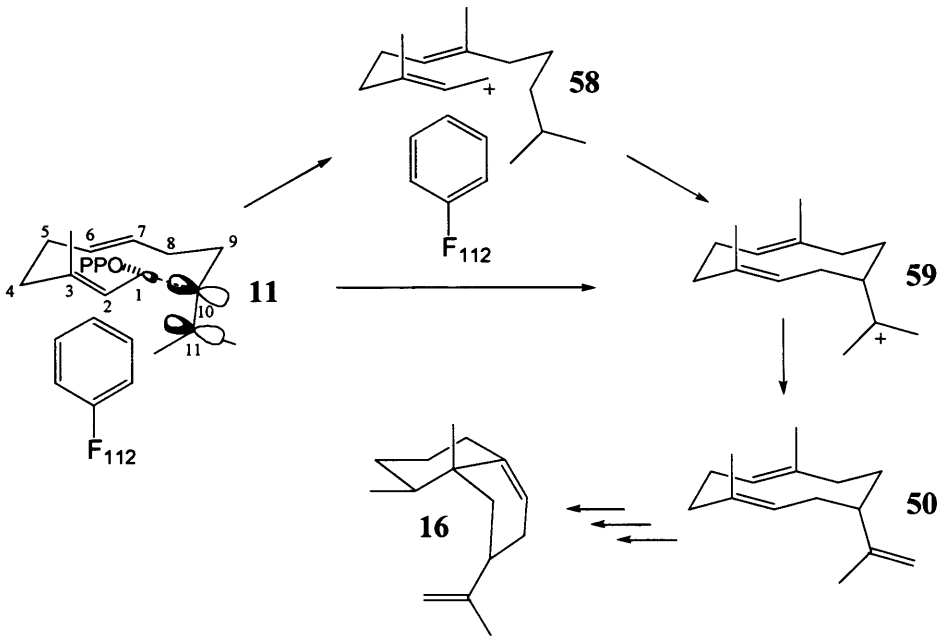


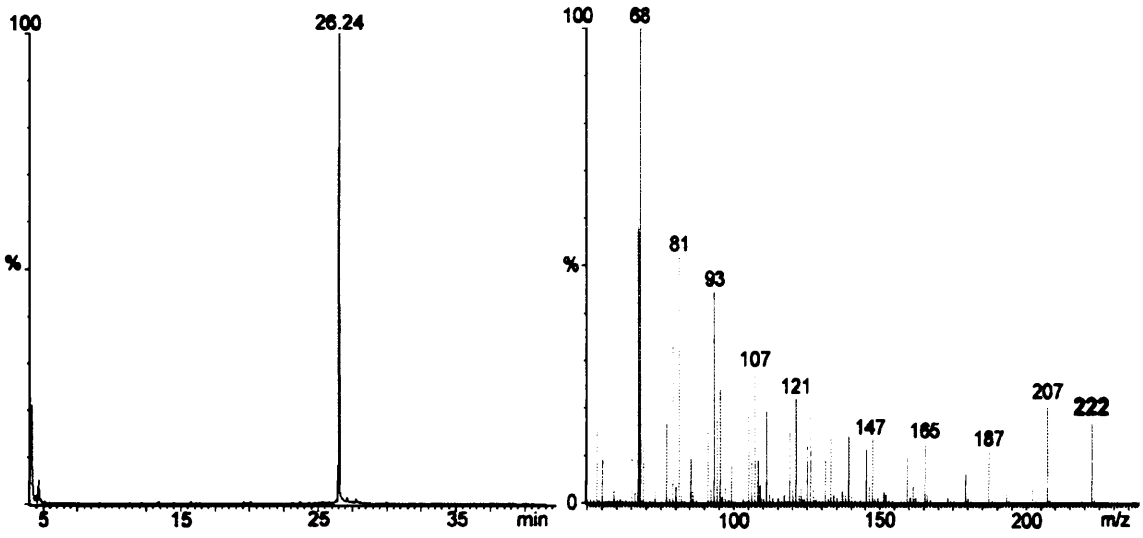
Figure 3.3: *Phe 112 may promote the formation of the reactive conformation.*

According to this argument, **154** should act as a substrate of the mutant enzyme AS-F112A since the less hindered rotation around the C1–O bond of **154** in the mutant should lead to the formation of 12,13-difluorofarnesyl cation (**307**) and hence of the difluorinated farnesenes, **308** and **309**. One major and two side products with the molecular ion peaks of *m/z* of 240 expected for difluorinated sesquiterpenoids were detected in the gas chromatogram of the hexane extractable products of incubations of the AS-F112A with **154**. While the low activity of AS-F112A even with FPP[58] allowed for the production of only a small amount of fluorinated products and prevented the detailed molecular characterization of the products, this experiment nevertheless confirmed that AS-F112A could indeed turnover **154**. This result strongly supported the proposal that AS catalyses the breaking of the ester bond in FPP and the formation of the cyclic germacryl cation (**59**), at least in part, by facilitating optimal orbital overlap between the C10–C11 and C1–O bonds in FPP through the templating effect of bulky active site residues such

as Phe 112.

3.4 Incubation results for 2-fluoro FPP (155)

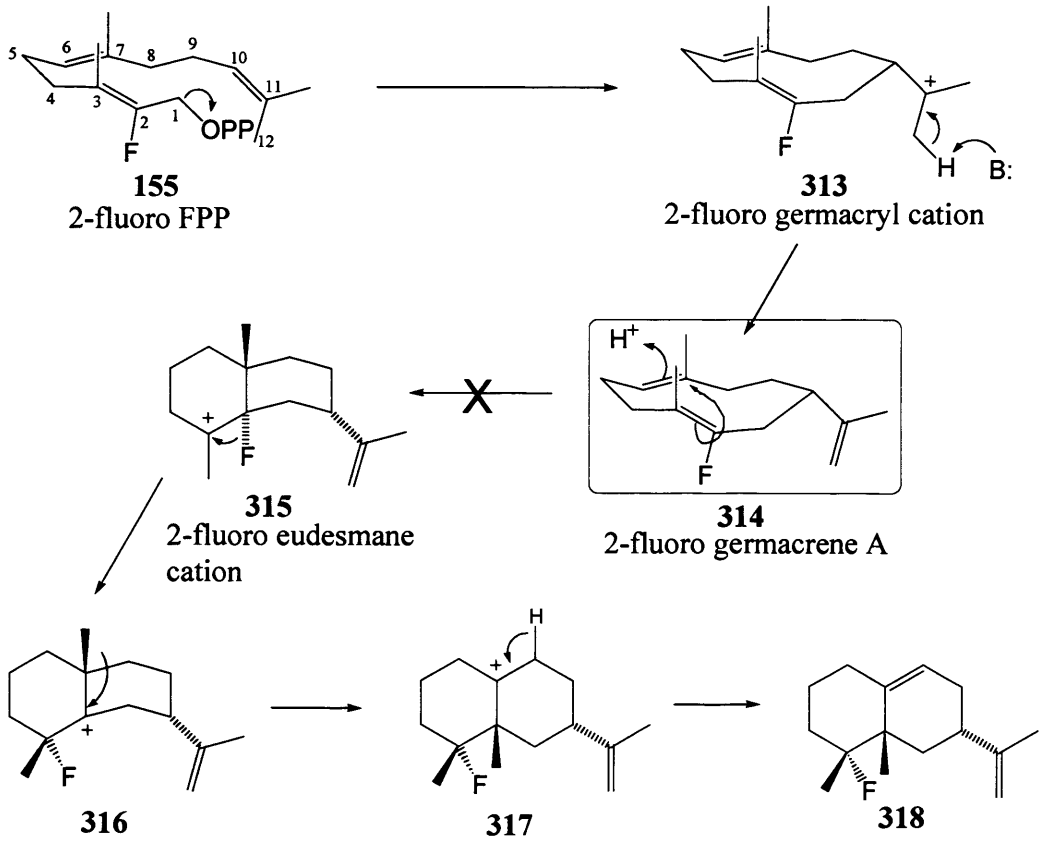
Small scale incubation of 2-fluoro FPP (**155**) with purified recombinant aristolochene synthase for 48 h under the reaction conditions previously used for the incubation of FPP with AS[64] gave a single pentane extractable product as judged by GC-MS analysis, that showed the molecular mass expected for a monofluorinated sesquiterpenoid (Figure 3.2).



Scheme 3.2: *GC-MS analysis of the pentane extractable product of 2-fluoro FPP* (**155**) *utilisation by AS.*

As the small scale incubation result was promising, the incubation was repeated on a preparative scale. Quantities were increased 50 fold (incubation of 25 mg of diphosphate **155** with AS gave 4 mg of product). GC mass spectral analysis of the pentane extractable material gave the same result.

Scheme 3.4 shows the outcome expected for the reaction of 2-fluoro FPP (**155**) when incubated with AS. Concurrent loss of pyrophosphate and cyclisation *via* attack by the C10-C11 double bond gives the 2-fluoro germacryl cation (**313**). Loss of the C12 proton should generate 2-fluoro germacrene A (**314**). The next cyclisation step should be disfavoured because of the inductive effect of the fluoride group reducing the nucleophilicity of the C2-C3 double bond and the destabilising effect of the positive charge on C3 in eudesmane cation **315**. These effects lead to an increase of the height of the energy barrier of the step following formation of compound **314**.



Scheme 3.4: *Prediction of the outcome from incubation of 2-fluoro FPP with AS.*

^{1}H-NMR spectroscopic analysis of the product at room temperature gave a rather poorly resolved NMR spectrum which was similar to the literature reference spectrum of germacrene A without

full assignment.[181] The ^{19}F NMR spectrum of the product showed two resonances (δ_F = –88.8 and –90.9 ppm) at room temperature. Because of the known flexibility of cyclodecadienes a variable temperature NMR study was carried out in d^8-toluene between –80 °C and 90 °C at 10 °C intervals. The compound proved to be stable over the whole temperature range as evidenced by the reversibility of the temperature dependent spectral changes. Significant line sharpening was observed both on lowering and increasing the temperature from 25 °C (Figure 2.5). The compound existed as two resolvable conformers at temperatures below 0 °C while at elevated temperatures the position of the sharp resonance signals indicated a fast equilibrium between the two conformers resulting in a weighted average of the NMR-resonances. A coalescence temperature of approximately 30 °C was observed.

COSY and HSQC NMR spectra were obtained both at –60 °C and 90 °C, which made it possible to assign the ^1H-resonance of the compound (see Chapter 3). Together with the room temperature HMBC spectrum these data also allowed us to assign the ^{13}C NMR spectrum of this compound. Based on these assignments the spectrum at room temperature could also be assigned and all were fully consistent with structure of compound **314**. As evident from the variable temperature NMR spectrum, ^1H-NMR signals of **314** were temperature dependent. According to the NMR peaks assignment, it was found that the change of resonances for the protons on C4, C6, C14 and the olefinic proton on C12 were most obvious, and which clearly indicated the slow exchange of the two major conformations (Figure 3.3). CH_3-14 for instance gives rise to two singlets at 1.67 and 1.78 ppm at –60 °C while at 90 °C only one singlet is observed at 1.65 ppm. H-6 presented two well-resolved signals at 5.05 and 5.36 ppm at –60 °C while at high temperature only a poorly

resolved double doublet is observed at 5.18 ppm. At low temperature, the proton on C6 in the two conformers showed two different splitting patterns; in the predominant conformer a relatively broad doublet is observed as its coupling to one of protons on C5 is presumably minimal due to the dihedral angle being close to 90°, however, in the minor conformer coupling to both protons on C5 is evident. Similarly, the patterns of the H4 resonances change with temperature as well. One of the two protons which leads to a complex multiplet at 90 °C is well resolved at low temperature to indicate a double double triplet splitting pattern due to its coupling with the geminal proton, the vicinal protons on C5 and the fluoro-substituent. The two resonances for the olefinic protons on C12 at 90 °C were resolved into an AB-system when temperature was lowed down to –60 °C.

Conformational isomerism of the germacradiene family of natural products, in particular the (*E,E*)-germacranes, has been studied previously both by NMR-spectroscopy and computationally.[182] The 10-membered ring can adopt four distinct conformations with up-up, down-down, up-down and down-up orientations of the methyl groups on C3 and C7 (Figure 3.4).[182] These conformations are interconvertible by rotation of each of the double bonds through the ring and inversion of the C9-C10 unit, the conformation of which is largely determined by the exocyclic subsistent on C10 which favors a pseudo-equatorial orientation.

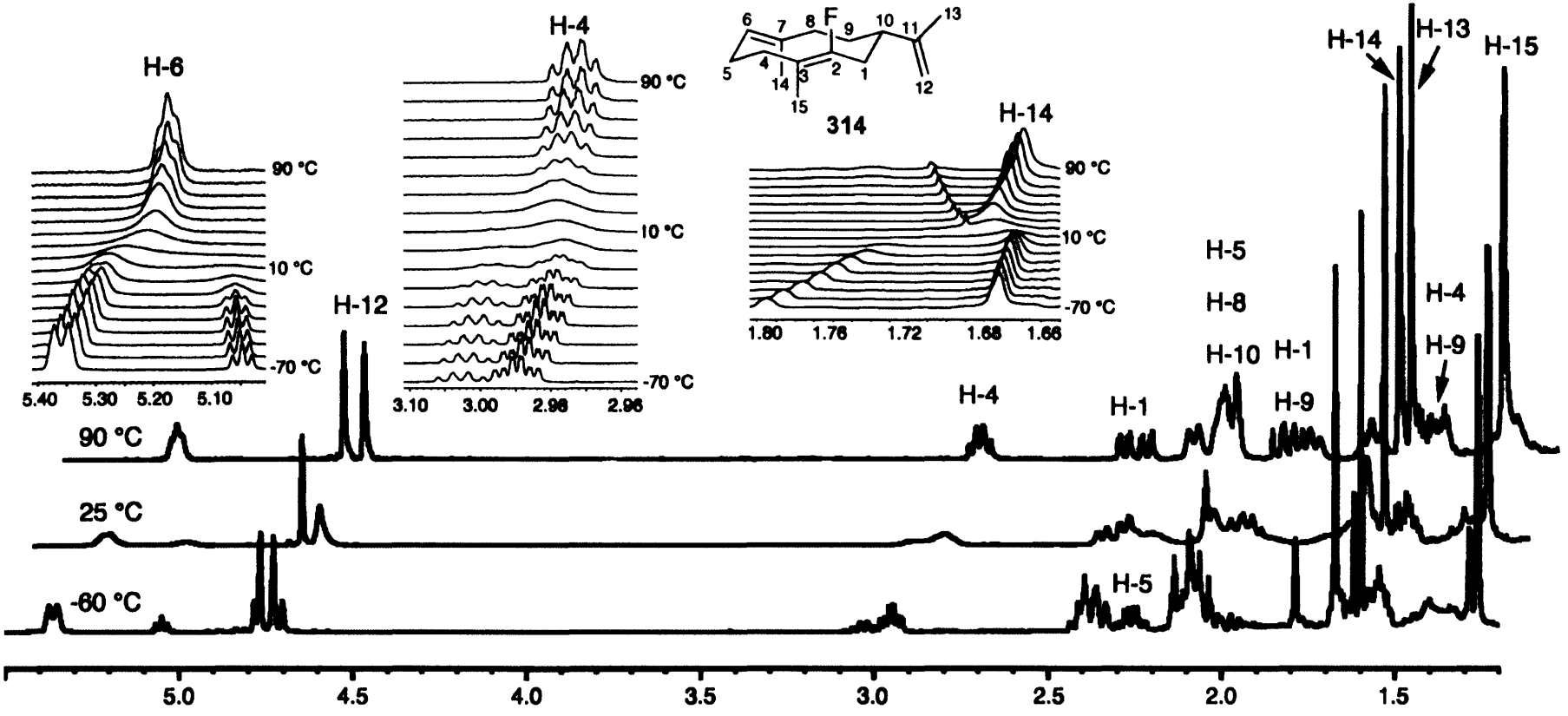


Figure 3.3. *500 MHz ^1H NMR spectra of* **314** *at -60, 25 and 90 °C with assignments shown. Insets show the temperature dependent change in the resonances for H6, one of the H4 protons and CH_3-14. Clearly two conformational isomers exist in a ratio of approximate 3:1 at the lower temperatures (integrals not shown) and the signals for the two coalesce at approximately 30 °C. The conformation of the down-down isomer of* **314** *is also shown.*

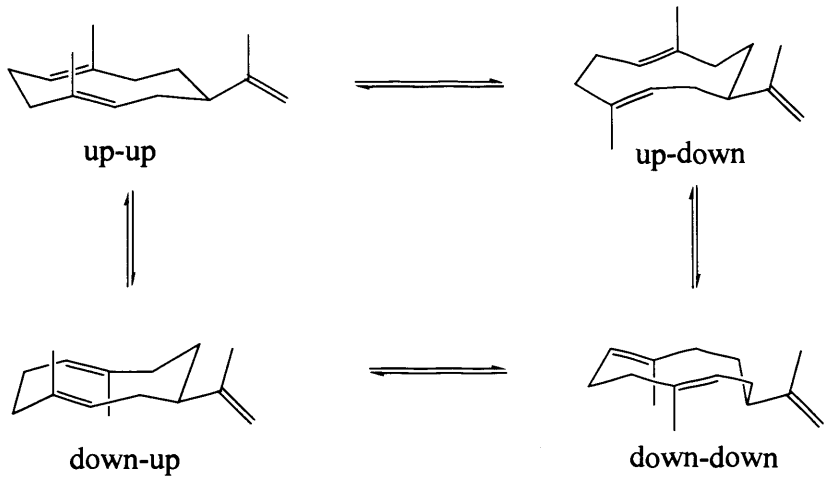


Figure 3.4. *Four distinct conformations of (+)-germacrene A.*

From the recent publication of the conformational analysis of (+)-germacrene A, it can be seen that the up-up conformer was predominant according to NMR studies (52% at –20°C).[183] As the AS from *Penicillium roqueforti* only produces (–)-germacrene A[58] and the relatively large isopropylidene substituent will predominately adopt the pseudo-equatorial position, it is reasonable to suggest that the mirror image of above illustrated up-up conformer is most stable and populated in compound **314**. And the minor conformer is expected to be one of the down-up or up-down conformers according to the published (+)-germacrene A structure.[183] The analysis of the relative intensities of the resonances for the proton on C6 in the –60 °C ^{1}H NMR spectrum indicate that the ratio between the major and the minor conformer of compound **314** was 3.22 : 1, which is closed to what had been observed for (+)-germacrene A.

3.4.1 Thermal rearrangement of 2-fluoro germacrene A

Germacrene A is known to undergo a Cope rearrangement to generate β-elemene[181] and it was

partly, as a result of the temperature induced Cope rearrangement in GC-MS experiments that germacrene A was first identified as a minor product of FPP turnover by AS.[64] Hence a GC-MS analysis with varying injector temperatures but under otherwise identical conditions was applied to the product as another experiment to further characterize compound **314** (Figure 3.5).

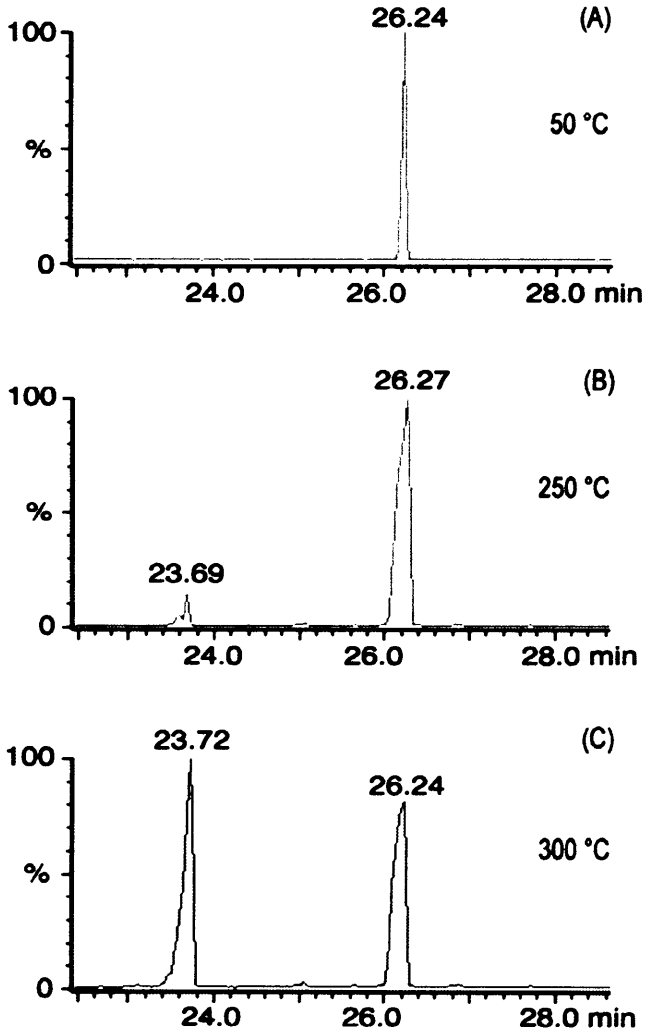


Figure 3.5: *GC analysis of the temperature induced reaction products of the pentane extractable products of 2-fluoroFPP* (**155**) *from the cyclization by AS. (A) GC analysis of the compound produced by AS from* **155** *at an injection port temperature of 50 °C. (B) GC analysis of the same compound at an injection port temperature of 250 °C; (C) GC analysis of the same compound at an injector port temperature of 300 °C.*

Germacrene A undergoes significant Cope rearrangement when the injector temperature is set to 200 °C.[64] In contrast, no thermal rearrangement of compound **314** was observed at this temperature. When higher injection temperatures were used, injection at 250 °C gave a small amount of rearranged product, and at 300 °C thermal rearrangement of compound **314** was dominant (Figure 3.6) and high-resolution mass spectrometry indicated that the two compounds had an identical elemental composition ($C_{15}H_{23}F$).

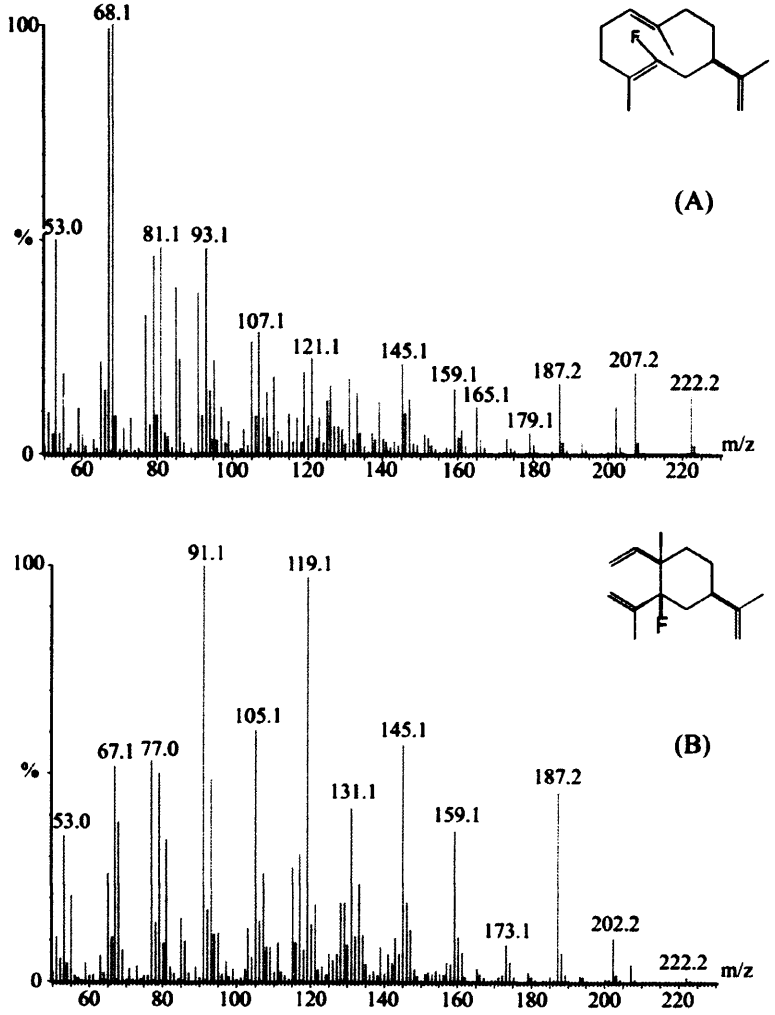


Figure 3.6: *Mass spectral analysis of the two isomers produced by thermal rearrangement of **314** in the GC injector. (A) EI^+ mass spectrum of the material eluting at 26.24 min, this is the presumed 2-fluoro-germacrene A derivative **314**; (B) EI^+ Mass spectrum of the material eluting at 23.72 min, this is the presumed β-elemene analogue **319**.*

From the above results, it can be concluded that the presence of the fluoro-substituent in **314** appears to slow the Cope rearrangement down relative to germacrene A. The effects of fluorine substituents on six electron electrocylic processes is unclear since steric and electronic effects often compete leading to unpredictable effects on the reaction kinetics,[184] hence a rationalization of the reduced reactivity of **314** is difficult.

The fragmentation patterns of both compounds were indicative of a loss of CH_3 (–15 *m/z*), HF (–20 *m/z*) and loss of both CH_3 and HF (–35 *m/z*). The parent molecular ion of compound **314** has greater intensity than that of the presumed fluoro-β-elemene (**319**), which is most likely because of facilitated loss of hydrogen fluoride from C1 and C2 of **319** to generate a conjugated diene, when compared to elimination of HF from **314**.

3.4.2 Discussion on 2-fluoro farnesyl pyrophosphate

As germacrene A does not act as a substrate for AS, it can be argued that germacrene A is not an on-path reaction product but a minor side product from a separate reaction pathway catalysed by AS.[64] However, the above results illustrated that the 2-fluorofarnesyl diphosphate **155** is a substrate of aristolochene synthase and is converted exclusively to 2F-germacrene A **314**, which provides strong evidence that germacrene A is generated as an on-path reaction product of the conversion of FPP to aristolochene. If germacrene A was produced in a minor, but separate pathway by AS, more than one product would be expected from the incubation with 2F-FPP. The observation that germacrene A doesn't act as a substrate for AS is most likely the consequence of its very poor solubility in aqueous reaction buffer.

3.5 Incubation results for 6-fluoro FPP

Incubation of 6-fluoro FPP (**156**) with purified recombinant aristolochene synthase under the standard reaction conditions (see Section 3.2) for 48 h led to two major hexane extractable products as judged by GC-MS analysis, and which showed the molecular mass expected for monofluorinated sesquiterpenoid (*m/z* = 222) (Figure 3.7). The mass spectrum of these two products have similar fragmentation pattern, so that we can assume these two molecules have similar structures.

In order to make clear what exactly these two products are, preparative scale (50 times scale up) incubation of 6-fluoro FPP (**156**) with recombinant AS (pH 7.5, 25 °C, 48 hrs) was also done with same GC-MS results.

As there were two major products from the incubation of 6-fluoro FPP (**156**) and AS, the separation of them was necessary before characterization. Two different methods of separation were tried. Firstly chromatography using silver nitrate coated silica gel as the stationary phase was attempted. This relies upon the coordination between silver and different double bonds, 3% (w/w) silver nitrate coated silica gel was made by suspending silica gel in aqueous silver nitrate solution and lyophilization, however the two products were not separable by this method. Analytical scale HPLC was also attempted, but also failed.

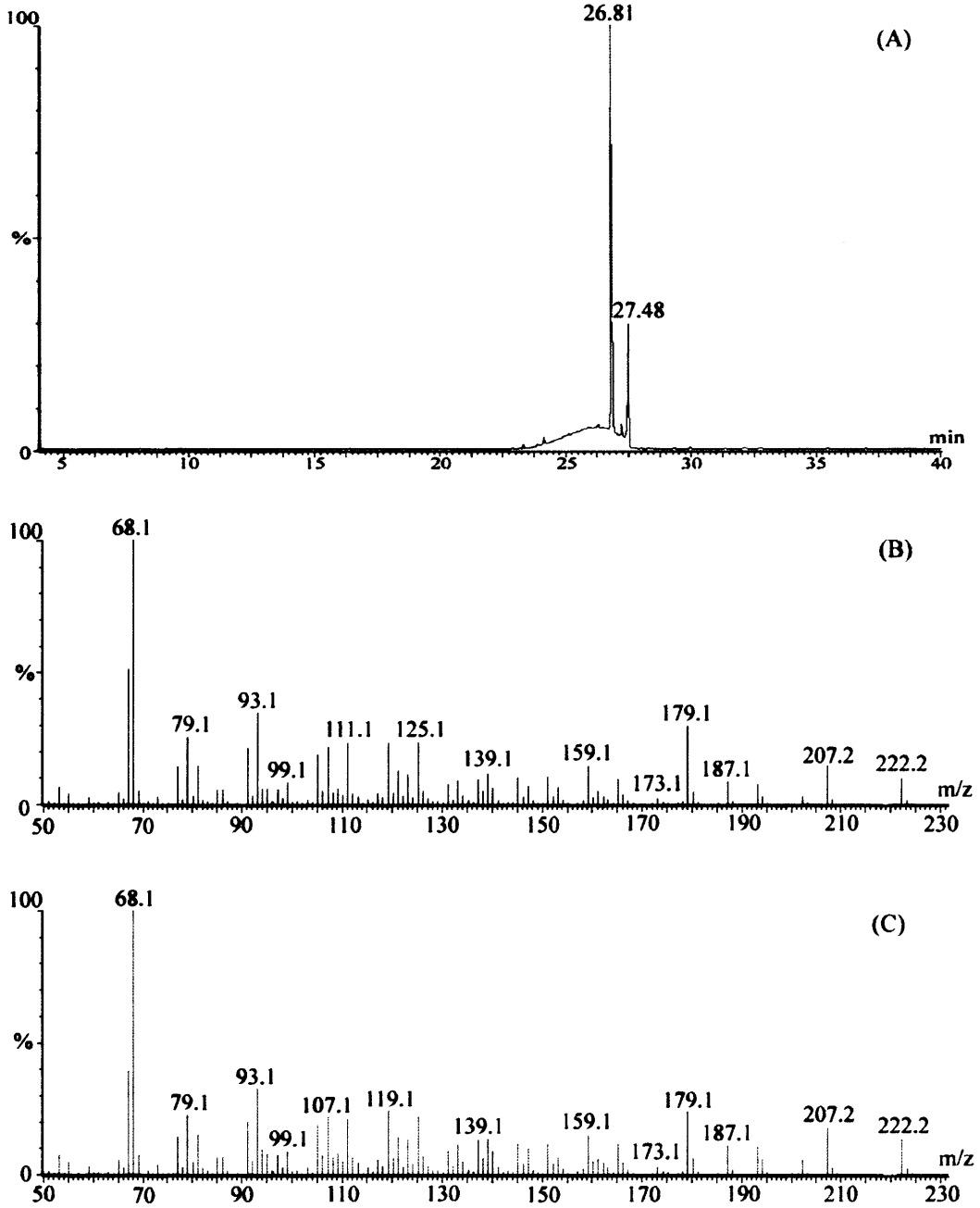
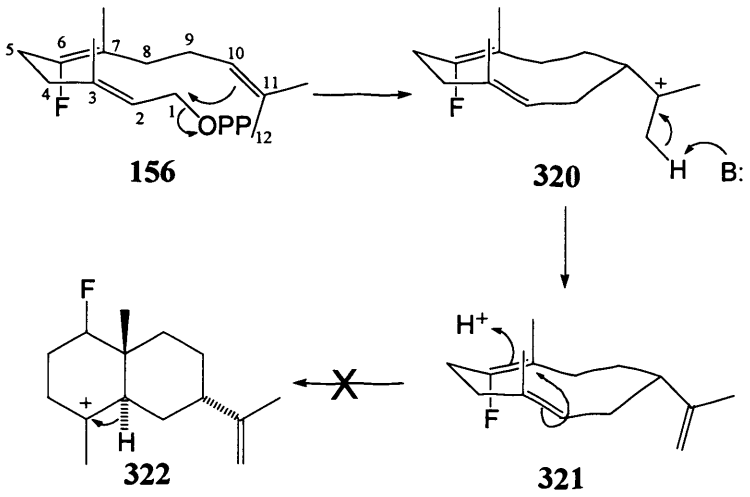


Figure 3.7: *GC-MS analysis of the pentane extractable products of 6-fluoroFPP* (**156**) *utilisation by AS, (A) GC analysis of the compounds produced by AS from* **156** *at an injection port temperature of 50 °C; (B) EI^+ mass spectrum of the material eluting at 26.81 min; (C) EI^+ mass spectrum of the material eluting at 27.48 min.*

As these two products were not separable using the above methods, another explanation is possible. It is possible the fluorine substituent reduces the basicity of C6-C7 double bond in 6-fluoro germacrene A, therefore further cyclization cannot carry on as it cannot easily protonate. There is probably only one major product from this incubation, which is 6-fluoro germacrene A (**321**) (Scheme 3.5). The two peaks observed in GC analysis are probably 6-fluoro germacrene A (**321**) and its Cope rearrangement product. 6-Fluoro germacrene A has been observed as a product when 6-fluoro FPP was incubated with epi-aristolochene synthase.[185]



Scheme 3.5: *Prediction of the incubation of 6-fluoro FPP with AS.*

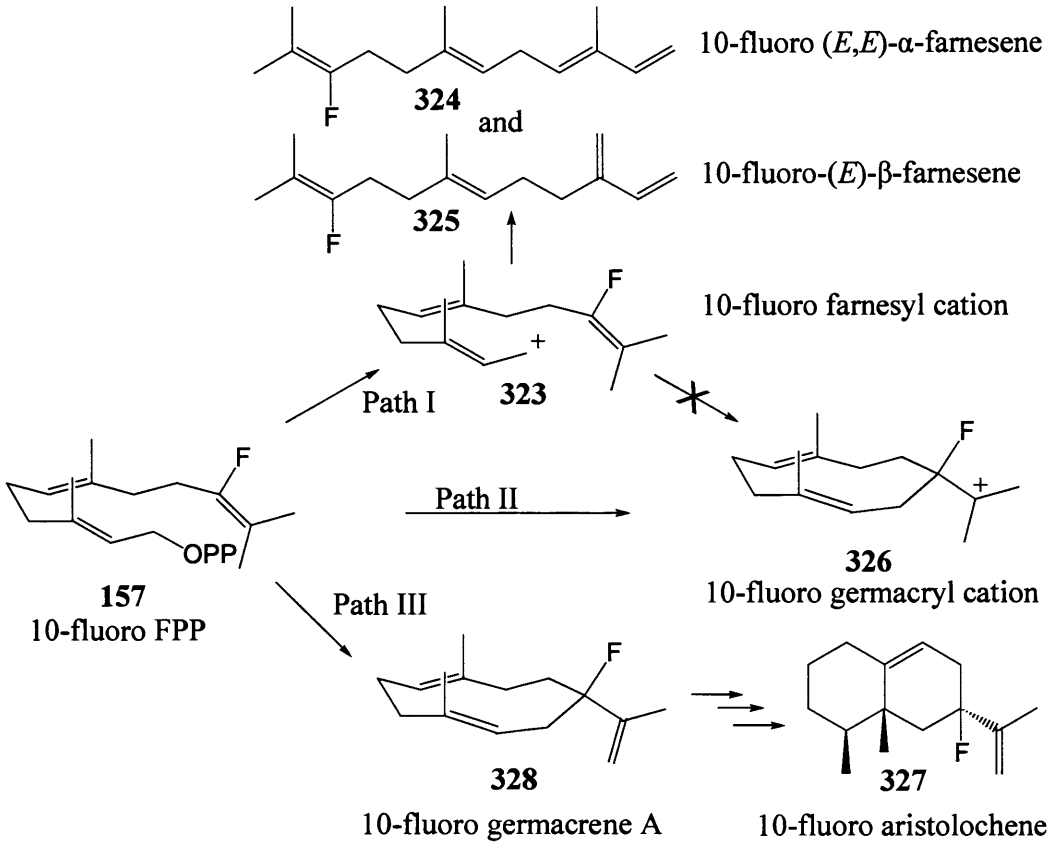
3.6 Incubation results for 10-fluoro FPP (157)

Incubation of 10-fluoro FPP (**157**) with purified recombinant AS under the standard reaction conditions used throughout this work led to no hexane extractable product as judged by GC-MS analysis.

This result can be compared to that obtained for 12,13-difluoro FPP (Scheme 3.2). In the first

step of the cyclisation of 10-fluoro FPP **157**, for path I,[57] the destabilizing effect of the C10 fluoro substituent on carbocation on C11 should lead to the accumulation of the 10-fluorofarnesyl cation **323** and the formation of 10-fluoro (*E*,*E*)-α-farnesene **324** and 10-fluoro-(*E*)-β-farnesene **325** through deprotonation from C4 or C15 since **326** is destabilised. Path III[89] will lead to the production of 10-fluoro aristolochene **327**. From our incubation results, there was no haxane extractable product, so path I and path III are not in agreement with the experimental results.

Even though the kinetic study of this analogue as an inhibitor of AS has not yet been done, the GC-MS result is also strong evidence for path II, which is a concerted reaction, in which diphosphate ionisation is accompanied by electrophilic attack of C1 by the 10, 11 π-system,[50] in this case, the result 10-fluoro germacryl cation is strongly destablised, which should make this analogue a competitive inhibitor of AS. All above results and discussion give further support for the reaction mechanism of the formation of the decalin ring system (Scheme 3.6).



Scheme 3.6: *Possible products for10-fluoro FPP during catalysis by AS.*

3.7 Incubation results for 14-fluoro FPP (158) and 15-fluoro FPP (159).

Incubation of 14-fluoro FPP (**158**) with purified recombinant AS under the standard reaction conditions used earlier for 48 hours led to 3 major hexane extractable products as judged by GC-MS analysis (Figure 3.8). One of those had the required mass (*m/z* = 222).

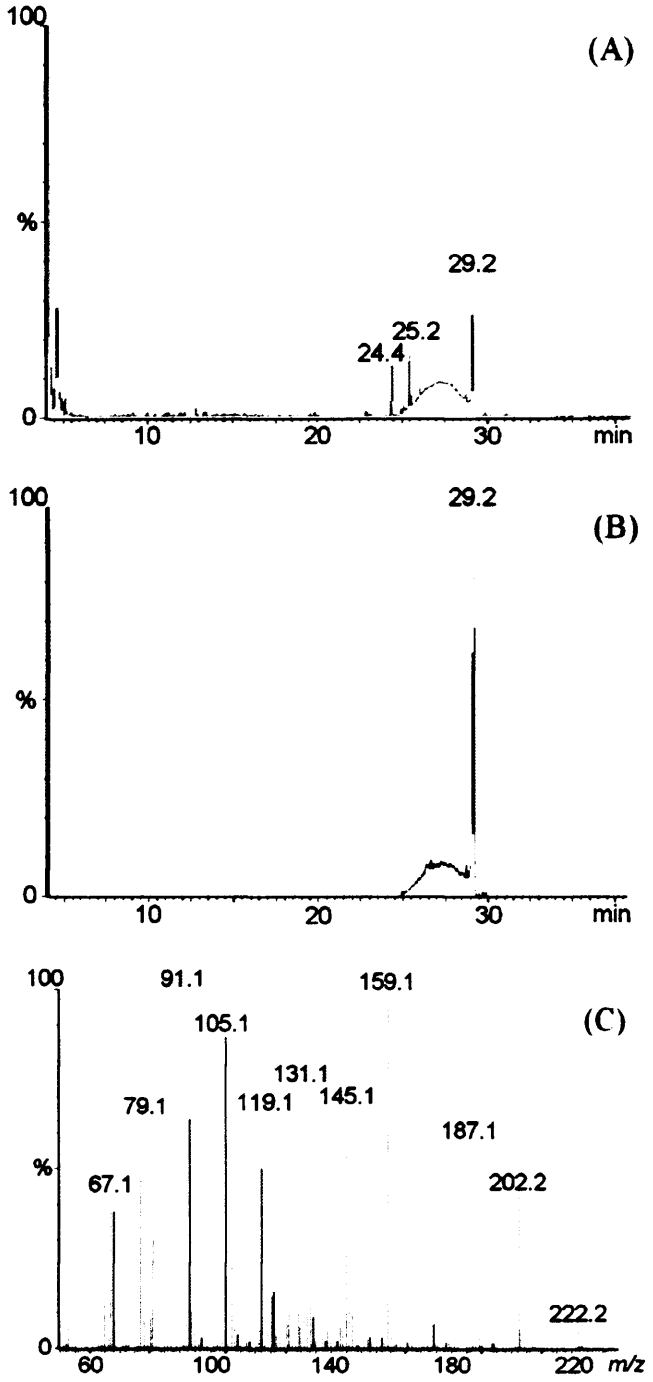


Figure 3.8: *GC-MS analysis of the pentane extractable products of 14-fluoroFPP* (**157**) *utilisation by AS, (A) GC analysis of the compounds produced by AS from* **157** *at an injection port temperature of 50 °C; (B) GC trace containing m/z=222; (C) EI^+ mass spectrum of the material eluting at 29.2 min.*

Similar incubation of 15-fluoro FPP (**159**) with purified recombinant AS led to four major hexane extractable products as judged by GC-MS analysis (Figure 3.9). One of the four products had the

required mass (m/z = 222).

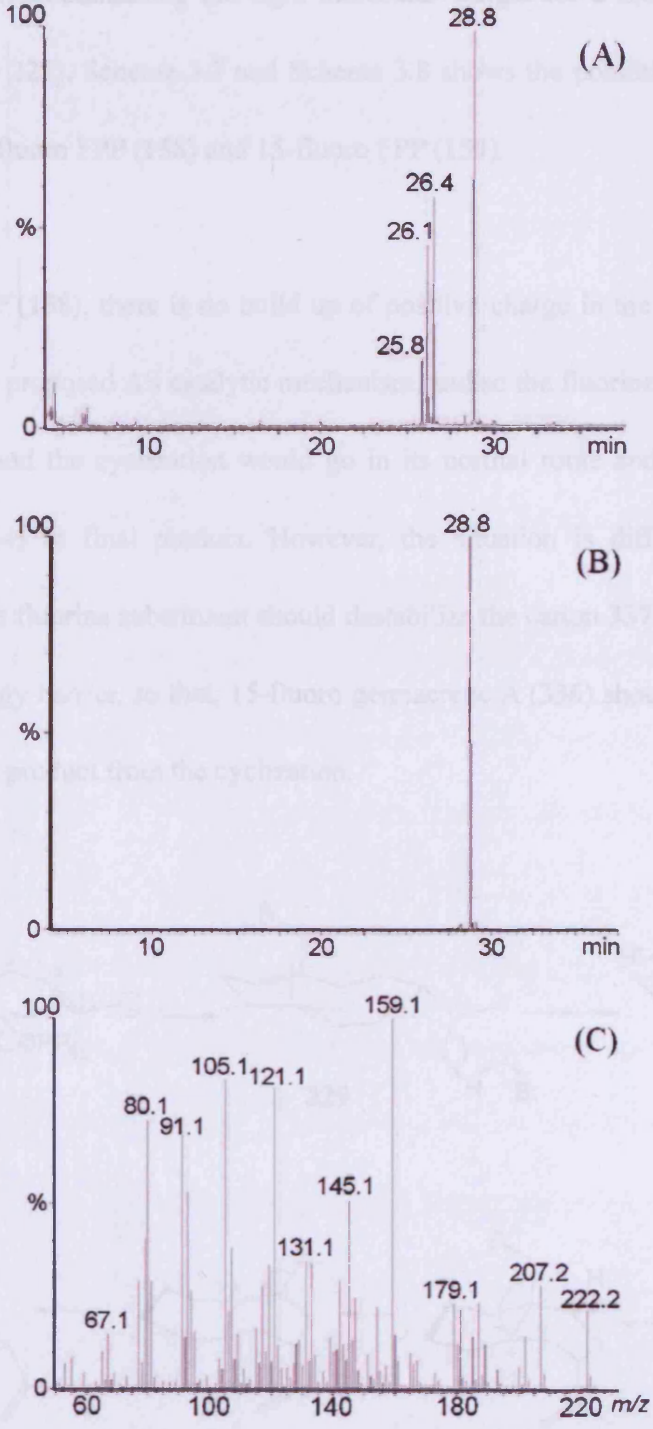
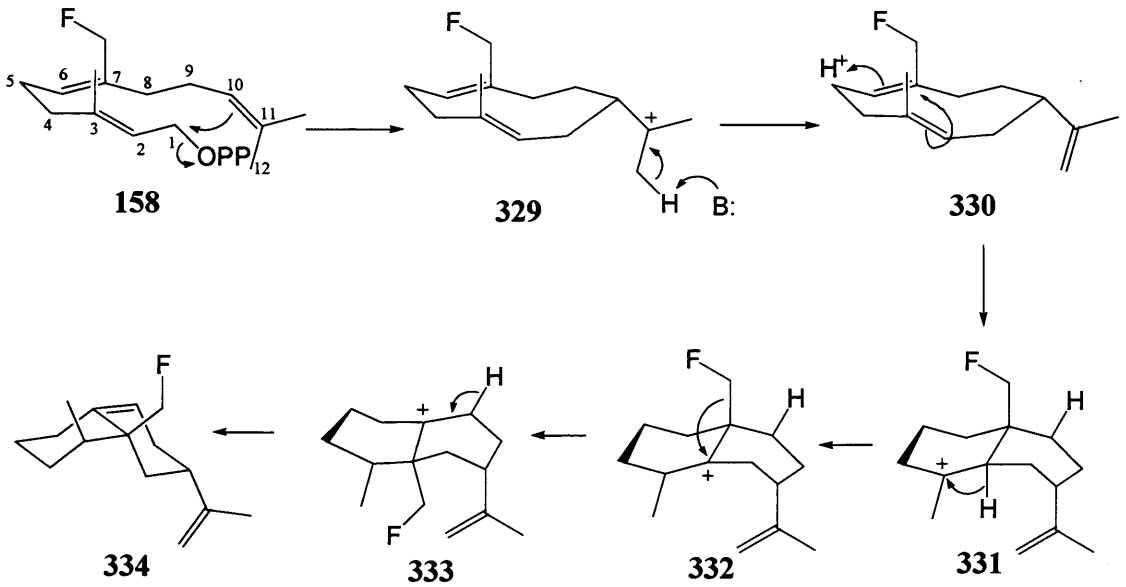


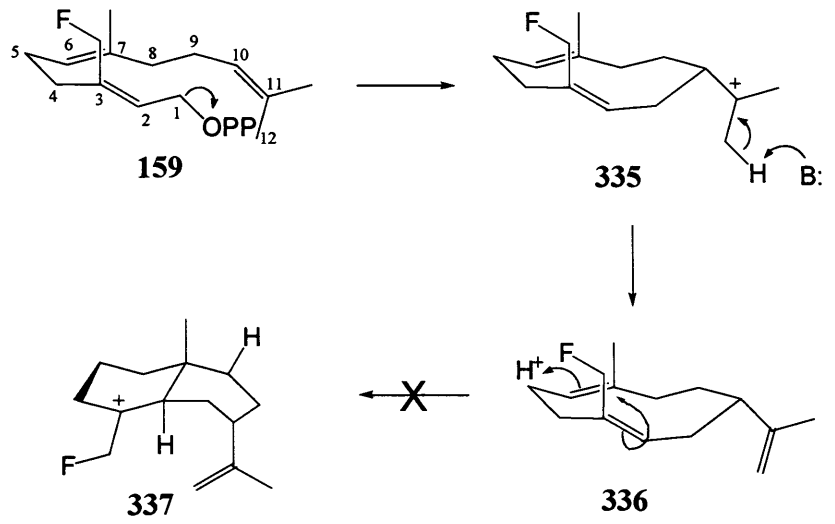
Figure 3.9: *GC-MS analysis of the pentane extractable products of 15-fluoroFPP* (**158**) *utilisation by AS, (A) GC analysis of the compounds produced by AS from* **158** *at an injection port temperature of 50 °C; (B) GC trace containing m/z=222; (C) EI^+ mass spectrum of the material eluting at 28.8 min.*

The above two results clearly show that these two analogues were substrates of AS. They both have one peak which containing the right molecular weight for a monofluoro sesquiterpenoid compound (*m/z* = 222). Scheme 3.7 and Scheme 3.8 shows the possible outcomes from the AS cyclization of 14-fluoro FPP (**158**) and 15-fluoro FPP (**159**).

For 14-fluoro FPP (**158**), there is no build up of positive charge in the vicinity of fluorine atom through the entire proposed AS catalytic mechanism, and so the fluorine atom may have no effect on the reaction, and the cyclization would go in its normal route and then generate 14-fluoro aristolochene (**334**) as final product. However, the situation is different for the other FPP analogue **159**. The fluorine substituent should destabilize the cation **337**. This effect increases the height of the energy barrier, so that, 15-fluoro germacrene A (**336**) should accumulate and hence become the major product from the cyclization.



Scheme 3.7: *Prediction of the incubation of 14-fluoro FPP with AS.*



Scheme 3.8: *Prediction of the incubation of 15-fluoro FPP with AS.*

3.8 Incubation results for Phenyl substituted FPP analogues

Each of the farnesyl diphosphate analogues prepared **160**, **161** and **162** was tested as a substrate for aristolochene synthase as previously described. None of the compounds tested in this study produced any hydrocarbon products of the correct mass as judged by GC-MS analysis. Each compound was therefore examined as an inhibitor of AS using a radiolabelled assay by Dr. David J. Miller. Double reciprocal plots indicated that all compounds were reversible competitive inhibitors of AS and hence are bound at the enzyme's active site in a way similar to that of the natural substrate (Figure 3.10). The K_I-values were determined as 0.8 ± 0.2, 1.2 ± 0.2 and 1.2 ± 0.1 μM for **160**, **161**, and **162**, respectively.

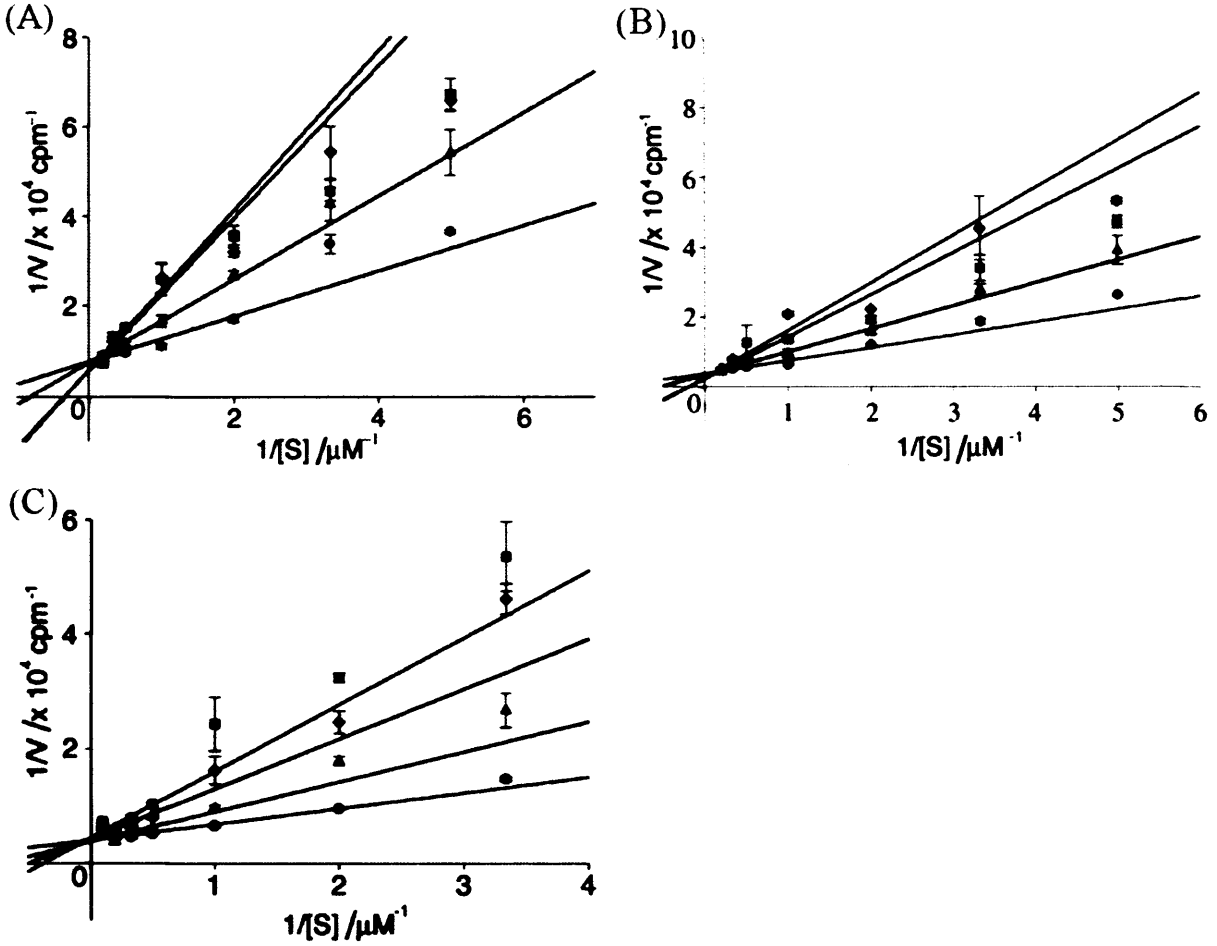


Figure 3.10: *Double reciprocal plots of initial rates versus the concentration of substrate for AS catalysed turnover of FPP in the presence of* **160**, **161** *and* **162** *are shown on panels A, B and C for increasing concentrations of inhibitor (0 μM (●), 1 μM (▲), 2 μM (♦) and 3 μM (■)). Intersection of the lines on the y-axis indicate that each compound is a competitive inhibitor of AS. All assays were carried out at 37 °C and pH 7.5.*

The three phenyl derivatives **160**, **161** and **162** prepared in this study were initially envisaged as proof of concept synthetic targets in order to test the Suzuki-Miyaura cross-coupling methodology and were not expected to bind with any great affinity to the enzyme. It was expected that the bulky phenyl groups would make the whole molecule too large to fit in the active site. It was therefore quite surprising when each of these compounds – although not a substrate for AS – were nevertheless binding to the active site with barely reduced affinity compared to natural substrate. Each inhibitor was therefore docked to the active site of AS using

the existing molecular model of FPP bound to AS[49] as a starting point by Neil J. Young. Energy minimisations of the docked structures were performed using the MMFF94 forcefield.[186] Amino acids within 6.5Å of the inhibitor molecule were allowed to move while the coordinates of all other residues were fixed. Each inhibitor appeared to fit well into the active site of AS and only minor reorganizations of active site residues were necessary to avoid steric clashes through the introduction of the bulky phenyl substituents (Figure 3.11).

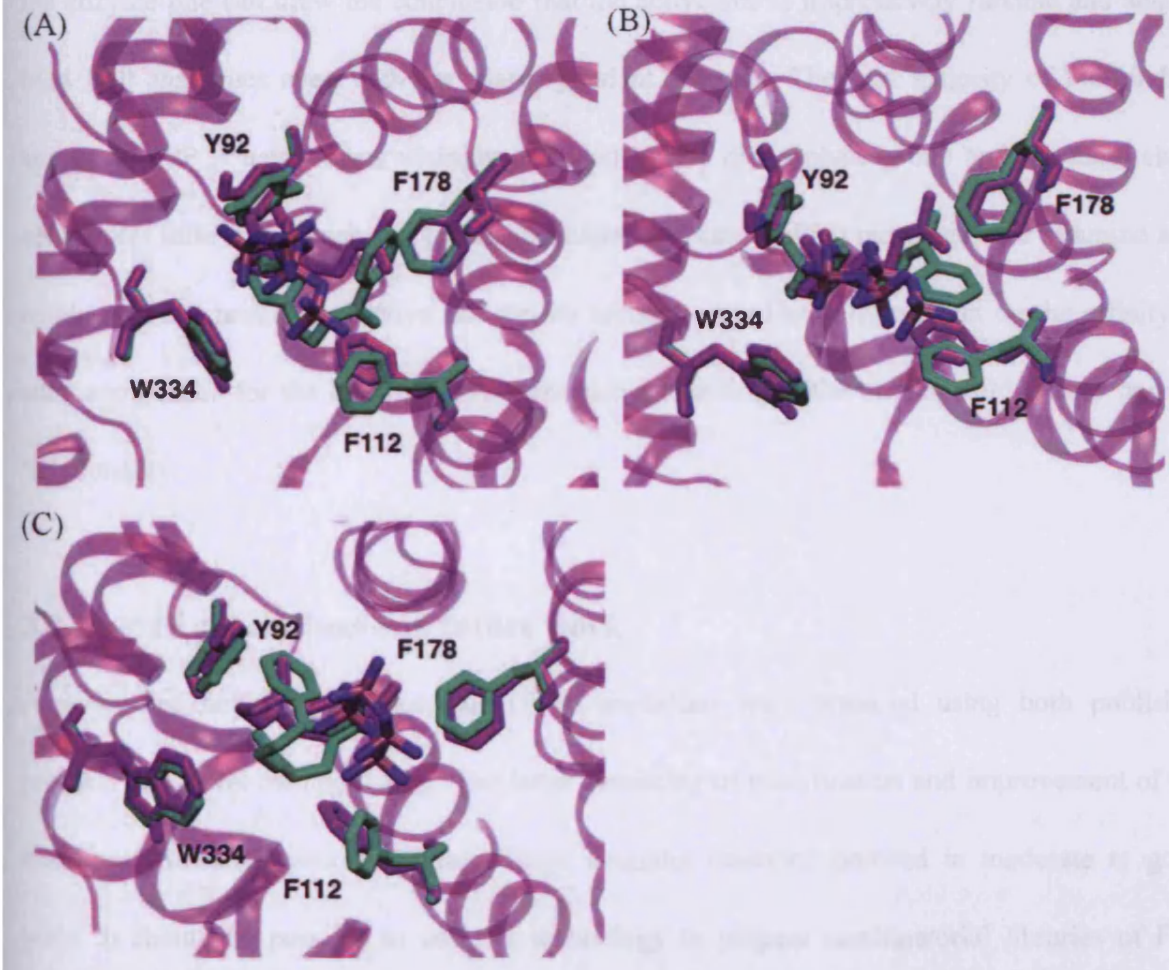


Figure 3.11: *Sketches from molecular mechanics simulations of the active sites of AS complexed with E-11-phenyl FPP* (**160**) *(A), Z-11-phenyl FPP* (**161**) *(B) and 3-phenyl FPP* (**162**) *(C). The substrate and the inhibitors as well as key amino acid residues are shown in purple for the original structure and in green for the energy minimised structures of the inhibitor complexes of AS.*

Particularly noteworthy is the case of the 3-phenyl FPP analogue **162** which binds to AS with K_i = 1.2 μM. Previous work has shown that a large tryptophan residue (W334) is positioned in the active site of AS to stabilise a positive charge build up on C-3 during formation of the eudesmane cation and so one would expect this residue to sterically hinder the approach of a phenyl group on C3.[49, 56] Clearly this does not occur although the enzyme does not produce any product from this compound. From this result and the fact that the other two phenyl derivatives bind effectively to the enzyme one can draw the conclusion that the active site is impressively flexible and able to bind FPP analogues even with very large pendant groups. The vast majority of the binding energy of FPP is hence unsurprisingly provided by the diphosphate group and the alkyl chain contributes little to this such that large substituents and concomitant rearrangement of amino acid residues in and around the active site can be accommodated with little effect on the affinity of such compounds for the enzyme. However, such alterations in the binding fold clearly prevent functionality.

3.8 Overall conclusions and future work

A variety of farnesyl pyrophosphate (FPP) analogues were prepared using both published protocol and novel methodologies. The latter consisting of modification and improvement of the traditional Suzuki-Miyaura coupling. These coupling reactions proceed in moderate to good yield. It should be possible to use this technology to prepare combinatorial libraries of FPP analogues in the future. Some members of such libraries may act as substrates for AS and other terpene cyclases thereby opening up the possibility of short economical routes to complex, synthetic unnatural analogues of terpenoids. These compounds were incubated with aristolochene

synthase (AS) in order to probe its mechanism of action and resulted in many interesting results.

The mechanisms of the enzyme-catalysed cyclisations of sesquiterpenoids are inherently difficult to study spectrometrically and their elucidation has been largely dependent on the use of substrate mimics and site directed enzyme mutants. Mechanistic studies with substrate analogues suggested that AS converts FPP into the bicyclic sesquiterpene aristolochene (**16**) through an initial cyclisation to generate the intermediate (–)-germacrene A (**50**) followed by protonation, 1,2 hydride and methyl shifts and stereospecific deprotonation from C8 (Scheme 1.6). Surprisingly little is known about the reaction mechanism of the formation of the decalin ring system that could be formed by several mechanistically distinct pathways (Scheme 3.1). First, the diphosphate ionisation led to the formation of farnesyl cation (**58**) which was attacked by the C10–C11 π-bond to generate germacryl cation (**59**). Alternatively, the formation of **59** could take place in a concerted reaction in which farnesyl diphosphate ionisation is accompanied by electrophilic attack of C1 of FPP by the C10, C11 π-bond. Also, cyclisation can be initiated by deprotonation from C12 and concurrent bond formation between C10 and C1. Since it has become increasingly apparent that only a few amino acid substitutions can be sufficient to remodel the active site template provided by terpene synthases, thereby often dramatically altering the reaction products, a detailed mechanistic understanding of the reaction catalysed by these enzymes is central to their further evolution in vitro or in vivo for the generation of novel products. The synthesis of 12,13-difluoro farnesyl pyrophosphate (**154**) was achieved in 13 steps using Suzuki-Miyaura chemistry. This compound proved to be potent inhibitor of aristolochene synthase (AS), which revealed that the initial cyclisation to germacryl cation occurs in a concerted fashion, in which farnesyl diphosphate

ionisation is accompanied by electrophilic attack of C1 by the C10, C11 π-bond with inversion of configuration at C1 resulting in the formation of germacryl cation.

As described above, it is known the mechanism of the conversion of FPP (**11**) to aristolochene (**16**) catalyzed by aristolochene synthase from *Penicillium roqueforti* has been proposed to proceed through the neutral intermediate germacrene A (**50**). However, much of the experimental evidence is also in agreement with a mechanism in which germacrene A is not an intermediate in the predominant mechanism that leads to the formation of aristolochene, but rather an off-pathway product that is formed in a side reaction. Hence, to elucidate the mechanism of FPP cyclisation the substrate analogue 2-fluoro FPP (**155**) was synthesized, and upon incubation with aristolochene synthase was converted to a single pentane extractable product according to GC-MS analysis. On the basis of NMR analyses this product was identified as 2-fluorogermacrene A (**314**). Variable temperature ^{1}H NMR spectroscopy indicated the existence of two conformers of **314** that were in slow exchange at –60 °C, while at 90 °C the two isomers gave rise to averaged NMR signals. In the major isomer (~75%) the methyl groups on C3 and C7 were most likely in the down - down orientation as had been observed for other (*E*,*E*)-germacranes. This work suggests that after an initial concerted cyclisation of FPP to germacryl cation deprotonation leads to the formation of germacrene A, and provides compelling evidence that germacrene A is indeed an on-pathway product of catalysis by aristolochene synthase.

6-Fluoro FPP (**156**) was prepared using the Weiler's chain extension method in 10 steps, and was

identified as substrate of AS. The inductive electron-withdrawing effect of the fluoro group decreases the π system basicity of the 6,7-double bond resulting in blockage of the subsequent proton-induced cyclization to the bicyclic eudesmane cation **322**. The product was postulated as 6-fluoro germacrene A, which is consistent with published results on epi-aristolochene synthase[185].

10-Fluoro FPP (**157**) was synthesized using the sulfonylation-alkylation-desulfonylation methodology and it was found not to act as substrates of the AS. This result is fully consistent with the conclusions drawn from the results with 12,13-difluoro FPP (**154**). Work is ongoing in the group to support this hypothesis. FPP analogues with one fluoro substituent at position C14 **158** and C15 **159** were made using methodology employing the Horner-Emmons Wittig condensation as a key step. These two compounds were tested with AS and both gave one major extractable terpene product according to GC/MS analysis. These two products are postulated as two different compounds - 14-fluoro aristolochene and 15-fluoro germacrene A respectively, because of the destabilizing effect of the β-substituted fluorine atom on the carbocation in their vicinity. Again, work to support this hypothesis is ongoing within the group.

From above mentioned work, it seems the strong influence on the reactivity exerted by fluoro substituents without significantly affecting size and shape may make fluorinated FPP analogues a very powerful method to explore the chemistry of terpene synthases.[187] However, only through a combination of experimental approaches will the intricate details of this masterpiece in combinatorial chemistry be deciphered.

Analogues of farnesyl pyrophosphate containing phenyl substituents in place of methyl groups were synthesized using the Suzuki-Miyaura reaction as the key step. These analogues were found not to act as substrates of the aristolochene synthase. However, they were potent competitive inhibitors of AS, which indicate that the active sites of AS is sufficiently flexible to accommodate even substrate analogues with large substituents. The plasticity of the terpene cyclases appears not only to provide the framework for the combinatorial production of many natural terpenoids through subtle alterations in the composition of the active site during evolution but may also allow modifications of the active site residues by site directed or random mutagenesis *in vitro* or *in vivo* for the production of functional enzymes that convert FPP analogues to unnatural "terpenoids".

In the future, it should be possible to make use of new Suzuki coupling technology to prepare combinatorial libraries of FPP analogues. Some of these compounds will no doubt act as substrates for AS and other terpene cyclase enzymes and thus open up the possibility of short economical routes to complex, synthetic modified terpenoids.

CHAPTER 4

EXPERIMENTAL

4.1 General experimental

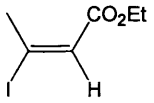
All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Tetrahydrofuran (THF) and diethyl ether were distilled from sodium/benzophenone ketyl under nitrogen. Acetonitrile, dichloromethane, toluene and triethylamine were distilled from calcium hydride under nitrogen. 9-BBN was used as the commercially available crystalline form as solutions were not found to work adequately in hydroboration reactions employed in this study. Ecoscint scintillation fluid was purchased from National Diagnostics. All other chemicals were of analar quality and used as received unless otherwise stated. Reactions were stirred at room temperature in air unless otherwise stated. All glassware was clean and dry before use.

^{1}H NMR spectra were measured at 500 MHz on a Bruker Avance 500 NMR spectrometer or a Bruker Avance DPX400 NMR spectrometer and are reported as chemical shifts in parts per million downfield from tetramethylsilane, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (to the nearest 0.5 Hz) and assignment respectively. ^{13}C NMR spectra were measured at 125 MHz on a Bruker Avance 500 NMR spectrometer and are reported as chemical shift downfield from tetramethylsilane, coupling constant where appropriate and assignment. Assignments are made to the limitations of COSY, DEPT 90/135, gradient HSQC and gradient HMBC spectra. ^{19}F and ^{31}P NMR spectra were recorded on a Jeol Eclipse +300 NMR spectrometer and are reported in chemical shift downfield from $CFCl_3$ and 85% H_3PO_4 respectively followed by multiplicity and coupling constant (to the nearest 0.5 Hz) if appropriate. IR spectra were recorded on a Perkin ELMER 1600 series FTIR spectrometer and samples were prepared as thin films of neat liquid on sodium chloride discs for oils and as KBr

disks for solids. EI^+ mass spectra were measured on a Micromass LCT premiere XE mass spectrometer. ES^- mass spectra were provided by the UK EPSRC mass spectrometry service, Swansea UK. Reverse phase HPLC was performed on a system comprising of a Dionex P680 pump and a Dionex UVD170U detector unit.

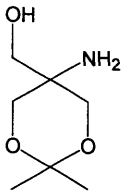
4.2 Experimental

(*E*)-3-Iodo-but-2-enoic acid ethyl ester (165)



The (*Z*)-3-iodo-but-2-enoic acid ethyl ester (4.63g, 19.3 mmol) was heated at 220 °C under argon in a sealed tube for 4 h, purification of the resulting brown oil by flash column chromatography directly to give the title compound as a pale yellow oil (2.16 g, 46%, recover 47%); R_f 0.21 (Hexane : EtOAc = 9 : 1); υ_{max}(thin film)/cm^{-1} 2980.1, 1718.3, 1617.3, 1424.7, 1366.3, 1330.1, 1264.7, 1185.7, 1074.4, 1036.2, and 861.2; δ_H (300 MHz; C^2HCl_3) 1.26 (3 H, t, *J* 7.0, CH_3CH_2O), 2.97 (3 H, d, *J* 1.5, CH_3Cl), 4.13 (2 H, q, *J* 7.0, CH_3CH_2O) and 6.61 (1 H, *J* 1.5, ICC*H*); δ_C (75.4 MHz; C^2HCl_3) 12.61 (CH_3CH_2O), 29.42 (CH_3Cl), 58.80 (CH_3CH_2O), 118.69 (quaternary C) and 129.94 (ICCH); *m/z* (EI^+) 240.1 (100%, M^+).

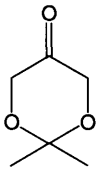
5-Amino-5-hydroxymethyl-2,2-dimethyl-1,3-dioxane (167)



To a stirred solution of tris(hydroxymethyl)aminomethane hydrochloride (7.9 g, 50 mmol) in dry

DMF (40 cm^3) was added *p*-toluenesulfonic acid (0.5g, 2.5mmol) monohydrate followed by 2,2-dimethoxypropane (6.8 cm^3, 55mmol). The resulting clear and colourless solution was allowed to stir overnight. Triethylamine (0.42 cm^3, 3mmol) was added and stirring continued for a further 10 min. The mixture was concentrated under reduced pressure and then treated with triethylamine (6.3 cm^3, 45 mmol) and ethyl acetate (150 cm^3). The white precipitate which was formed upon addition of base was removed via filtration and the fitrate purified by bulb-to-bulb distillation (85 °C/ 0.7 Torr) to afford a white solid (1.83 g, 23%); υ_{max} (KBr disk)/cm^{-1} 3324.0, 3270.2, 1993.0, 2863.1, 2361.5, 1611.6, 1454.4, 1369.3, 1153.6, 1052.1 and 826.8; δ_H (300 MHz; C^2HCl_3) 1.38, 1.41 [6 H, 2 × s, $(CH_3)_2CO_2$], 2.36 (3 H, br, OH and NH_2), 3.45 (2 H, s, HOCH_2C) and 3.53 and 3.78 [4 H, 2 × d, *J* 12.0 and *J* 12.0, $(CH_2)_2C$]; δ_C (75.4 MHz; C^2HCl_3) 22.88 and 25.56 [$(CH_3)_2CO_2$], 50.89 ($HOCH_2$), 65.43 and 67.85 (CH_2CCH_2) and 99.21 [$(CH_3)_2CO_2$]; *m/z* (EI^+) 161.7 (8%, M^+), 145.7 (43, $[M - NH_2]^+$), 72.8 (100).

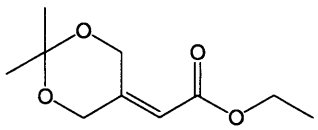
2,2-Dimethyl-5-oxo-1,3-dioxane (168)



To a stirred solution of 5-amino-5-hydroxymethyll-2,2-dimethyl-1,3-dioxane (crude) and potassium dihydrogenphosphate (6.8 g, 50 mmol) in water (100 cm^3) at 5 °C was added dropwise, over 3 h a solution of sodium periodate (10.7 g, 50 mmol) in water (100 cm^3). The mixture was allowed to stir for an additional hour at 5 °C and then for 15 h at R.T. before being extracted with DCM (10 × 15 cm^3). The combined organic extractions were dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The crude ketone was then purified by bulb-to-bulb

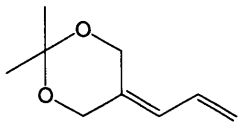
distillation (85 °C/ 20 Torr) to give the title compound as a colorless liquid (4.4g, 68% over two steps); R_f 0.25 (Hexane : EtOAc = 4 : 1); υ_{max} (thin film)/cm^{-1} 2993.1, 2937.3, 1751.4, 1679.5, 1381.3, 1232.6, 1088.6, 1047.9 and 831.5; δ_H (300 MHz; C^2HCl_3) 1.29 (6 H, s, 2 × CH_3) and 3.97 (4 H, s, 2 × CH_2); δ_C (75.4 MHz; C^2HCl_3) 25.05 (2 × CH_3), 68.38 (2 × CH_2), 101.88 [$(CH_3)_2C$] and 209.72 [$(CH_2)_2C$=O]; *m/z* (EI^+) 130 (17 %, M^+), 115 (35, $[M - CH_3]^+$), 100 (30, $[M - (CH_3)_2]^+$) and 43 (100).

2-(2,2-Dimethyl-1,3-dioxan-5-ylidene)-acetic acid ethyl ester (169)



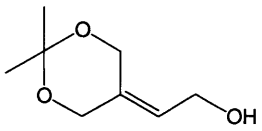
Fleshly cut sodium (0.68 g, 29 mmol) was dissolved in dry ethanol (50 cm^3). (Carbethoxymethyl)triphenyl-phosphonium bromide (12.7 g, 29 mmol) was added and the reaction mixture allowed to stir for 1 h. The ketone **168** (2.21 g, 17 mmol) was added and after 15 h, the mixture was extracted with DCM (3 × 15 cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (4 : 1) as eluent gave the title compound as a light yellow oil (2.6 g, 77%); R_f 0.36 (Hexane : EtOAc = 4 : 1); υ_{max}(KBr disk)/cm^{-1} 2984.1, 2939.8, 1778.9, 1736.7, 1639.8, 1443.0, 1375.3, 1225.0, 1022.8 and 890.8; δ_H (500 MHz; C^2HCl_3) 1.29 (3 H, t, *J* 7.0, OCH_2CH_3), 1.41 [6 H, s, $(CH_3)_2C$], 4.16 (2 H, q, *J* 7.0, OCH_2CH_3), 4.28 and 4.87 (4 H, 2 × s, OCH_2C) and 5.62 (1 H, s, C=CHC=O); δ_C (75.4 MHz; C^2HCl_3) 14.42 (OCH_2CH_3), 24.13 [$(CH_3)_2C$], 60.32 (OCH_2CH_3), 61.83 and 63.04 [$(CH_2)_2C$], 100.31 [$(CH_3)_2C$], 112.49 (C=CHC=O), 158.04 (C=CHC=O) and 166.02 (C=CHC=O); *m/z* (EI^+) 185 (28%, $[M - CH_3]^+$) and 97 (100).

5-Allylidene-2,2-dimethyl-1,3-dioxane (172)



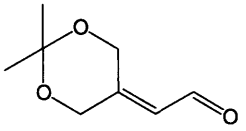
Methyl triphenylphosphonium bromide (20.9g, 58.7 mmol) was dissolved in dry THF (100 cm^3), then BuLi (29.4 cm^3, 58.7 mmol) was added dropwise to the stirred solution under an argon atmosphere. After stirring under argon for about 30 min at room temperature, the aldehyde **176** (3.05g, 19.6 mmol) was added dropwise in the deep yellow solution and the reaction mixture was stirred under argon for 18h. The result mixture was extracted with diethyl ether (3 × 30 cm^3), and the organic phases were combined, washed with brine (30 cm^3) and dried ($MgSO_4$), filtered and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (9 : 1) as eluent gave the title compound as a pale yellow oil (1.85 g, 61.5 % yield); R_f 0.36 (Hexane : EtOAc = 9 : 1); υ_{max}(thin film)/cm^{-1} 2988.3, 2849.6, 1596.6, 1447.7, 1380.9, 1221.7, 1154.9, 1083.0, 1047.1, 903.3 and 831.4; δ_H (500 MHz; C^2HCl_3) 1.37 [6 H, s, $(CH_3)_2C$], 4.22 and 4.45 (4 H, 2 × s, OCH_2), 5.06 (1 H, d, *J* 10.0 $CHCHCH_{trans}H_{cis}$), 5.12 (1 H, d, *J* 16.5, $CHCHCH_{trans}H_{cis}$), 5.75 (1 H, d, *J* 11.0, $CHCHCH_2$) and 6.31 (1 H, dt, *J* 10.5, *J* 16.5, $CHCHCH_2$); δ_C (75.4 MHz; C^2HCl_3) 25.07 [$(CH_3)_2C$], 60.91 and 65.18 [$(CH_2)_2C$], 100.30 [$(CH_3)_2C$], 119.32 ($C=CHCH_2$), 123.92 and 131.59 (2 × CH), 135.83 ($C=CHCH_2$); *m/z* (EI^+) 154 (5%, M^+) and 67 (100).

2-(2,2-Dimethyl-1,3-dioxan-5-ylidene)-1-ol (175)



To a stirred solution of ester **169** (2.11 g, 10.6 mmol) in dry THF (50 cm^3) at 0 °C (ice bath), was added dropwise (over 3 min) diisobutylaluminum hydride (1.5 M, 17.7 mL, 26.5 mmol). The reaction mixture was allowed to stir at 0 °C for 1 h, quenched with sat. sodium potassium tartrate (30 cm^3), and then extracted with diethyl ether (3 × 15 cm^3). The combined organic extracts were dried ($MgSO_4$), and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (1 : 2) as eluent gave the title compound as a pale yellow oil (1.63 g, 97%); R_f 0.27 (Hexane : EtOAc = 1 : 2); υ_{max} (thin film)/cm^{-1} 3357.3, 2935.8, 2882.3, 1720.7, 1652.2, 1411.6, 1215.5, 1167.6, 1090.2 and 1002.5; δ_H (300 MHz; C^2HCl_3) 1.49 (6 H, s, $(CH_3)_2C$), 2.32 (1 H, br, $CHCH_2O\mathit{H}$), 4.06 (2 H, d, *J* 6.5, $CHC\mathit{H}_2OH$), 4.22 and 4.38 (2 H, s, OCH_2C) and 5.42 (1 H, t, *J* 6.5, $C{=}C\mathit{H}CH_2OH$); δ_C (75.4 MHz; C^2HCl_3) 25.59 [$(\mathit{C}H_3)_2C$], 59.33 and 61.15 [$(\mathit{C}H_2)_2C$], 65.69 ($CH\mathit{C}H_2OH$), 100.98 [$(CH_3)_2\mathit{C}$], 123.62 ($C{=}\mathit{C}HCH_2OH$) and 136.93 ($\mathit{C}CHCH_2OH$); *m/z* (EI^+) 143 (40%, $[M - CH_3]^+$) and 83 (100).

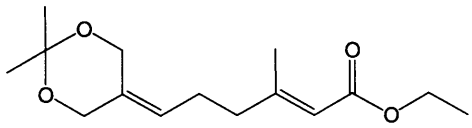
(2,2-Dimethyl-1,3-dioxan-5-ylidene)-acetaldehyde (176)



To a stirred suspension of pyridinium chlorochromate (9.2g, 42.5mmol) in anhydrous DCM was added a solution of alcohol **175** (5.6g, 35.4 mmol) in DCM (100 cm^3) in one portion. After 4 h, dry diethyl ether (100 cm^3) was added and the supernatant liquid was decanted from the black gum. The insoluble residue was washed with dry diethyl ether (100 cm^3) and became a black granular solid. The organic phases were combined, washed with brine (30 cm^3) and then dried

($MgSO_4$), filtered and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (2 : 1) as eluent gave the title compound as a yellow oil (3.05 g, 55% yield); R_f 0.30 (Hexane : EtOAc = 2 : 1); υ_{max}(thin film)/cm^{-1} 2988.3, 2844.5, 1678.7, 1611.9, 1442.5, 1375.8, 1216.6, 1077.9 and 831.4; δ_H (300 MHz; C^2HCl_3) 1.35 [6 H, s, $(CH_3)_2C$], 4.28 and 4.77 (4 H, 2 × s, OCH_2) and 5.79 (1 H, m, C=C*H*CHO) and 9.71 (1 H, d, *J* 6.0, CHO); δ_C (75.4 MHz; C^2HCl_3) 22.17 [(CH_3)$_2$C], 58.58 and 61.16 [(CH_2)$_2$C], 98.79 [$(CH_3)_2C$], 119.33 (C=*C*HCHO), 158.45 (*C*=CHCHO) and 187.50 (CH*C*HO); *m/z* (EI^+) 156.1 (33%, M^+) and 43.0 (100).

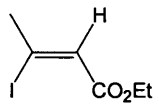
(*E*)-6-(2,2-Dimethyl-1,3-dioxan-5-ylidene)-3-methyl-hex-2-enoic acid ethyl ester (177)



A mixture of the diene **172** (0.144g, 1.00 mmol) and 9-BBN solution (6.0 cm^3, 3.0 mmol) in dry THF (10 cm^3) was stirred at room temperature until all the starting material had been consumed. Following this the iodide **165** (0.22g, 0.9 mmol), triphenyl arsine (0.031g, 0.1mmol), $PdCl_2$dppf (0.041g, 0.05 mmol) and 6 M NaOH (0.67 cm^3, 4 mmol) were added, the complete solution was then stirred under a inert atmosphere for 15 h at 50 °C. TLC (eluting with Hexane:EtOAC, 9:1) after this time showed that the iodide had been consumed. H_2O_2 (8.0 cm^3, 8 mmol) was then added to quench the reaction. The result mixture was extracted with diethyl ether (3 × 30 cm^3), and the organic phases were combined, washed with brine (30 cm^3) and dried ($MgSO_4$), filtered and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (9 : 1) as eluent gave the title compound as a pale yellow

oil (0.16g, 62 % yield); R_f 0.18 (Hexane : EtOAc = 9 : 1); HRMS (ES^+, $[M + Na]^+$) found 291.1578. $C_{15}H_{24}O_4Na$ requires 291.1572; υ_{max}(thin film)/cm^{-1} 2978.0, 2849.6, 1709.8, 1642.8, 1442.5, 1365.5, 1216.6, 1139.5, 1072.8 and 831.4; δ_H (300 MHz; C^2HCl_3) 1.25 (3 H, t, *J* 7.0, OCH_2CH_3), 1.40 [6 H, s, $(CH_3)_2C$], 2.12 (3 H, d, *J* 1.0, CH_3C=CHC=O), 2.15 (4 H, b, CH_2CH_2), 4.11 (2 H, q, *J* 7.0, OCH_2CH_3), 4.20 and 4.36 (4 H, 2 × s, OCH_2), 5.17 (1 H, m, C=C*H*CH_2CH_2) and 5.62 (1 H, d, *J* 1.0, C=CHC=O); δ_C (75.4 MHz; C^2HCl_3) 12.35 (OCH_2CH_3), 16.73 (CH_3C=CH), 22.06 [$(CH_3)_2C$], 22.59 and 38.34 (CH_2CH_2), 57.60 and 57.79 [$(CH_2)_2C$], 62.43 (OCH_2CH_3), 97.14 [$(CH_3)_2C$], 114.18 and 119.74 (2 × CH), 131.00, 156.51 and 165.00 (quaternary C); *m/z* (ES^+) 291.1 (100%, $[M + Na]^+$).

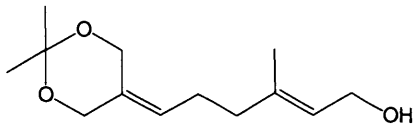
(*Z*)-3-Iodo-but-2-enoic acid ethyl ester (179)



An oil bath was preheated at 115 °C for 15 min, a flask containing a mixture of ethyl 2-butynoate (2.33 cm^3, 20.0 mmol) , sodium iodide (4.80 g, 3.0 mmol) and glacial acetic acid (7.70 cm^3, 134 mmol) was placed in the oil bath and the mixture was stirred at 115 °C for 90 min. the bath was removed and the brown mixture was transferred while hot to a separatory funnel containing water. The phases were separated and the aqueous phase was washed with diethyl ether (3 × 30 cm^3), the combined organic phase was washed sequentially with saturated aqueous sodium bicarbonate (30 cm^3), satd. sodium metabisulfite (30 cm^3) and brine (30 cm^3). Then dried ($MgSO_4$) and concentrated under reduced pressure to give the title compound as a yellow oil (4.75 g, 98%); R_f 0.26 (Hexane : EtOAc = 9 : 1); υ_{max}(thin film)/cm^{-1} 2979.1, 1727.2, 1628.3, 1431.8, 1365.8, 1308.2, 1275.2, 1180.1, 1094.7, 1046.1, 948.4 and 846.3; δ_H (300 MHz; C^2HCl_3) 1.27 (3 H, t, *J*

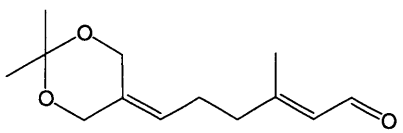
7.0, CH_3CH_2O), 2.71 (3 H, d, *J* 1.5, CH_3Cl), 4.18 (2 H, q, *J* 7.0, CH_3CH_2O) and 6.26 (1 H, *J* 1.5, ICC*H*); δ_C (75.4 MHz; C^2HCl_3) 13.55 (CH_3CH_2O), 35.85 (CH_3Cl), 59.83 (CH_3CH_2O), 112.48 and 163.50 (quaternary C) and 124.95 (ICCH); *m/z* (EI^+) 240.1 (100%, M^+).

(*E*)-6-(2,2-Dimethyl-1,3-dioxan-5-ylidene)-3-methyl-hex-2-en-1-ol (180)



This compound was prepared from **177** in a manner similar to that for the alcohol **175**; purification by silica column chromatography using hexane and ethyl acetate (1: 2) as eluent gave alcohol **180** as a pale yellow oil (0.76 g, 91 % yield); R_f 0.32 (Hexane : EtOAc = 1 : 2); HRMS (ES^+, $[M + Na]^+$) found 249.1478. $C_{13}H_{22}O_3Na$ requires 249.1467; υ_{max}(thin film)/cm^{-1} 3416.0, 2987.2, 2934.7, 2852.2, 1666.9, 1449.4, 1370.4, 1222.6, 1194.5, 1155.8, 1063.8, 1015.1 and 831.2; δ_H (300 MHz; C^2HCl_3) 1.36 [6 H, s, $(CH_3)_2C$], 1.60 (3H, s, CH_3C=CHCH$_2$OH), 2.00 (4H, b, CH_2CH_2), 2.32 (1H, b, OH), 3.94 (2 H, d, *J* 7.0, C=CHCH_2OH), 4.16 and 4.30 (4 H, 2 × s, 2 × OCH_2), 5.16 (1H, m, C=C*H*CH$_2$CH$_2$) and 5.33 (1 H, dt, *J* 7.0, C=C*H*CH$_2$OH); δ_C (75.4 MHz; C^2HCl_3) 18.46 (CH_3C=CHCH$_2$OH), 26.16 [$(CH_3)_2C$], 27.01 and 40.96 (CH_2CH_2), 59.15 (C=CHCH$_2$OH), 61.25 and 62.00 [$(OCH_2)_2C$], 101.24 [$(CH_3)_2C$], 124.99 and 126.61 (2 × CH), 134.16 and 140.23 (quaternary C); *m/z* (ES^+) 249.1 (15%, $[M + Na]^+$), 133.1 (100).

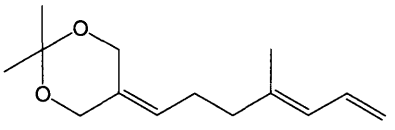
(*E*)-6-(2,2-Dimethyl-1,3-dioxan-5-ylidene)-3-methyl-hex-2-enal (181)



A mixture of alcohol **180** (0.76 g, 3.36 mmol), *N*-methylmorpholine-*N*-oxide (0.56 g, 4.70 mmol)

and freshly activated powdered 4 Å molecular sieves (0.30 g) in anhydrous acetonitrile (40 cm^3) was stirred for 10 min whereupon tetra-*n*-propyl perruthenete (0.06 g, 0.17 mmol) was added. The reaction became warm and was then stirred at room temperature for 16 h. The mixture was filtered through Celite® and the solvent was concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (3 : 2) as eluent gave **181** as a pale yellow oil (0.59 g, 78%); R_f 0.33 (Hexane : EtOAc = 3 : 2); HRMS (ES^+, $[M + Na]^+$) found 247.1316. $C_{13}H_{20}O_3Na$ requires 247.1310; υ_{max} (thin film)/cm^{-1} 2990.1, 1672.3, 1448.2, 1370.2, 1222.1, 1194.1, 1066.5 and 831.6; δ_H (500 MHz, C^2HCl_3) 1.50 [6 H, s, $(CH_3)_2C$], 1.58 (3 H, s, CH_3), 1.73 (4 H, b, $CHCH_2CH_2C$), 4.20 and 4.30 [4 H, 2 × s , $(CH_2)_2C$], 4.83 (1 H, b, $C=CHCH_2CH_2$), 5.82 (1 H, d, *J* 7.5, $CHCHO$) and 9.95 (1 H, d, *J* 7.5, $CHCHO$); δ_C (125 MHz, C^2HCl_3) 16.60 (CH_3), 23.94 and 39.47 (CH_2CH_2C), 24.04 and 24.08 [$(CH_3)_2C$], 59.41 and 64.04 [$(CH_2)_2C$], 98.98 [$(CH_3)_2C$], 120.39 ($C=CHCH_2CH_2$), 127.51 ($CHCHO$), 134.21 (quaternary C) and 189.59 ($CHCHO$); *m/z* (ES^+) 247.1 (20%, $[M + Na]^+$) and 131.1 (100).

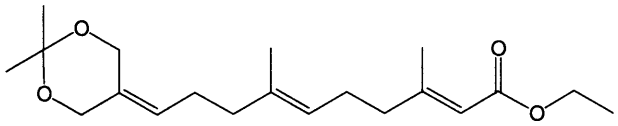
(*E*)-2,2-Dimethyl-5-(4-methyl-hepta-4,6-dienylidene)-1,3-dioxane (183)



This compound was prepared from **181** in a manner similar to that for the triene **172**; purification by silica column chromatography using hexane and ethyl acetate (9: 1) as eluent gave triene **183** as a light yellow oil (0.89 g, 91%); R_f 0.36 (Hexane : EtOAc = 9 : 1); HRMS (EI^+, M^+) found 222.1624. $C_{14}H_{22}O_2$ requires 222.1620; υ_{max} (thin film)/cm^{-1} 2987.4, 2847.0, 1649.2, 1448.9, 1369.7, 1222.4, 1194.1, 1156.2, 1067.6, 898.3 and 832.5; δ_H (500 MHz, C^2HCl_3) 1.45 [6 H, s,

$(CH_3)_2C$], 1.73 (3 H, s, CH_3), 2.10 (4 H, m, C=CHCH_2CH_2), 4.23 and 4.41 [4 H, 2 × s, $(CH_2)_2C$], 5.01 (1 H, dd, *J* 10.0, *J* 1.5, CHCH=C$H_{trans}H_{cis}$), 5.11 (1 H, dd, *J* 17.0, *J* 1.5, CHCH=C$H_{trans}H_{cis}$), 5.24 (1 H, m, C=C*H*CH_2CH_2), 5.85 (1 H, d, C*H*=$CHCH_2$) and 6.58 (1 H, dt, *J* 17.0, *J* 10.0, CHC*H*=CH_2); δ_C (125 MHz, C^2HCl_3) 16.60 (CH_3), 24.05 [(CH_3)$_2$C], 25.00 and 39.28 (C=CCH_2CH_2), 59.87 and 64.56 [CH_3]$_2$C], 99.02 [$(CH_3)_2C$], 115.12 (CHCH=CH_2), 122.84 (C=$CHCH_2CH_2$), 125.97 ($CHCH=CH_2$), 131.96 and 138.42 (quaternary C) and 133.18 (CH$CH=CH_2$); *m/z* (EI^+) 222.2 (1%, M^+) and 177.1 (100).

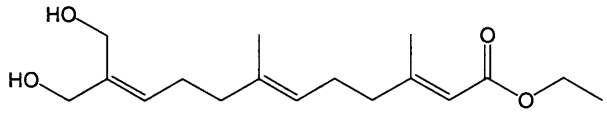
(2*E*,6*E*)-10-(2,2-Dimethyl-1,3-dioxan-5-ylidene)-3,7-dimethyl-deca-2,6-dienoic acid ethyl ester (184)



This compound was prepared from **183** in a manner similar to that for the ester **177**; purification by silica column chromatography using hexane and ethyl acetate (9: 1) as eluent gave ester **184** as a light yellow oil (0.48g, 38%); R_f 0.26 (Hexane : EtOAc = 9 : 1); HRMS (ES^+, $[M + NH_4]^+$) found 354.2638. $C_{20}H_{36}O_4N$ requires 354.2639; υ_{max}(thin film)/cm^{-1} 2933.9, 2849.1, 1715.0, 1647.5, 1448.4, 1368.7, 1222.0, 1145.1, 1089.8, 1055.7 and 833.0; δ_H (500 MHz; C^2HCl_3) 1.21 (3 H, t, *J* 7.0, OCH_2CH_3), 1.36 [6 H, s, $(CH_3)_2C$], 1.52 (3 H, s, CH_3), 1.93 [4 H, m, $(OCH_2)_2C$=CHCH_2CH_2], 2.04 (7 H, m, CH_3 and CH_2CH_2CCHC=O), 4.06 (2 H, q, *J* 7.0, OCH_2CH_3), 4.15 and 4.32 (4 H, 2 × s, 2 × OCH_2), 5.01 (1 H, m, CH_3C=C*H*CH_2CH_2), 5.14 [1 H, t, *J* 7.0, $(OCH_2)_2C$=C*H*CH_2CH_2] and 5.59 (1 H, s, C=C*H*C=O); δ_C (125 MHz; C^2HCl_3) 14.34 (OCH_2CH_3), 15.95 and 18.79 (CH_3C=CH), 24.07 [(CH_3)$_2$C], 25.15, 25.81, 39.12 and 40.80 (2 ×

CH_2CH_2), 59.48 (OCH_2CH_3), 59.90 and 64.54 [$(CH_2)_2C$], 99.01 [$(CH_3)_2C$], 115.73, 123.02 and 123.61 (3 × CH), 131.82, 135.27, 159.54 and 166.85 (quaternary C); *m/z* (CI^+) 353.3 (20%, $[M + NH_4]^+$) and 279.2 (100).

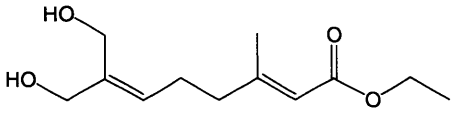
(2*E*,6*E*)-12-Hydroxy-11-hydroxymethyl-3,7-dimethyl-dodeca-2,6,10-trienoic acid ethyl ester (185)



The ester **184** (0.53 g, 1.58 mmol) was dissolved in ethanol (30 cm^3), then 3 drops of TFA was added and the mixture was stirred at room temperature for 3h. The reaction mixture was diluted with diethyl ether (30 cm^3), then washed with sodium bicarbonate (20 cm^3) and brine (20 cm^3), the aqueous layer was extracted with diethyl ether, The organic phases were combined, washed with brine and dried ($MgSO_4$), filtered and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (1 : 9) as eluent gave the title compound **185** as a pale yellow oil (0.37g, 79%); R_f 0.34 (Hexane : EtOAc = 1 : 9); HRMS (ES^+, $[M + H]^+$) found 297.2059. $C_{17}H_{29}O_4$ requires 297.2060; υ_{max}(thin film)/cm^{-1} 3348.4, 2931.1, 1714.2, 1645.9, 1445.9, 1382.8, 1223.4, 1446.5, 1011.9 and 864.9; δ_H (500 MHz; C^2HCl_3) 1.21 (3 H, t, *J* 7.0, OCH_2CH_3), 1.53 (3 H, s, CH_3), 1.96–2.14 (11 H, m, CH_3 and 2 × CH_2CH_2), 2.27 (2 H, b, 2 × OH), 4.06 (2 H, q, *J* 7.0, OCH_2CH_3), 4.14 and 4.24 (4 H, 2 × s, 2 × $HOCH_2$), 5.02 (1 H, m, $CH_3C=CHCH_2CH_2$), 5.44 [1 H, t, *J* 7.0, $(HOCH_2)_2C=CHCH_2CH_2$] and 5.59 (1 H, s, C=CHC=O); δ_C (125 MHz; C^2HCl_3) 14.33 (OCH_2CH_3), 16.01 and 18.80 ($CH_3C=CH$), 24.89, 25.81, 39.23 and 40.81 (2 × CH_2CH_2), 59.58 (OCH_2CH_3), 60.03 and 67.53 (2 × CH_2OH), 115.79, 123.66 and 130.41 (3 × CH), 135.28, 137.28, 159.58 and 166.99

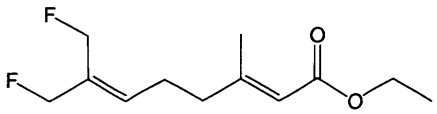
(quaternary C); *m/z* (CI^+) 314.2 (50%, $[M + NH_4]^+$) and 297.2 (100, $[M + H]^+$).

(*E*)-8-Hydroxy-7-hydroxymethyl-3-methyl-octa-2,6-dienoic acid ethyl ester (186)



This compound was prepared from **177** in a manner identical to that for the diol **185**; purification by silica column chromatography using hexane and ethyl acetate (1: 9) as eluent gave **186** as a light yellow oil (0.20g, 97%); R_f 0.31 (Hexane : EtOAc = 1 : 9); HRMS (ES^+, $[M + H]^+$) found 229.1433. $C_{12}H_{21}O_4$ requires 229.1434; υ_{max}(thin film)/cm^{-1} 3357.6, 2934.1, 1713.0, 1647.2, 1368.5, 1224.4, 1146.5, 1010.0 and 865.2; δ_H (500 MHz; C^2HCl_3) 1.21 (3 H, t, *J* 7.0, OCH_2CH_3), 2.08 (3 H, d, *J* 1.0, CH_3C=CHC=O), 2.13–2.24 (4 H, m, CH_2CH_2), 4.07 (2 H, t, *J* 7.0, OCH_2CH_3), 4.13 and 4.23 (4 H, 2 × s, 2 × $HOCH_2$), 5.44 (1 H, t, *J* 7.0, C=C*H*CH_2CH_2) and 5.59 (1 H, q, *J* 1.0, C=CHC=O); δ_C (125 MHz; C^2HCl_3) 14.31 (OCH_2CH_3), 15.27 (CH_3CCH), 25.24 and 40.42 (CH_2CH_2), 59.71 (OCH_2CH_3), 59.83 and 67.28 [$(CH_2)_2C$], 116.18 (C=C*H*CH_2CH_2), 129.02 (C=CHC=O), 138.16, 158.48 and 166.85 (quaternary C). *m/z* (CI^+) 246.2 (100%, $[M + NH_4]^+$) and 229.1 (30, $[M + H]^+$).

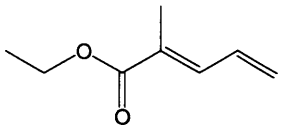
(*E*)-8-Fluoro-7-fluoromethyl-3-methyl-octa-2,6-dienoic acid ethyl ester (187)



To a stirred solution of DAST (0.23 cm^3, 1.76 mmol) in anhydrous DCM (30 cm^3) at –78 °C under argon was added a solution of the diol **186** (0.20 g, 0.88 mmol) in anhydrous DCM (2 cm^3), this solution was stirred for 1h at –78 °C, then 4h at room temperature. Water was carefully added

to quench the reaction, and the separated aqueous layer was extracted with DCM (3 × 10 cm^3). The organic phases were combined, washed with brine and dried ($MgSO_4$) and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (4 : 1) as eluent gave the title compound as a pale yellow oil (39 mg, 19%); R_f 0.36 (Hexane : EtOAc = 4 : 1); HRMS (ES^+, $[M + NH_4]^+$) found 250.1614. $C_{12}H_{22}O_2NF_2$ requires 250.1613; υ_{max}(thin film)/cm^{-1} 2982.3, 1713.8, 1649.1, 1445.7, 1369.4, 1223.6, 1146.7, 1060.3, 969.1 and 864.8; δ_H (500 MHz; C^2HCl_3) 1.21 (3 H, t, *J* 7.5, OCH_2CH_3), 2.11 (3 H, d, *J* 1.0, CH_3CCHCO), 2.16-2.33 (4H, m, CH_2CH_2), 4.08 (2H, t, *J* 7.5, OCH_2CH_3), 4.76 and 4.88 (4 H, 2 × d, J_{H-F} 47.5, 2 × FCH_2), 5.60 (1 H, q, *J* 1.0, C=CHCO) and 5.59 (1 H, m, $C=CHCH_2CH_2$); δ_C (125 MHz; C^2HCl_3) 14.31 (OCH_2CH_3), 18.67 ($CH_3C=CH$), 25.43 and 40.04 (CH_2CH_2), 59.67 (OCH_2CH_3), 78.05 (d, J_{C-F} 165.0, CH_2F), 85.09 (d, J_{C-F} 165.0, CH_2F), 116.44 ($C=CHC=O$), 131.67 (t, J_{C-F} 14.5, FCH_2C), 135.77 (dd, J_{C-F} 9.0, J_{C-F} 10.0, $C=CHCH_2CH_2$), 157.73 and 166.58 (quaternary C); δ_F (250 MHz, proton decoupled, C^2HCl_3) –217.07 (d, J_{F-F} 7.5) and –212.87 (d, J_{F-F} 7.5); *m/z* (ES^+) 250.2 (100%, $[M + NH_4]^+$).

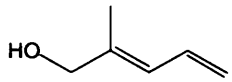
(*E*)-2-Methyl-penta-2,4-dienoic acid ethyl ester (193)



Acrolein (3.28 cm^3, 50 mmol) and carbethoxyethylidene triphenyl phosphorane (27.2 g, 75 mmol) were dissolved in DCM (150 cm^3), the reaction mixture was stirred at room temperature for 5h, then the water was added to quench the reaction, the organic phase was separated. The aqueous layer was extracted with diethyl ether (3 ×10 cm^3). The combined organic phases were washed with brine (10 cm^3) and dried ($MgSO_4$), filtered and then concentrated under reduced pressure.

Purification by flash column chromatography on silica gel with hexane and ethyl acetate (9 : 1) as eluent gave the title compound as a yellow oil (5.51g, 79%); R_f 0.34 (Hexane : EtOAc = 9 : 1); HRMS (EI^+, M^+) found 140.0836. $C_8H_{12}O_2$ requires 140.0837; υ_{max}(thin film)/cm^{-1} 2981.3, 1708.8, 1632.6, 1596.1, 1366.6, 1246.9, 1176.1, 1101.9, 990.5, 928.1 and 757.5; δ_H (500 MHz; C^2HCl_3) 1.24 (3 H, t, *J* 7.0, CH_3CH_2O), 1.88 (3 H, d, *J* 0.5, CH_3C=CHCH), 4.14 (2 H, t, *J* 7.0, CH_3CH_2O), 5.37 (1 H, dd, *J* 10.0, *J* 0.5, CHCHCHH_{cis}), 5.48 (1 H, dd, *J* 17.0, *J* 0.5, CHCHCH_{trans}H), 6.55 (1 H, ddd, *J* 10.0, *J* 11.0, *J* 17.0, CHCHCH$_2$) and 7.09 (1 H, dd, *J* 11.0, *J* 0.5, CHCHCH$_2$); δ_C (125 MHz; C^2HCl_3) 12.66 (CH_3C=CH), 14.29 (CH_3CH_2O), 60.63 (CH_3CH_2O), 124.01 (CHCHCH_2), 128.19 (C=O), 132.24 and 138.21 (CH), 168.36 (quaternary C); ***m/z*** (EI^+) 140.1 (65%, M^+) and 67.1 (100).

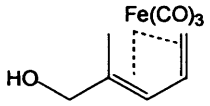
(*E*)-2-Methyl-penta-2,4-dien-1-ol (195)



To a stirred solution of ester **193** (1.63 g, 11.6 mmol) in dry THF (80 cm^3) at –78 °C, was added dropwise (over 3 min) diisobutylaluminum hydride (1.0 M, 25.6 mL, 25.6 mmol). The reaction mixture was allowed to stir at –78 °C for 1 h, then 0 °C for 1h, quenched with sat. sodium potassium tartrate (30 cm^3), and then extracted with diethyl ether (3 × 20 cm^3). The combined organic extracts were dried ($MgSO_4$), filtered and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (2 : 1) as eluent gave the title compound as a pale yellow oil (0.86 g, 76%); R_f 0.33 (Hexane : EtOAc = 2 : 1); HRMS (EI^+, M^+) found 98.0729. $C_6H_{10}O$ requires 98.0732; υ_{max} (thin film)/cm^{-1} 3333.9, 2915.7, 1658.4, 1601.1, 1421.2, 1144.2, 989.3, 901.5 and 650.0; δ_H (500 MHz; C^2HCl_3) 1.80 (3 H,

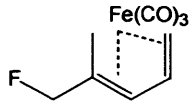
s, C*H*$_3$CCHCH), 4.08 (2 H, s, HOC*H*$_2$C), 5.13 (2 H, dd, *J* 10.0, *J* 1.0, CHCHCH*H*$_{cis}$), 5.21 (2 H, dd, *J* 17.0, *J* 1.0, CHCHC*H*$_{trans}$H), 6.11 (1 H, dd, *J* 10.0, *J* 1.0, C*H*CHCH$_2$) and 6.61 (1 H, dt, *J* 17.0, 10.0, CHC*H*CH$_2$); δ_C (125 MHz; C^2HCl_3) 14.08 (*C*H$_3$CCHCHCH$_2$), 68.21 (CH_2OH), 117.02 (CHCH*C*H$_2$), 125.35 (*C*HCHCH$_2$), 132.56 (CH*C*HCH$_2$) and 137.83 (quaternary C); *m/z* (EI^+) 98.1 (80%, M^+) and 83.1 (100).

[(*E*)-2-Methyl-penta-2,4-dien-1-ol]tricarbonyliron (196)



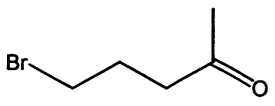
Diiron nonacarbonyl (1.17 g, 3.21 mmol) was added to a solution of the diene **195** (0.21 g, 2.14 mmol) in degassed THF (20 cm^3) and the mixture was refluxed under argon for 15h until the solid carbonyl had disappeared. The resulting mixture was concentrated under reduced pressure and column chromatography of the residue on silica separated the required complex form any inorganic species. Further purification by flash column chromatography on silica gel with hexane and ethyl acetate (2 : 1) as eluent gave the title compound as a yellow oil (0.41 g, 81%); R_f 0.30 (Hexane : EtOAc = 2 : 1); HRMS (EI^+, M^+) found 237.9917. $C_9H_{10}O_4{}^{56}Fe$ requires 237.9928; υ_{max}(thin film)/cm^{-1} 3327,3, 2931.8, 2043.4, 1961.2, 1411.7, 995.7 and 610.6; δ_H (500 MHz; C^2HCl_3) 1.19 (3 H, br, C*H*$_3$C=CHCH), 1.68 and 1.89 (2 H, br, CHCHC*H*$_2$), 3.64 (2 H, br, C*H*$_2$OH) and 5.36 (2 H, br, 2 × CH); δ_C (125 MHz; C^2HCl_3) 15.79 (CH_3), 41.52 (CHCH*C*H$_2$), 73.37 (CH_2OH), 86.51 and 91.14 (2 × CH) and 211.10 (C=O); *m/z* (EI^+) 238.0 (2%, M^+), 210.0 (10, [M – CO]$^+$), 182.0 (10, [M – 2 × CO]$^+$), 154.0 (12, [M – 3 × CO]$^+$) and 134.0 (100).

[(*E*)-5-Fluoro-4-methyl-penta-1,3-diene]tricarbonyliron (197)



To a stirred solution of DAST (0.45 cm^3, 3.40 mmol) in dry DCM (50 cm^3) at –78°C under argon atmosphere was added a solution of the alcohol **196** (0.67 g, 2.84 mmol) in dry DCM (2 cm^3), this solution was stirred for 1h at –78°C, then 4h at room temperature. Water was carefully added to quench the reaction, and the separated aqueous layer was extracted with DCM (3 × 10 cm^3). The organic phases were combined, washed with brine (10 cm^3) and dried ($MgSO_4$), filtered and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (9 : 1) as eluent gave the title compound as a pale yellow oil (0.38g, 56 %); R_f 0.33 (Hexane : EtOAc = 9 : 1); υ_{max}(thin film)/cm^{-1} 2895.6, 2044.7, 1963.8, 1446.3, 1386.0, 1111.8, 1056.2 and 607.0; δ_H (500 MHz; C^2HCl_3) 0.38 (2 H, d, $J_{H\text{-}F}$ 687.0, CH_2F), 0.79 (1 H, br, CHC*H*CH_2), 2.08 (3 H, s, C*H*$_3$C=CHCH), 3.31 (1 H, br d, *J* 42.5, CHCHC*H*$_{trans}$H), 3.52 (1 H, br d, *J* 33.0, CHCHCH*H*$_{cis}$) and 5.17 (1 H, br, C*H*CHCH$_2$); δ_C (125 MHz; C^2HCl_3) 22.68 (CH_3), 43.53 (d, $J_{C\text{-}F}$ 10.0, CH_2F), 55.16 (d, $J_{C\text{-}F}$ 24.0, CH*C*HCH$_2$), 72.23 (d, $J_{C\text{-}F}$ 12.5, CHCH*C*H$_2$), 87.04 (d, $J_{C\text{-}F}$ 27.5, *C*HCHCH$_2$), 100.48 (d, $J_{C\text{-}F}$ 12.5, quaternary C) and 211.31 (C=O); δ_F (250 MHz, proton decoupled, C^2HCl_3) –158.03; *m/z* (EI^+) 240.1 (1%, M^+), 212.1 (12, $[M - CO]^+$), 184.1 (13, $[M - 2 \times CO]^+$), 156.1 (10, $[M - 3 \times CO]^+$) and 136.0 (100).

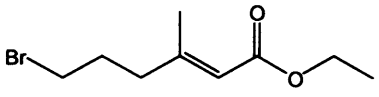
5-Bromopentan-2-one (202).



A solution of α-acetylbutyrolactone (6.46 cm^3, 60.0 mmol) in toluene (40 cm^3) was stirred and

hydrobromic acid (48% in H_2O, 10.2 cm^3, 90.0 mmol) was added. The whole reaction mixture was heated at 80 °C for 14 h under N_2. Water (30 cm^3) and diethyl ether (30 cm^3) were added, and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 20 cm^3). The combined ethereal extracts were washed with water (2 × 20 cm^3) and brine (20cm^3), dried over $MgSO_4$, filtered and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (4 : 1) as eluent gave bromoketone **202** as a pale yellow oil (1.83 g, 23%); R_f 0.25 (Hexane : EtOAc = 4 : 1); HRMS (EI^+, $[M(^{79}Br)]^+$) found 163.9829. $C_5H_9O^{79}Br$ requires 163.9831; υ_{max} (thin film)/cm^{-1} 2963.8, 1714.7, 1434.6, 1367.2, 1301.3, 1247.1, 1179.0, 908.6 and 735.0; δ_H (500 MHz, C^2HCl_3) 2.12 (2 H, quintet, *J* 7.0, $CH_2CH_2CH_2$), 2.17 (3 H, s, CH_3), 2.65 (2 H, t, *J* 7.0, CH_2C) and 3.45 (2 H, t, *J* 6.5, $BrCH_2$); δ_C (125 MHz, $CDCl_3$) 26.37 ($CH_2CH_2CH_2$), 30.07 (CH_3), 33.28 ($BrCH_2$), 41.43 (CH_2C) and 207.30 (quaternary C); *m/z* (EI^+) 165.9 (40%, $[M(^{81}Br)]^+$), 164.0 (40, $[M(^{79}Br)]^+$) and 92.8 (100).

(*E*)-Ethyl 6-bromo-3-methylhex-2-enoate (203)



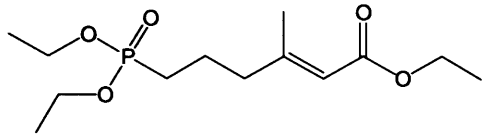
To a suspension of sodium hydride (0.12 g, 4.80 mmol) in anhydrous THF (10 cm^3) at 0 °C, triethyl phosphonoacetate (0.95 cm^3, 4.80 mmol) and a solution of bromo ketone **202** (0.66 g, 4.00 mmol) in anhydrous THF (5 cm^3) were sequentially added dropwise and with stirring. The reaction mixture was stirred for 14 h under N_2. Water (20 cm^3) and diethyl ether (20 cm^3) were added, and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 15 cm^3). The combined ethereal extracts were washed with water (2 × 10 cm^3) and brine (10

cm^3), dried over $MgSO_4$, filtered and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (9 : 1) as eluent gave the *Z* isomer of **203** as a minor by-product (56 mg, 6 %) followed by *E* isomer as a pale yellow oil (640 mg, 69%).

E isomer; R_f 0.27 (Hexane : EtOAc = 9 : 1); HRMS (ES^+, $[M + H]^+$) found 235.0327. $C_9H_{16}O_2{}^{79}Br$ requires 235.0328; υ_{max} (thin film)/cm^{-1} 2977.6, 1713.6, 1648.9, 1438.4, 1368.1, 1272.6, 1217.4, 1147.1, 1041.7, 951.3, 865.9 and 810.7; δ_H (500 MHz, C^2HCl_3) 1.30 (3 H, t, *J* 7.0, CH_2CH_3), 2.06 (2 H, m, $BrCH_2CH_2$), 2.18 (3 H, d, *J* 1.0, CCH_3), 2.32 (2 H, t, *J* 8.0, $BrCH_2CH_2CH_2$), 3.42 (2 H, t, *J* 7.5, $BrCH_2$), 4.16 (2 H, q, *J* 7.0, CH_2CH_3) and 5.72 (1 H, m, CCH); δ_C (125 MHz, C^2HCl_3) 14.30 (CH_2CH_3), 18.67 (CH_3), 30.25 ($BrCH_2CH_2$), 32.61 ($BrCH_2$), 38.99 ($BrCH_2CH_2CH_2$), 59.61 (CH_2CH_3), 116.65 (CH), 157.51 (quaternary C) and 166.57 (C=O); *m/z* (CI^+) 252.0 (100%, $[M(^{79}Br) + NH_4]^+$) and 254.0 (100, $[M(^{81}Br) + NH_4]^+$).

Z-isomer; R_f 0.35 (Hexane : EtOAc = 9 : 1); HRMS (ES^+, $[M + H]^+$) found 235.0328. $C_9H_{16}O_2{}^{79}Br$ requires 235.0328; υ_{max} (thin film)/cm^{-1} 2978.7, 1713.4, 1649.9, 1439.6, 1366.7, 1349.0, 1222.7, 1149.6, 1044.4 and 868.0; δ_H (500 MHz, C^2HCl_3) 1.28 (3 H, t, *J* 7.0, CH_2CH_3), 1.91 (3 H, d, *J* 1.5, CCH_3), 2.05 (2 H, m, $BrCH_2CH_2$), 2.74 (2 H, t, *J* 8.0, $BrCH_2CH_2CH_2$), 3.44 (2 H, t, *J* 7.0, $BrCH_2$), 4.15 (2 H, q, *J* 7.0, CH_2CH_3) and 5.71 (1 H, d, *J* 1.5, CCH); δ_C (125 MHz, C^2HCl_3) 14.30 (CH_2CH_3), 18.65 (CH_3), 31.39 ($BrCH_2CH_2$), 32.24 ($BrCH_2CH_2CH_2$), 33.24 ($BrCH_2$), 59.58 (CH_2CH_3), 117.19 (CCH), 158.22 (quaternary C) and 166.16 (C=O); *m/z* (CI+) 254.0 (60, $[M(^{81}Br) + NH_4]^+$), 252.0 (60%, $[M(^{79}Br) + NH_4]^+$)and 174.0 (100).

(*E*)-6-(Diethoxy-phosphoryl)-3-methyl-hex-2-enoic acid ethyl ester (199)



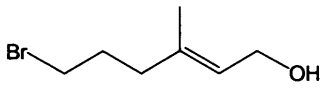
The bromo ester **203** (1.18 g, 5.0 mmol) was added to fresh distilled triethyl phosphite (1.03 cm^3, 6 mmol) and the mixture was stirred and heated to 130 °C for 3.5 h under N_2. The resulting material was purified by flash column chromatography on silica gel with hexane and ethyl acetate (1 : 9) as eluent gave the title compound as light yellow oil (1.19 mg, 81%).

E-isomer: R_f 0.17 (Hexane : EtOAc = 1 : 9); HRMS (ES^+, $[M + H]^+$) found 293.1514. $C_{13}H_{26}O_5P$ requires 293.1512; υ_{max} (thin film)/cm^{-1} 2980.8, 2360.6, 1714.6, 1648.4, 1444.7, 1391.8, 1368.1, 1223.8, 1151.7, 1096.9, 1029.1, 959.7, 851.1 and 810.5; δ_H (500 MHz, C^2HCl_3) 1.29 (3 H, t, *J* 7.0, $CO_2CH_2CH_3$), 1.34 (6 H, *J* 7.0, 2 × $POCH_2CH_3$), 1.70–1.83 (4 H, m, $PCH_2CH_2CH_2$), 2.16 (3 H, d, *J* 1.0, CCH_3), 2.24 (2 H, t, *J* 7.5, $PCH_2CH_2CH_2$), 4.08–4.18 (6 H, m, 3 × CH_2CH_3) and 5.68 (1 H, d, *J* 1.0, CH); δ_C (125 MHz, C^2HCl_3) 14.31 ($CO_2CH_2CH_3$), 16.45 and 16.50 (2 × $POCH_2CH_3$), 18.50 (CCH_3), 20.25 (d, $J_{C\text{-}P}$ 5.0, $PCH_2CH_2CH_2$), 24.50 (d, $J_{C\text{-}P}$ 140.0, $PCH_2CH_2CH_2$), 41.19 (d, $J_{C\text{-}P}$ 16.0, $PCH_2CH_2CH_2$), 59.57, 61.51 and 61.56 (3 × OCH_2CH_3), 116.54 (CH), 158.04 (quaternary C) and 166.60 (C=O); δ_P (500 MHz, C^2HCl_3) 31.50; *m/z* (CI^+) 310.2 (25%, $[M + NH_4]^+$) and 293.2 (100, $[M + H]^+$).

Z-isomer: R_f 0.14 (Hexane : EtOAc = 1 : 9); HRMS (ES^+, $[M + H]^+$) found 293.1511. $C_{13}H_{26}O_5P$ requires 293.1512; υ_{max} (thin film)/cm^{-1} 2982.4, 2908.5, 1713.3, 1649.8, 1443.9, 1391.9, 1368.6, 1225.8, 1150.6, 1096.6, 1024.8, 963.3, 811.9 and 701.9; δ_H (500 MHz, C^2HCl_3) 1.21 (3 H, t, *J* 7.0, $CO_2CH_2CH_3$), 1.26 (6 H, *J* 7.0, 3 × $POCH_2CH_3$), 1.61 (4 H, m, $PCH_2CH_2CH_2$), 2.08 (3H, d, *J* 1.0, CCH_3), 2.16 (2H, t, *J* 7.5, $PCH_2CH_2CH_2$), 4.01-4.10 (6H, m, 3 × OCH_2CH_3) and 5.60 (1H, d, *J*

1.0, CH); δ_C (125 MHz, C^2HCl_3) 14.30 ($CO_2CH_2CH_3$), 16.44 and 16.49 (3 × $POCH_2CH_3$), 18.49 (CCH_3), 20.24 (d, $J_{C\text{-}P}$ 5.0, $PCH_2CH_2CH_2$), 24.49 (d, $J_{C\text{-}P}$ 140.0, $PCH_2CH_2CH_2$), 41.17 (d, $J_{C\text{-}P}$ 17.5, $PCH_2CH_2CH_2$), 59.56, 61.51 and 61.56 (3 × CH_2CH_3), 116.54 (CH), 158.01 (quaternary C) and 166.58 (C=O); δ_P (500 MHz, C^2HCl_3,) 31.66; *m/z* (CI^+) 293.2 310.2 (25%, $[M + NH_4]^+$) and (100, $[M + H]^+$).

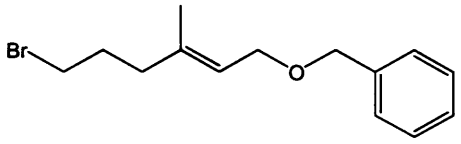
(*E*)-6-Bromo-3-methyl-hex-2-en-1-ol (206)



A solution of **203** (1.50 g, 6.40 mmol) in anhydrous THF (60 cm^3) was stirred under N_2 and cooled to –78 °C and then diisobutylaluminium hydride (1 M solution in hexane, 15.4 cm^3, 15.4 mmol) was added dropwise. The resulting mixture was stirred at –78 °C for 2 h and then allowed to warm to 0 °C at which time the reaction was judged complete by TLC analysis. Saturated potassium sodium tartrate solution (50 cm^3) and diethyl ether (50 cm^3) were added. The mixture was stirred at room temperature for another 30 min, and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 30 cm^3). The combined ethereal extracts were washed with brine (30 cm^3), dried over $MgSO_4$, filtered and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (2 : 1) as eluent gave **206** as a light yellow oil (1.20 g, 98%); R_f 0.29 (Hexane : EtOAc = 2 : 1); υ_{max} (thin film)/cm^{-1} 3327.9, 2936.0, 1668.1, 1437.5, 1382.1, 1284.6, 1244.0, 1202.3, 1092.2, 1000.9 and 865.9; δ_H (500 MHz, C^2HCl_3) δ 1.40 (1 H, b, OH), 1.61 (3H, s, CCH_3), 1.92 (2 H, quintet, *J* 7.0, $BrCH_2CH_2$), 2.11 (2 H, t, *J* 7.0, $BrCH_2CH_2CH_2$), 3.33 (2 H, t, *J* 7.0, $BrCH_2$), 4.10 (2 H, d, *J* 7.0, CH_2OH) and 5.39 (1 H, m, CH); δ_C (125 MHz, C^2HCl_3) 16.19 (CCH_3), 30.63

($BrCH_2CH_2$), 33.18 ($BrCH_2$), 37.71 ($BrCH_2CH_2CH_2$), 59.29 (CH_2OH), 124.61 (*C*H) and 137.76 (quaternary *C*); *m/z* (EI^+) 194.0 (100% , $[M(^{81}Br)]^+$) and 192.0 (100%, $[M(^{79}Br)]^+$).

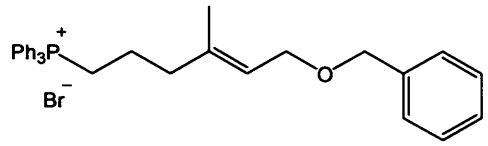
1-{[(*E*)-6-bromo-3-methylhex-2-enyloxy]methyl}benzene (207)



To a suspension of sodium hydride (1.02 g, 25.5 mol) in anhydrous THF (80 cm^3) at 0 °C, benzyl bromide (3.03 cm^3, 25.5 mmol) and a solution of alcohol **206** (3.28 g, 17.0 mmol) in anhydrous THF (10 cm^3) were sequentially added dropwise and with stirring. The whole reaction mixture was allowed to warm to RT and stirred for 3 h under N_2. Water (40 cm^3) and diethyl ether (40 cm^3) were added, and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 30 cm^3). The combined ethereal extracts were washed with water (2 × 30 cm^3) and satd. NaCl (1 × 40 cm^3), dried over $MgSO_4$ and then concentrated under reduced pressure. Purification of the crude product by flash column chromatography on silica gel with hexane and ethyl acetate (9 : 1) as eluent gave the title benzyl ether as a pale yellow oil (3.51 g, 73%); R_f 0.36 (Hexane : EtOAc = 9 : 1); HRMS (CI^+, $[M + NH_4]^+$) found 300.0956. $C_{14}H_{23}ON^{79}Br$ requires 300.0958; υ_{max} (thin film)/cm^{-1} 2925.0, 2845.1, 1668.9, 1495.6, 1452.7, 1363.2, 1245.3, 1203.8, 1069.1, 1027.7, 736.2 and 697.6; δ_H (500 MHz, C^2HCl_3) 1.69 (3H, s, CH_3), 2.02 (2 H, quintet, *J* 7.0, $CH_2CH_2CH_2$), 2.22 (2 H, t, *J* 7.0, CH_2C), 3.43 (2H, t, *J* 6.5, $BrCH_2$), 4.07 (2H, d, *J* 6.5, $CHCH_2O$), 4.55 (2 H, s, OCH_2Ph), 5.50 (1 H, m, $CCHCH_2$) and 7.34 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 16.49 (CH_3), 30.66 ($CH_2CH_2CH_2$), 33.38 ($BrCH_2$), 37.80 (CH_2C), 66.53 ($CCHCH_2$), 72.21 (OCH_2Ph), 122.15 ($CCHCH_2$), 123.23 (quaternary C), 127.65, 127.88 and

128.44 (Ar-CH) and 138.48 (quaternary C); *m/z* (CI^+) 302.1 (95%, $[M(^{81}Br) + NH_4]^+$) and 301.1 (100, $[M(^{79}Br) + NH_4]^+$).

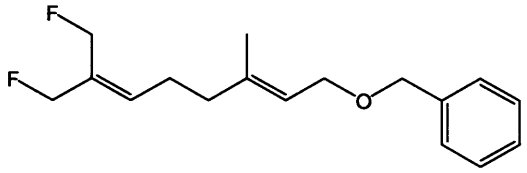
(*E*)-[6-(benzyloxy)-4-methyl-4-hexen-1-yl]triphenylphosphonium bromide (208)



A mixture of bromo benzyl ether **207** (3.51 g, 12.4 mmol) and triphenyl phosphine (4.88 g, 18.6 mmol) in acetonitrile (100 cm^3) under a nitrogen atmosphere was refluxed for 14 h and cooled to 25 °C. The solvent was evaporated to afford an oily mixture. Excess triphenyl phosphine was removed by continuously washing the mixture with anhydrous diethyl ether (3 × 20 cm^3) and triturating the mixture until a solid precipitated. Diethyl ether (20 cm^3) was added to the solid, and mixture was kept refrigetated overnight. The phosphonium salt was collect and washed with diethyl ether (2 × 20 mL) to afford the title product as a white solid (5.81 g, 87%); HRMS (ES^+, M^+) found 465.2338. $C_{32}H_{34}OP$ requires 465.2347; υ_{max} (thin film)/cm^{-1} 3386.2, 3030.1, 2859.8, 2356.8, 1652.0, 1585.2, 1483.6, 1437.3, 1111.6, 1066.6, 995.5, 922.1, 733.9 and 691.0; δ_H (500 MHz, C^2HCl_3) 1.46 (3 H, s, CH_3), 1.72 (2 H, m, $CH_2CH_2CH_2$), 2.31 (2 H, t, *J* 7.0, CH_2C), 3.69 (2 H, br, PCH_2), 3.89 (2 H, d, *J* 6.5, $CHCH_2O$), 4.38 (2 H, s, OCH_2Ph), 5.27 (1 H, dt, *J* 6.5, *J* 1.0, $C=CHCH_2$), 7.18–7.25 (5 H, m, Ar-H) and 7.60–7.77 (15H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 16.61 (CH_3), 20.32 (d, $J_{C\text{-}P}$ 4.0, $CH_2CH_2CH_2$), 21.86 (d, $J_{C\text{-}P}$ 51.5, PCH_2), 39.13 (d, $J_{C\text{-}P}$ 15.0, CH_2CH_2C), 66.50 ($C=CHCH_2$), 72.35 (OCH_2Ph), 117.87 (d, $J_{C\text{-}P}$ 86.5, Ar-CH), 122.41 ($CCHCH_2$), 127.60, 127.82 and 128.35 (Ar-CH), 130.49 (d, $J_{C\text{-}P}$ 12.5, Ar-CH), 133.60 (d, $J_{C\text{-}P}$ 10.0, Ar-CH), 135.06 (d, $J_{C\text{-}P}$ 2.5, Ar-CH) and 138.31 (quaternary C); *m/z* (ES^+) 465.2 (100%,

M^+).

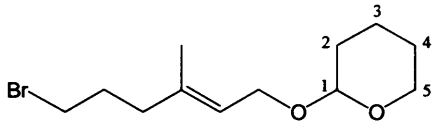
1-{[(*E*)-8-fluoro-7-(fluoromethyl)-3-methylocta-2,6-dienyloxy]methyl}benzene (209)



To a suspension of triphenylphosphonium bromide **208** (5.45 g, 10.0 mol) in anhydrous THF (50 cm^3) at –78 °C, LiHMDS (1 M, 11.0 cm^3, 11.0 mmol) was added dropwise and with stirring. The reaction mixture was allowed to warm to –20 °C for 30 min before it was cooled to –78 °C again. Difluoro acetone (0.82 cm^3, 10.0 mmol) was then added dropwise. The whole reaction mixture was then allowed to warm to RT over 1 h. Water (20 cm^3) and diethyl ether (20 cm^3) were added, and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 15 cm^3). The combined ethereal extracts were washed with satd. NaCl (1 × 20 cm^3), dried over $MgSO_4$ and then concentrated under reduced pressure. Purification of the crude product by flash column chromatography on silica gel with hexane and ethyl acetate (9 : 1) as eluent gave the difluoride as a colorless oil (2.67 g, 93%); R_f 0.28 (Hexane : EtOAc = 9 : 1); HRMS (ES^+, $[M + NH_4]^+$) found 298.1978. $C_{17}H_{26}ONF_2$ requires 298.1977; υ_{max} (thin film)/cm^{-1} 2925.4, 2855.4, 1617.7, 1495.9, 1453.7, 1371.6, 1237.5, 1202.1, 1068.6, 969.6, 737.9 and 698.4; δ_H (500 MHz, C^2HCl_3) 1.50 (3 H, s, CH_3), 1.89 (2 H, t, *J* 8.0, CH_2CH_2C), 2.16 (2 H, m, CH_2CH_2C), 3.87 (2 H, d, *J* 7.5, CH_2O), 4.35 (2 H, s, OCH_2Ph), 4.66 (2 H, d, $J_{H\text{-}F}$ 47.5, CH_2F), 4.80 (2 H, d, $J_{H\text{-}F}$ 48.0, CH_2F), 5.27 (1 H, dt, *J* 7.0, *J* 1.0, $CHCH_2O$), 5.70 (1 H, m, $CCHCH_2CH_2$) and 7.10–7.20 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 16.44 (CH_3), 25.77 ($CHCH_2CH_2C$), 38.78 (t, $J_{C\text{-}F}$ 2.5, CH_2CH_2C), 66.49 (CH_2O), 72.18 (CH_2Ph), 76.87 (d, $J_{C\text{-}F}$161.5, CH_2F), 83.94 (d, $J_{C\text{-}F}$ 163.5, CH_2F), 121.98

(*C*HCH$_2$O), 127.59, 127.81 and 128.39 (Ar-CH), 131.05 (t, $J_{C\text{-}F}$ 15.0, *C*CH$_2$F), 137.11 (t, $J_{C\text{-}F}$ 9.0, CH$_2$FC*C*H), 138.48 and 138.74 (quaternary C); δ_F (282 MHz, C^{2}HCl$_3$) –210.37 (t, $J_{H\text{-}F}$ 48.0) and –215.10 (t, $J_{H\text{-}F}$ 48.5); *m/z* (CI$^+$) 298.3 (100%, [M + NH$_4$]$^+$).

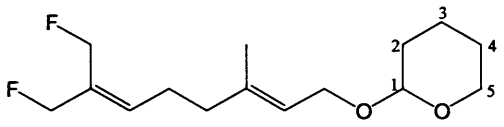
2-((*E*)-6-bromo-3-methylhex-2-enyloxy)-tetrahydro-2*H*-pyran (213)



To a stirred solution of **206** (2.82 g, 14.6 mmol) and 3,4-dihydro-*2H*-pyran (2.68 cm^3, 29.2 mmol) in CH$_2$Cl$_2$ (80 cm^3) under N$_2$ at 0 °C was added *p*-toluenesulfonic acid (0.14 g, 0.74 mmol), and the mixture stirred for 16 h whilst warming to room temperature. The solution was then diluted with diethyl ether (80 cm^3) and washed with a saturated NaHCO$_3$ solution (50 cm^3). The aqueous layer was extracted with diethyl ether (2 × 50 cm^3) and the combined ethereal extracts were washed with water (50 cm^3) and brine (50 cm^3), dried over MgSO$_4$, filtered and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (9 : 1) as eluent to gave **158** as a yellow oil (3.72 g, 93%); R$_f$ 0.23 (Hexane : EtOAc = 9 : 1); HRMS (ES$^+$, [M + NH$_4$]$^+$) found 294.1065. C$_{12}$H$_{25}$O$_2$N^{79}Br requires 294.1063; υ_{max} (thin film)/cm^{-1} 2940.1, 2865.2, 1669.3, 1439.8, 1383.6, 1353.0, 1245.5, 1199.8, 1116.8, 1076.2, 1023.2, 905.6, 868.8 and 813.8; δ_H (500 MHz, C^{2}HCl$_3$) 1.60–1.85 [6 H, m, CH(C*H*$_2$)$_3$], 1.69 (3 H, s, CCH$_3$), 2.00 (2 H, quintet, *J* 7.0, BrCH$_2$C*H*$_2$), 2.19 (2 H, t, *J* 7.0, BrCH$_2$CH$_2$C*H*$_2$), 3.40 (2 H, t, *J* 7.0, BrCH$_2$), 3.51 and 3.88 [2 H, m, (CH$_2$)$_2$C*H*$_2$O], 4.01 and 4.23 (2 H, m, CHC*H*$_2$O), 4.62 (1 H, m, ^{1}C*H*CH$_2$CH$_2$CH$_2$) and 5.42 (1 H, m, C*H*CH$_2$O); δ_C (125 MHz, C^{2}HCl$_3$) 16.34 (C*C*H$_3$), 19.61, 25.49, and 30.66 [(*C*H$_2$)$_3$CH$_2$O], 30.71 (BrCH$_2$*C*H$_2$), 33.25 (BrCH$_2$), 37.80

($BrCH_2CH_2CH_2$), 62.32 [$(CH_2)_3CH_2O$], 63.58 ($CHCH_2O$), 97.95 (OCHO), 121.98 ($CHCH_2O$) and 138.13 (CH_3C=CH); *m/z* (EI^+) 296.2 (50, $[M(^{81}Br) + NH_4]^+$), 294.2 (50%, $[M(^{79}Br) + NH_4]^+$) and 102.1 (100).

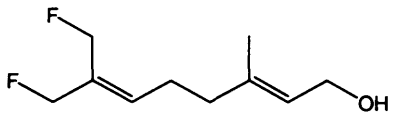
(*E*)-2-(8-Fluoro-7-fluoromethyl-3-methyl-octa-2,6-dienyloxy)-tetrahydro-2*H*-pyran (210)



A mixture of **213** (1.10 g, 3.97 mmol) and triphenylphosphine (1.57 g, 5.99 mmol) in anhydrous acetonitrile (20 cm^3), under a nitrogen atmosphere, was heated under reflux for 14 h. After cooling, the solvent was concentrated under reduced pressure to afford an oily mixture. Excess triphenyl phosphine was removed by triturating the mixture with anhydrous diethyl ether, and the sticky residue was dried *in vacuo* for 5 h to afford the intermediate phosphonium salt **214** as a viscous oil, which was used directly without further purification. To a stirred solution of crude **214** (1.96 g, 3.63 mmol) in anhydrous THF (40 cm^3) under N_2 at –78 °C, was added lithium hexamethyldisilazide (1.0 M solution in THF, 4.00 cm^3, 4.00 mmol), the mixture was stirred for 30 min at –78 °C, and then 1,3-difluoro acetone (0.30 cm^3, 3.64 mmol) was added dropwise. The whole reaction mixture was allowed to warm to –20 °C over 1 h, and maintained at –20 °C for a further 2 h. The reaction was then quenched by addition of diethyl ether (40 cm^3) and water (40 cm^3). The aqueous layer was extracted with diethyl ether (3 × 30 cm^3) and the combined ethereal extracts were washed brine (30 cm^3), dried over $MgSO_4$, filtered and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (6 : 1) as eluent gave **210** as a yellow oil (0.66 g, 66%); R_f 0.27 (Hexane : EtOAc = 6 : 1); HRMS (CI^+, $[M + NH_4]^+$) found 292.2081. $C_{15}H_{28}O_2NF_2$ requires 292.2083; υ_{max} (thin

film)/cm^{-1} 2942.1, 2364.1, 1672.0, 1441.6, 1317.6, 1261.0, 1200.1, 1117.4, 1076.4, 1203.8, 970.8, 904.9, 868.8 and 813.9; δ_H (500 MHz, C^2HCl_3) 1.39–1.68 [6 H, m, $(CH_2)_3CH_2O$], 1.53 (3 H, s, CCH_3), 1.98 (2 H, t, *J* 7.5, $FCH_2CCHCH_2CH_2$), 2.14 (2 H, m, FCH_2CCHCH_2), 3.35 and 3.71 [2 H, m, $(CH_2)_3CH_2O$], 3.85 and 4.07 (2 H, m, $CHCH_2O$), 4.47 (1 H, m, OCHO), 4.67 (2 H, d, J_{HF} 47.5, CH_2F), 4.80 (2 H, d, J_{HF} 47.5, CH_2F), 5.23 (1 H, dt, *J* 7.5, *J* 1.0, $C{=}CHCH_2O$) and 5.70 (1 H, m, $CH_2FC{=}CH$); δ_C (125 MHz, C^2HCl_3) 16.35 (CH_3), 19.61, 25.48 and 30.70 [$(CH_2)_3CH_2O$], 25.76 ($FCH_2C{=}CHCH_2$), 38.77 (t, J_{CF} 2.5, $CH_2FCCHCH_2CH_2$), 62.32 [$(CH_2)_3CH_2O$], 63.54 ($CHCH_2O$), 76.86 (d, J_{CF} = 161, CH_2F), 83.92 (d, J_{CF} = 164, CH_2F), 97.95 (OCHO), 121.81 ($CHCH_2O$), 130.89 (t, J_{CF} = 14.0, CCH_2F), 137.12 (t, J_{CF} = 9, CH_2FCCH) and 138.49 (CH_2CCH_3); δ_F (283 MHz, C^2HCl_3) –210.13 (t, J_{HF} 47.0) and –214.88 (t, J_{HF} 48.5); *m/z* (CI^+) 292.3 (100%, $[M + NH_4]^+$) and 102.2 (95).

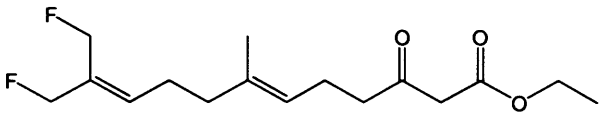
(*E*)-8-Fluoro-7-fluoromethyl-3-methylocta-2,6-dien-1-ol (211).



A solution of **210** (0.48 g, 1.75 mmol) and pyridinium *p*-toluenesulfonate (0.04 g, 0.17 mmol) in ethanol (20 cm^3) was stirred at 55 °C for 3 h. The solvent was concentrated under reduced pressure to give a yellow oil. Water (20 cm^3) and diethyl ether (20 cm^3) were added, and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 10 cm^3). The combined organic layers were washed with brine (10 cm^3), dried over $MgSO_4$, filtered and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (2 : 1) as eluent gave **211** as a pale yellow oil (0.30 g, 91%); R_f 0.24 (hexane : EtOAc = 2 : 1); HRMS (CI^+, $[M + NH_4]^+$) found 208.1508. $C_{10}H_{20}ONF_2$ requires

208.1507; υ_{max} (thin film)/cm^{-1} 3364.5, 2932.0, 2361.2, 1670.5, 1443.4, 1373.6, 1236.9 and 987.1; δ_H (500 MHz, C^2HCl_3) 1.65 (1 H, s, OH), 1.69 (3 H, s, CH_3), 2.14 (2 H, t, *J* 7.5, C=CHCH$_2$C*H*$_2$), 2.32 (2 H, m, C=CHC*H*$_2$CH$_2$), 4.16 (2 H, d, *J* 7.0, C*H*$_2$OH), 4.84 (2 H, d, $J_{H\text{-}F}$ 47.5, C*H*$_2$F), 4.96 (2 H, d, $J_{H\text{-}F}$ 47.5, CH$_2$F), 5.43 (1 H, dt, *J* 7.0, *J* 1.0, C*H*CH$_2$OH) and 5.86 (1 H, m, CC*H*CH$_2$CH$_2$); δ_C (125 MHz, C^2HCl_3) 16.19 (CH_3), 25.76 (C=CHCH$_2$*C*H$_2$), 38.63 (C=CH*C*H$_2$CH$_2$), 59.22 (CH$_2$OH), 76.88 (d, J_{CF} 161.0, CH$_2$F), 83.94 (d, J_{CF} 164.0, CH$_2$F), 124.54 (*C*HCH$_2$OH), 131.08 (t, J_{CF} 15.0, *C*CH$_2$F), 137.09 (t, $J_{C\text{-}F}$ 9.0, CH$_2$FC=*C*H) and 137.92 (CH$_2$*C*CH$_3$); δ_F (283 MHz, C^2HCl_3) –209.94 (t, J_{HF} 47.5) and –214.59 (t, J_{HF} 47.5); *m/z* (CI^+) 208.2 (100, $[M + NH_4]^+$) and 190.2 (90%, M^+).

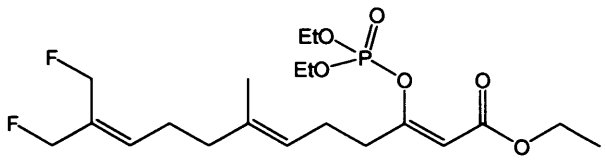
(*E*)-ethyl 12-fluoro-11-(fluoromethyl)-7-methyl-3-oxododeca-6,10-dienoate (216)



A solution of alcohol **211** (0.24 g, 1.26 mmol) and triethylamine (0.35 cm^3, 2.53 mmol) in anhydrous THF (15 mL) was stirred and cooled at –45 °C as MsCl (0.13 cm^3, 1.64 mmol) was added. The resulting milky mixture was stirred at –45 °C for 45 min. a solution of LiBr (0.44 g, 4.05 mmol) in THF (5 mL) was added via cannula at –45 °C. The suspension was allowed to warm to 0 °C and stirred for addition 1 h at which time the reaction was judged complete by TLC analysis. Cold water (20 cm^3) and hexane (20 cm^3) were added. The two layers were separated, and the aqueous layer was extracted with hexane (2 × 15 cm^3). The combined organic layers were washed with satd. $NaHCO_3$ (1 × 15 cm^3) and satd. NaCl (1 × 15 cm^3) and dried over $NaSO_4$. Evaporation of the solvent gave the bromide as a light yellow oil which was used without further purification.

To a suspension of NaH (0.10 g, 4.16 mmol) in anhydrous THF (15 cm^3) was added ethyl acetoacetate (0.48 cm^3, 3.78 mmol) dropwise at 0 °C. After 10 min, n-BuLi (2.5 M, 1.60 cm^3, 3.97 mmol) was added slowly over 3 min, during which time the colorless solution gradually turned yellow. It was stirred for additional 10 min at 0 °C, as a solution of above bromide **212** in THF (5 cm^3) was added. The clear solution turned to a cloudy yellow suspension. After 30 min, TLC analysis showed the reaction was complete and HCl (3 M, 2.0 cm^3) was added, water (10 cm^3) and diethyl ether (10 cm^3) were added, and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 10 cm^3). The combined ethereal extracts were washed with water (2 × 10 cm^3) and satd. NaCl (10 cm^3), dried over $MgSO_4$ and then concentrated under reduced pressure. Purification of the crude product by flash column chromatography on silica gel with hexane and ethyl acetate (4 : 1) as eluent gave the title β-keto ester **216** as a pale yellow oil (0.13 g, 89% for two steps); R_f 0.21 (Hexane : EtOAc = 6 : 1); υ_{max} (thin film)/cm^{-1} 2981.9, 1743.5, 1716.2, 1644.2, 1443.4, 1408.2, 1368.4, 1314.1, 1236.9, 1178.5, 1096.9, 1034.8, 968.0 and 855.9; δ_H (500 MHz, C^2HCl_3) 1.30 (3 H, t, *J* 7.0, CH_2CH_3), 1.63 (3 H, s, CCH_3), 2.08 (2 H, m, CH_2CH_2CO), 2.28 (4 H, m, $CH_2CH_2CCH_3$), 2.58 (2 H, t, *J* 7.5, CH_2CH_2CO), 3.44 (2 H, s, $COCH_2CO$), 4.20 (2 H, q, *J* 7.0, CH_2CH_3), 4.83 (2 H, d, $J_{H\text{-}F}$ 47.5, CH_2F), 4.98 (2 H, d, $J_{H\text{-}F}$ 47.5, CH_2F), 5.10 (1 H, td, *J* 7.0, *J* 1.0, CH_3CCHCH_2) and 5.83 (1 H, m, $CH_2FCCHCH_2CH_2$); δ_C (125 MHz, C^2HCl_3) 14.08 (CH_2CH_3), 15.88 (CH_3), 22.04, 25.91, 38.76 and 42.83 (2 × CH_2CH_2), 49.33 ($COCH_2CO$), 61.33 (CH_2CH_3), 76.88 (d, $J_{C\text{-}F}$ 161.5, CH_2F), 83.96 (d, $J_{C\text{-}F}$ 163.5, CH_2F), 123.42 (CH_3CCHCH_2), 130.87 (t, $J_{C\text{-}F}$ 15.0, CCH_2F), 135.15 (CH_3C), 137.39 (t, $J_{C\text{-}F}$ 8.5, CH_2FCCH), 167.18 and 202.38 (2 × C=O); δ_F (250 MHz, proton decoupled, C^2HCl_3) –211.9 (d, $J_{F\text{-}F}$ 5.0) and –216.8 (t, $J_{F\text{-}F}$ 5.0); *m/z* (EI^+) 302.2 (20%, M^+) and 189.1 (100).

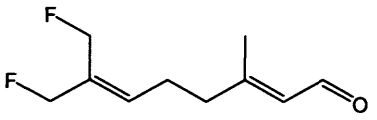
(1*Z*,5*E*)-1-(ethoxycarbonyl)-11-fluoro-10-(fluoromethyl)-6-methylundeca-1,5,9-trien-2-yl diethyl phosphate (217)



To a stirred suspension of NaH (0.011 g, 0.48 mmol) in anhydrous diethyl ether (10 cm^3) at 0 °C was added a solution of β-keto ester **216** (0.12 g, 0.40 mmol). The mixture was then stirred at 0 °C for 30 min until the bubbling stopped. Diethyl chlorophosphate (0.09 cm^3, 0.60 mmol) was added slowly to the resulting clear colorless solution. Stirring was continued at 0 °C for additional 15 min at which time, TLC showed that all the β-keto ester was consumed. The reaction was quenched by adding satd. NH_4Cl (5 cm^3). Water (10 cm^3) and diethyl ether (10 cm^3) were added, and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 10 cm^3). The combined ethereal extracts were washed with satd. NaCl (1 × 10 cm^3), dried over $MgSO_4$ and then concentrated under reduced pressure. Purification of the crude product by flash column chromatography on silica gel with hexane and ethyl acetate (1 : 1) as eluent gave the title compound as a pale yellow oil (0.14 mg, 88%); R_f 0.29 (Hexane : EtOAc = 1 : 1); HRMS (ES^+, $[M + H]^+$) found 439.2058. $C_{20}H_{34}O_6F_2P$ requires 439.2056; υ_{max} (thin film)/cm^{-1} 2982.6, 2358.2, 1726.0, 1663.3, 1445.9, 1370.8, 1280.1, 1204.9, 1147.6, 1097.9, 1034.5, 988.1 and 803.3; δ_H (500 MHz, C^2HCl_3) 1.28 (3 H, t, *J* 7.0, CH_2CH_3), 1.37 (6 H, t, *J* 7.0, 2 × $POCH_2CH_3$), 1.63 (3 H, s, CCH_3), 2.09 (2 H, t, *J* 7.5, CH_2CCH_3), 2.26 (4 H, m, 2 × $CHCH_2CH_2$), 2.47 (2 H, t, *J* 7.50, CH_2CH_2CO), 4.20 (2 H, q, *J* 7.0, CH_2CH_3), 4.27 (4 H, quintet, *J* 7.0, 2 × $POCH_2CH_3$), 4.83 (2 H, d, $J_{H\text{-}F}$ 47.5, CH_2F), 4.95 (2 H, d, $J_{H\text{-}F}$ 47.5, CH_2F), 5.13 (1 H, td, *J* 7.0, *J* 1.0, CH_3CCHCH_2), 5.35 (1 H, s, C=CHC=O) and 5.84 (1 H, m, $CH_2FC=CHCH_2CH_2$);

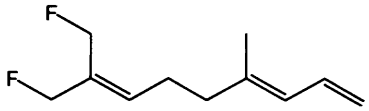
δ_C (125 MHz, C^2HCl_3) 14.22, 15.97, 16.03 and 16.09 (4 × CH_3), 24.85 and 25.95 (2 × C=CHCH_2CH$_2$), 35.09 (CH_2CH_2CO), 38.79 ($CH2CCH_3$), 59.89 (CH_2CH_3), 64.73 and 64.78 (2 × POCH_2CH$_3$), 76.86 (d, $J_{C\text{-}F}$ 161.5, CH_2F), 83.96 (d, $J_{C\text{-}F}$ 165.0, CH_2F), 105.37 (d, $J_{C\text{-}P}$ 7.5, C=C*H*CO), 123.04 (CH_3C=$CHCH_2$), 130.89 (t, $J_{C\text{-}F}$ 15.0, CCH_2F), 135.56 (CH_3C), 137.34 (t, $J_{C\text{-}F}$ 8.5, CH_2FC=CH), 161.12 (d, $J_{C\text{-}P}$ 7.5, CH_2COP) and 163.73 ($CO_2CH_2CH_3$); δ_P (500 MHz, C^2HCl_3,) –8.78; δ_F (282 MHz, C^2HCl_3) –211.98 (t, $J_{H\text{-}F}$ 47.0) and 216.73 (t, $J_{H\text{-}F}$ 47.0). *m/z* (CI^+) 456.3 (100%, $[M + NH_4]^+$) and 439.2 (80, $[M + H]^+$).

(*E*)-8-Fluoro-7-fluoromethyl-3-methylocta-2,6-dienal (221).



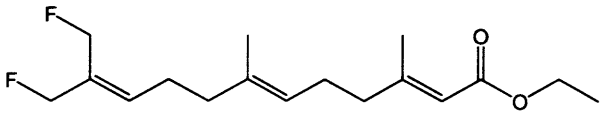
This compound was prepared from **211** in a manner identical to that for the aldehyde **181**; purification by silica column chromatography using hexane and ethyl acetate (2: 1) as eluent gave aldehyde **221** as a pale yellow oil (1.40 g, 90%); R_f 0.29 (Hexane : EtOAc = 2 : 1); HRMS (CI^+, $[M + NH_4]^+$) found 206.1354. $C_{10}H_{18}ONF_2$ requires 206.1351; υ_{max} (thin film)/cm^{-1} 2952.3, 2359.2, 1672.8, 1438.4, 1381.1, 1194.9, 1123.2, 967.7 and 865.9; δ_H (500 MHz, C^2HCl_3) 1.57 (3 H, s, CH_3), 1.67 (2 H, m, CH_2CH_2C), 1.83 (2 H, m, CH_2CH_2C), 4.62 (2 H, d, J_{HF} 47.5, CH_2F), 4.68 (2 H, d, J_{HF} 47.5, CH_2F), 5.35 (1 H, b, $CCHCH_2CH_2$), 5.78 (1 H, d, *J* 7.5, *C*HCHO) and 9.92 (1 H, d, *J* 7.5, CHC*H*O); δ_C (125 MHz, C^2HCl_3) 16.54 (CH_3), 24.69 (CH_2CH_2C), 39.03 (CH_2CH_2C), 76.29 (d, J_{CF} 162.5, CH_2F), 83.14 (d, J_{CF} 166.5, CH_2F), 127.53 (*C*HCHO), 131.96 (t, J_{CF} 15.0, CCH_2F), 134.59 (t, J_{CF} 7.5, CH_2FCCH), 159.61 (CH_2CCH_3) and 189.50 (CHCHO); δ_F (283 MHz, C^2HCl_3) –212.04 (t, J_{HF} 47.5) and –216.79 (t, $J_{H\text{-}F}$ 47.5); *m/z* (CI^+) 206.2 (100%, $[M + NH_4]^+$).

(*E*)-9-Fluoro-8-fluoromethyl-4-methylnona-1,3,7-triene (220).



This compound was prepared from **221** in a manner identical to that for the triene **172**; purification by silica column chromatography using hexane and ethyl acetate (9: 1) as eluent gave triene **220** as a light yellow oil (1.27 g, 92%); R_f 0.34 (Hexane : EtOAc = 9 : 1); HRMS (CI^+, $[M + H]^+$) found 187.1290. $C_{11}H_{17}F_2$ requires 187.1293; υ_{max} (thin film)/cm^{-1} 2925.6, 2359.8, 1674.4, 1651.5, 1448.4, 1372.9, 1217.4, 986.8 and 901.0; δ_H (500 MHz, C^2HCl_3) 1.56 (3 H, s, CH_3), 1.95 (2 H, t, *J* 7.5, CH_2CH_2C), 1.83 (2 H, m, $C{=}CHCH_2CH_2$), 4.62 (2 H, d, J_{HF} 47.5, CH_2F), 4.75 (2 H, d, J_{HF} 47.5, CH_2F), 4.80 (1 H, dd, *J* 10.0, *J* 1.5, $CHCH{=}CH_{trans}H_{cis}$), 4.90 (1 H, dd, *J* 17.0, *J* 1.5, $CHCH{=}CH_{trans}H_{cis}$), 5.64 (2 H, m, $C{=}CHCH_2CH_2$ and $CH{=}CHCH_2$) and 6.35 (1 H, dt, *J* 17.0, *J* 10.0, $CHCH{=}CH_2$); δ_C (125 MHz, C^2HCl_3) 16.56 (CH_3), 25.91 ($C{=}CHCH_2CH_2$), 39.01 ($C{=}CCH_2CH_2$), 76.86 (d, J_{CF} 161.0, CH_2F), 83.92 (d, $J_{C\text{-}F}$ 165.0, CH_2F), 115.44 ($CHCH{=}CH_2$), 126.34 ($CHCH{=}CH_2$), 131.07 (t, $J_{C\text{-}F}$ 15.0, CCH_2F), 133.04 ($CHCH{=}CH_2$), 136.98 (dd, $J_{C\text{-}F}$ 10.0, $J_{C\text{-}F}$ 7.5, $FCH_2C{=}CH$) and 137.62 ($CH_3C{=}CH$); δ_F (300 MHz, C^2HCl_3) –211.97 (t, $J_{H\text{-}F}$ 47.5) and –216.70 (t, $J_{H\text{-}F}$ 47.5); *m/z* (CI^+) 204.2 (100%, $[M + NH_4]^+$) and 187.1 (45, $[M + H]^+$).

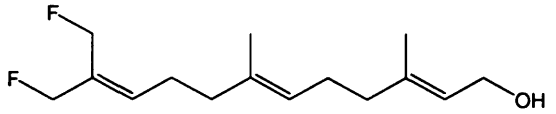
(2*E*,6*E*)-Ethyl 12-fluoro-11-fluoromethyl-3,7-dimethyldodeca-2,6,10-trienoate (219).



This compound was prepared from **220** in a manner identical to that for the ester **177**; purification by silica column chromatography using hexane and ethyl acetate (9: 1) as eluent gave ester **219** as a light yellow oil (0.55 g, 29%); R_f 0.32 (Hexane : EtOAc = 9 : 1); HRMS (ES^+, $[M + H]^+$) found

318.2239. $C_{17}H_{30}O_2NF_2$ requires 318.2239; υ_{max} (thin film)/cm^{-1} 2932.0, 1713.8, 1648.7, 1446.7, 1370.8, 1350.3, 1274.9, 1222.6, 1145.3, 1096.0, 1040.0, 970.6, 865.3 and 735.4; δ_H (500 MHz, C^2HCl_3) 1.28 (3 H, t, *J* 7.0, CH_2CH_3), 1.62 and 2.17 (6 H, s, 2 × CH_3), 2.07-2.30 (8 H, m, 2 × CH_2CH_2), 4.14 (2 H, q, *J* 7.0, CH_2CH_3), 4.83 (2 H, d, J_{HF} 47.5, CH_2F), 4.96 (2 H, d, J_{HF} 47.5, CH_2F), 5.12 and 5.85 (2 H, m, 2 × C=C*H*CH_2CH_2) and 5.67 (1 H, s, C=C*H*CO_2); δ_C (125 MHz, C^2HCl_3) 14.31 (CH_2CH_3), 15.91 and 18.74 (2 × CH_3), 25.85 (CH_2), 25.99 (CH_2), 38.82 (t, J_{CF} 2.5, FCH_2C=CH*C*H2), 40.75 (CH_2), 59.48 (CH_2CH_3), 76.87 (d, J_{CF} 161.5, CH_2F), 83.95 (d, J_{CF} 164.0, CH_2F), 115.77 (C=*C*HCO_2), 124.11 (CH_3C=*C*HCH_2CH_2), 130.84 (t, $J_{C\text{-}F}$ 15.0, FCH_2C=CH), 137.37 (dd, J_{CF} 10.0, J_{CF} 7.5, FCH_2C=*C*H), 134.65 and 159.41 (quaternary C) and 166.81 (C=O); δ_F (283 MHz, C^2HCl_3) –211.76 (t, J_{HF} 48.0) and –216.68 (t, J_{HF} 48.0); *m/z* (CI^+) 318.3 (100%, $[M + NH_4]^+$), 301.3 (20, $[M + H]^+$) and 281.2 (40, $[M - F]^+$).

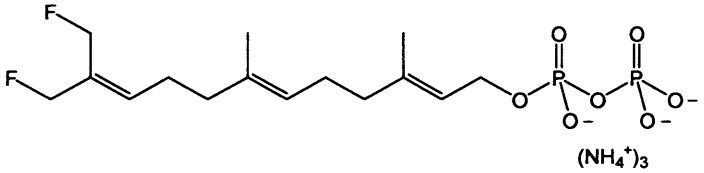
(2*E*,6*E*)-12-Fluoro-11-(fluoromethyl)-3,7-dimethyldodeca-2,6,10-trien-1-ol (222).



This compound was prepared from **219** in a manner identical to that for the alcohol **175**; purification by silica column chromatography using hexane and ethyl acetate (1: 2) as eluent gave alcohol **222** as a light yellow oil (0.12 g, 68%); R_f 0.26 (Hexane : EtOAc = 2 : 1); HRMS (ES^+, $[M + NH_4]^+$) found 276.2132. $C_{15}H_{28}ONF_2$ requires 276.2133; υ_{max} (thin film)/cm^{-1} 3349.3, 2922.0, 2361.2, 1668.2, 1445.4, 1376.8, 1235.0, 987.5 and 569.1; δ_H (500 MHz, C^2HCl_3) 1.37 (1 H, s, OH), 1.53 and 1.61 (6 H, s, 2 × CH_3), 1.95-2.22 (8 H, m, 2 × CH_2CH_2), 4.08 (2 H, d, *J* 7.0, CH_2OH), 4.75 (2 H, d, J_{HF} 47.5, CH_2F), 4.88 (2 H, d, J_{HF} 47.5, CH_2F), 5.04 (1 H, dt, *J* 7.0, *J* 1.0, CH_3C=C*H*CH_2CH_2), 5.34 (1 H, dt, *J* 7.0, *J* 1.0, C*H*CH_2OH) and 5.78 (1 H, m, FCH_2C=C*H*); δ_C

(125 MHz, C^2HCl_3) 15.91 and 16.25 (2 × CH_3), 26.03 (CH_2), 26.21 (CH_2), 38.84 (t, J_{CF} 2.5, $FCH_2C{=}CHCH_2$), 39.38 (CH_2), 59.36 (CH_2OH), 76.93 (d, J_{CF} 161.5, CH_2F), 84.00 (d, J_{CF} 165.0, CH_2F), 123.52 ($C{=}CHCH_2OH$), 125.00 ($CH_3C{=}CHCH_2CH_2$), 130.74 (t, J_{CF} 15.0, $FCH_2C{=}CH$), 133.86 and 139.44 (2 x $CH_3C{=}CH$) and 137.61 (dd, J_{CF} 10.0, J_{CF} 9.0, $FCH_2C{=}CH$); δ_F (283 MHz, C^2HCl_3) –211.62 (t, $J_{H\text{-}F}$ 47.5) and –216.60 (t, $J_{H\text{-}F}$ 47.5); *m/z* (CI^+), 276.2 (60%, $[M + NH_4]^+$), 258.2 (20, M^+) and 241.1 (100, $[M - OH]^+$)

(2*E*,6*E*)-12-Fluoro-11-fluoromethyl-3,7-dimethyldodeca-2,6,10-trien-1-yl diphosphate tris-ammonium salt (154)

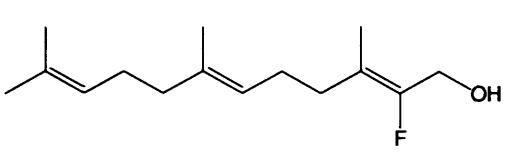


A stirred solution of **222** (0.12 g, 0.45 mmol) and triethylamine (0.13 cm^3, 0.90 mmol) in anhydrous THF (5 cm^3) was cooled to –45 °C and then methansulfonyl chloride (45 mm^3, 0.59 mmol) was added. The resulting milky mixture was stirred at –45 °C for 45 min and then a solution of lithium bromide (0.16 g, 1.80 mmol) in THF (5 cm^3) was added via a cannula. The resulting suspension was allowed to warm to 0 °C and stirred for additional 1 h. Cold water (10 cm^3) and hexane (10 cm^3) were added and the two layers were separated. The aqueous layer was extracted with hexane (2 × 10 cm^3) and the combined organic layers were washed with saturated $NaHCO_3$ solution (10 cm^3) and brine (10 cm^3) then dried over Na_2SO_4 and filtered. The solvent was concentrated under reduced pressure to give bromide **223** as a light yellow oil, which was used directly without further purification. To a stirred solution of crude bromide **223** in anhydrous acotonitrile (10 cm^3) under N_2 was added freshly recrystallized tris(tetra-n-butyammonium)

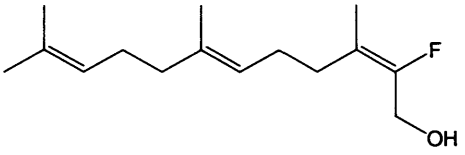
hydrogenpyrophosphate (0.81 g, 0.90 mmol). The reaction mixture was stirred for 2 h, the solvent removed under reduced pressure and the resulting opaque residue dissolved in 2 cm^3 of 1 : 49 (v/v) isopropyl alcohol and 25 mM ammonium bicarbonate (ion-exchange buffer) . The pale yellow solution was slowly passed through a column containing 30 equiv. of DOWEX 50W-X8 (100-200 mesh) cation-exchange resin that had been equilibrated with two column volumes of above mentioned ion-exchange buffer. The column was eluted with two column volumes of same buffer at a flow rate of one column volume per 15 min. The clear light yellow eluent was lyophilized to dryness to give a solid, which 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 cm^3/min, detecting at 220 nm) to give the title compound **154** as a white solid (21.2 mg, 10% over two steps); HPLC t_R 36.58 min; Purity 94.90 % by analytical RP HPLC detecting at 220nm; HRMS (ES^-, $[M - H]^-$) found 417.1044. $C_{15}H_{25}O_7F_2P_2$ requires 447.1049; υ_{max}(KBr disc)/cm^{-1} 2924.0, 2362.7, 1704.8, 1494.4, 1457.1, 1412.6, 1201.7, 1123.5, 1091.4, 1036.1, 911.4, 826.1 and 721.4; δ_H (500 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$)[188] 1.48 (3 H, s, CH_3), 1.57 (3 H, s, CH_3), 1.93-2.20 (8 H, m, 2 × CH_2CH_2), 4.33 (2 H, t, *J* 6.5, CH_2O), 4.76 (2 H, d, J_{HF} 47.5, CH_2F), 4.91 (2 H, d, J_{HF} 47.5, CH_2F), 5.07 (1 H, t, *J* 7.0, CH_3C=C*H*CH_2CH_2), 5.31 (1 H, t, *J* 7.0, C=C*H*CH_2O) and 5.87 (1 H, m, FCH_2C=C*H*); δ_C (125 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 15.15 and 15.65 (2 × CH_3), 25.58 (CH_2), 25.63 (CH_2), 38.08 (CH_2), 38.80 (CH_2), 62.71 (d, J_{CP} 5.0, CH_2O), 78.58 (d, J_{CF} 154.0, CH_2F), 85.67 (d, J_{CF} 156.5, CH_2F), 119.72 (C=*C*HCH_2O), 125.11 (CH_3C=*C*HCH_2CH_2), 129.97 (t, $J_{C\text{-}F}$ 14.0, FCH_2C=CH), 135.38 and142.87 (2 x CH_3C=CH) and 140.61 (t, J_{CF} 9.5, FCH_2C=*C*H); δ_F (283

MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) –207.21 (d, J_{HF} 47.5) and –216.83 (d, $J_{F\text{-}F}$ 47.5); δ_P (122 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) –6.61 (d, J_{PP} 21.0) and –10.47 (d, J_{PP} 21.0); *m/z* (ES^-) 417.1 (100%, $[M-H]^-$).

(2*Z*,6*E*)-2-Fluoro-3,7,11-trimethyldodeca-2,6,10-trien-1-ol (226) and (2*E*,6*E*)-2-Fluoro-3,7,11-trimethyldodeca-2,6,10-trien-1-ol (227)



and



To a suspension of sodium hydride (60% dispersion in mineral oil, 0.10 g, 4.13 mol) in anhydrous THF (10 cm^3) at 0 °C under and N_2 atmosphere, triethyl 2-fluoro-2-phosphonoacetate (0.84 cm^3, 4.13 mmol) and a solution of geranylacetone (1.02 cm^3, 4.54 mmol) in anhydrous THF (5 cm^3) were sequentially added, dropwise, with stirring. The complete reaction mixture was stirred for 14 h under N_2. Water (20 cm^3) and diethyl ether (20 cm^3) were added, and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 15 cm^3). The combined ethereal extracts were washed with water (2 × 10 cm^3) and satd. NaCl solution (10 cm^3), dried ($MgSO_4$), filtered and then concentrated under reduced pressure. The resulting ester was a 1:1 mixture of *E* and *Z* isomers and was reduced without further purification.

To a stirred solution of the crude ester in anhydrous THF (30 cm^3) at –78 °C, was added, dropwise (over 3 min), diisobutylaluminum hydride (1.0 M solution in hexanes, 10.33 cm^3, 10.33 mmol). The reaction mixture was allowed to stir at –78 °C for 1 h, then 0 °C for 1 h and was then quenched with satd. sodium potassium tartrate solution (30 cm^3). The resulting mixture was then extracted with diethyl ether (3 × 20 cm^3). The combined organic extracts were dried over

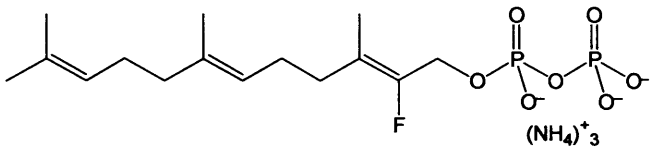
anhydrous $MgSO_4$, filtered and then concentrated under reduced pressure. Purification of the residue by flash column chromatography on silica gel with hexane and ethyl acetate (4:1) as eluent gave the *Z* isomer (0.48 g, 48%) as a colorless oil followed by *E* isomer (0.46 g, 46%) as a colourless oil.

E isomer: TLC R_f 0.28 (4:1 hexane-ethyl acetate); HRMS (EI^+, M^+) found 240.1890. $C_{15}H_{25}OF$ requires 240.1889; υ_{max}(thin film)/cm^{-1} 3358.1, 2920.4, 1703.7, 1444.7, 1380.2, 1266.9, 1148.9, 1105.3 and 1012.5; δ_H (500 MHz, C^2HCl_3) 1.62 (6 H, s, 2 × CH_3), 1.70 (3 H, d, *J* 1.0, CH_3), 1.71 (3 H, d, $J_{H\text{-}F}$ 3.48, CH_3C=CF), 1.79 (1 H, broad s, OH), 1.99–2.14 (8 H, m, 2 × CH_2CH_2), 4.19 (2 H, dd, $J_{H\text{-}F}$ 23.0, *J* 6.0, CH_2OH) and 5.09 (2 H, m, 2 × CCH); δ_C (125 MHz, C^2HCl_3) 13.48 (d, $J_{C\text{-}F}$ 9.0, CH_3C=CF), 15.99 (CH_3), 17.67 (CH_3), 25.67 (CH_3), 26.36 (d, $J_{C\text{-}F}$ 4.0, CH_2CH_2C=CF), 26.62 (CH_2), 31.71 (d, $J_{C\text{-}F}$ 5.0, CH_2CH_2C=CF), 39.67 (CH_2), 57.67 (d, $J_{C\text{-}F}$ 31.0, C=CFCH_2OH), 115.74 (d, $J_{C\text{-}F}$ 14.0, CH_3C=$CFCH_2$), 123.00 and 124.14 (2 × C=CH), 131.52 and 136.52 (quaternary C) and 152.57 (d, $J_{C\text{-}F}$ 242.5, CH_3C=CF); δ_F (283 MHz, C^2HCl_3) –119.5 (t, $J_{H\text{-}F}$ 23.0); ***m/z*** (EI^+) 240.2 (2%, M^+), 81.1 (20) and 69.1 (100).

Z isomer: TLC R_f 0.20 (4:1 hexane-ethyl acetate); HRMS (EI^+, M^+) found 240.1897. $C_{15}H_{25}OF$ requires 240.1889; υ_{max}(thin film)/cm^{-1} 3349.0, 2931.5, 2361.3, 1703.1, 1667.2, 1450.6, 1380.5, 1270.8, 1238.0, 1158.9, 1105.5, 1012.8 and 910.9; δ_H (500 MHz, C^2HCl_3) 1.62 (6 H, s, 2 × CH_3), 1.70 (3 H, d, $J_{H\text{-}F}$ 2.5, CH_3CCF), 1.70 (3 H, s, CH_3), 1.82 (1 H, b, OH), 1.98–2.15 (8 H, m, 2 × CH_2CH_2), 4.23 (2 H, dd, $J_{H\text{-}F}$ 22.5, *J* 5.5, CH_2OH) and 5.11 (2 H, m, 2 × CCH); δ_C (125 MHz, C^2HCl_3) 15.37 (d, $J_{C\text{-}F}$ 5.0, CH_3CCF), 15.96 (CH_3), 17.67 (CH_3), 25.67 (CH_3), 25.87 (d, $J_{C\text{-}F}$ 1.25, CH_2CH_2C=CF), 26.70 (CH_2), 29.77 (d, $J_{C\text{-}F}$ 6.25, CH_2CH_2C=CF), 39.69 (CH_2), 57.94 (d, $J_{C\text{-}F}$ 32.5, CH_2OH), 116.02 (d, $J_{C\text{-}F}$ 16.25, CH_3C=$CFCH_2$), 123.53 and 124.32 (2 × C=CH), 131.36

and 135.73 (quaternary C) and 151.79 (d, $J_{C\text{-}F}$ 240.0, $CH_3C{=}CF$); δ_F (283 MHz, C^2HCl_3) 121.13 (t, $J_{H\text{-}F}$ 22.5); *m/z* (EI^+) 240.2 (4%, M^+), 81.1 (35) and 69.1 (100).

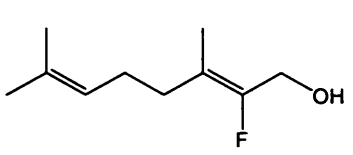
(2*Z*,6*E*)-2-fluoro-3,7,11-trimethyldodeca-2,6,10-trien-1-yl diphosphate tris ammonium salt (155)



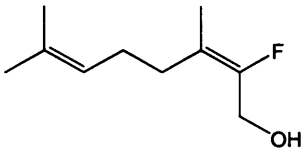
This compound was prepared from alcohol **226** and purified in a manner identical to that for the diphosphate **154** to give diphosphate **155** as a white solid (0.15 g, 36%); HPLC t_R 36.99 min; Purity 96.33% by analytical RP HPLC 220 nm; HRMS (EI^-, $[M - H]^-$) found 399.1135. $C_{15}H_{26}O_7FP_2$ requires 399.1143; υ_{max}(KBr disc)/cm^{-1} 2963.4, 1701.8, 1494.4, 1455.4, 1411.8, 1204.0, 1164.6, 1122.1, 1091.9, 1034.7, 908.7, 825.9, 723.3, 595.4 and 552.7; δ_H (500 MHz, ^2H_2O) 1.47 (3 H, s, CH_3), 1.48 (3 H, s, CH_3), 1.54 (3 H, s, CH_3), 1.59 (3 H, d, $J_{H\text{-}F}$ 2.5, $CH_3C{=}CF$), 1.86–2.01 (8 H, m, 2 × CH_2CH_2), 4.42 (2 H, dd, $J_{H\text{-}F}$ 24.0, $J_{H\text{-}P}$ 6.0, $CCFCH_2OP$), 5.02 (1 H, t, *J* 7.0, C=C*H*) and 5.06 (1 H, m, C=C*H*); δ_C (125 MHz, ^2H_2O) 4.82 (d, $J_{C\text{-}F}$ 5.0, $CH_3C{=}CF$), 15.33, 17.06 and 24.98 (3 × CH_3), 25.33 and 26.04 (2 × CH_2), 29.38 (d, $J_{C\text{-}F}$ 6.0, $CH_2CH_2C{=}CF$), 39.04 (CH_2), 60.54 (dd, $J_{C\text{-}F}$ 31.25, $J_{C\text{-}P}$ 5.0, $C{=}CFCH_2OP$), 119.33 (d, $J_{C\text{-}F}$ 14.0, $CH_3C{=}CFCH_2$), 123.92 and 124.44 (2 × C=*C*H), 132.82 and 136.62 (quaternary C) and 149.25 (dd, $J_{C\text{-}F}$ 237.5, $J_{C\text{-}P}$ 9.0, CH_3CCFCH_2OP); δ_F (283 MHz, ^2H_2O) –120.23 (t, $J_{H\text{-}F}$ 24.0); δ_P (122 MHz, $2H_2O$) –6.29 (d, J_{PP} 21.0) and 10.50 (d, $J_{P\text{-}P}$ 21.0); *m/z* (ES^-) 399.2 (15%, $[M - H]^-$) and 74.9 (100).

(*Z*)-2-Fluoro-3,7-dimethylocta-2,6-dien-1-ol (231) and

(*E*)-2-fluoro-3,7-dimethylocta-2,6-dien-1-ol (232)



and



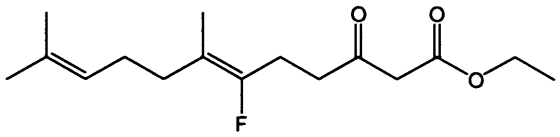
These two compounds were prepared from 6-methyl-5-hepten-2-one in a manner identical to that for the alcohol **226** and **227**; purification by silica column chromatography using hexane and ethyl acetate (4: 1) as eluent gave the title compound (*Z* isomer) as a colourless oil (1.79 g, 46%) and the *E* isomer (1.72 g, 44%).

Z isomer: TLC R_f 0.14 (Hexane : EtOAc = 4 : 1); HRMS (ES^+, $[M + NH_4]^+$) found 190.1602. $C_{10}H_{21}ONF$ requires 190.1602; υ_{max} (thin film)/cm^{-1} 3341.8, 2918.9, 1704.5, 1449.9, 1378.1, 1157.6 and 1011.2; δ_H (500 MHz, C^2HCl_3) 1.63 (3 H, s, CH_3), 1.69 (3 H, d, $J_{H\text{-}F}$ 7.5, CH_3CCF), 1.71 (3 H, d, *J* 0.5, CH_3), 1.81 (1 H, b, OH), 2.10 (4 H, m, CH_2CH_2), 4.23 (2 H, d, $J_{H\text{-}F}$ 25.0, CH_2OH) and 5.13 (1 H, t, *J* 6.03, $CHCH_2CH_2$); δ_C (125 MHz, C^2HCl_3) 15.32 (d, $J_{C\text{-}F}$ 6.25, $CH_3C{=}CF$), 17.62 and 25.64 (2 × CH_3), 25.97 and 29.78 (d, $J_{C\text{-}F}$ 6.5, CH_2CH_2), 57.95 (d, $J_{C\text{-}F}$ 31.5, CH_2OH), 116.00 (d, $J_{C\text{-}F}$ 15.0, $CH_3C{=}CF$), 123.67 (CH), 132.08 (quaternary C) and 151.79 (d, $J_{C\text{-}F}$ 240.0, $CH_3C{=}CF$); δ_F (250 MHz, proton decoupled, C^2HCl_3) –121.33; *m/z* (CI^+) 190.1 (100%, $[M + NH_4]^+$) and 172.2 (20, M^+).

E isomer: TLC R_f 0.21 (Hexane : EtOAc = 4 : 1); HRMS (ES^+, $[M + NH_4]^+$) found 190.1600. $C_{10}H_{21}ONF$ requires 190.1602; υ_{max} (thin film)/cm^{-1} 3345.4, 2920.1, 2860.4, 2359.0, 1704.4, 1449.8, 1378.3, 1267.8, 1149.3, 1021.2, 908.3 and 830.2; δ_H (500 MHz, C^2HCl_3) 1.61 (3 H, s, CH_3), 1.70 and 1.71 (6 H, CH_3CCF and terminal CH_3 overlap), 1.88 (1 H, b, OH), 2.04–2.12 (4 H, m, CH_2CH_2), 4.18 (2 H, d, $J_{H\text{-}F}$ 25.00, CH_2OH) and 5.10 (1 H, tt, *J* 7.0, *J* 1.5, $CHCH_2CH_2$); δ_C

(125 MHz, C^2HCl_3) 13.46 (d, $J_{C\text{-}F}$ 9.0, CH_3CCF), 17.62 and 25.61 (2 × CH_3), 26.40 (d, $J_{C\text{-}F}$ 2.5) and 29.78 (d, $J_{C\text{-}F}$ 5.0, CH_2CH_2), 57.63 (d, $J_{C\text{-}F}$ 31.25, CH_2OH), 115.62 (d, $J_{C\text{-}F}$ 15.0, CH_3C=CF), 123.26 (CH), 132.96 (quaternary C) and 152.66 (d, $J_{C\text{-}F}$ 242.5, CH_3C=CF); δ_F (250 MHz, proton decoupled, C^2HCl_3) –119.5; *m/z* (CI^+) 190.1 (100%, $[M + NH_4]^+$) and 172.2 (15, M^+).

(*Z*)-Ethyl 6-fluoro-7,11-dimethyl-3-oxododeca-6,10-dienoate (234)

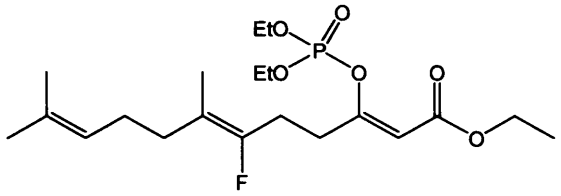


A solution of the alcohol **231** (0.62 g, 3.62 mmol) and triethylamine (1.03 cm^3, 7.40 mmol) in anhydrous THF (40 cm^3) under N_2 was stirred and cooled at –45 °C as MsCl (0.37 cm^3, 4.80 mmol) was added. The resulting milky mixture was stirred at –45 °C for 45 min, and a solution of LiBr (1.29 g, 14.8 mmol) in THF (10 cm^3) was added via cannula at –45 °C. The suspension was allowed to warm to 0 °C and stirred for an additional 1 h, and then cold water (20 cm^3) and hexane (20 cm^3) were added. The two layers were separated, and the aqueous layer was extracted with hexane (2 × 15 cm^3). The combined organic layers were washed with satd. $NaHCO_3$ solution (15 cm^3) and then brine (15 cm^3), dried over $NaSO_4$ and filtered. Concentration of the solvent gave the bromide **233** as a light yellow oil which was used without further purification.

To a stirred suspension of NaH (0.27 g, 11.3 mmol) in anhydrous THF (100 cm^3) was added ethyl acetoacetate (1.30 cm^3, 10.2 mmol) dropwise at 0 °C. After 10 min, n-BuLi (2.5 M, 4.29 cm^3, 10.7 mmol) was added slowly over 3 min, during which time the colourless solution gradually turned yellow. Stirring was continued for an additional 10 min at 0 °C, and then a solution of the above bromide in THF (2 cm^3) was added. The clear solution turned to a cloudy yellow

suspension. After stirring for 30 min at 0 °C, HCl (3 M, 5.0 cm^3) was added followed by water (20 cm^3) and diethyl ether (20 cm^3) then the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 15 cm^3). The combined ethereal extracts were washed with water (2 × 15 cm^3) and brine (15 cm^3), dried over $MgSO_4$ then filtrated and concentrated under reduced pressure. Purification of the crude product by flash column chromatography on silica gel with hexane and ethyl acetate (4 : 1) as eluent gave the title compound as a pale yellow oil (0.95g, 98%). TLC R_f 0.40 (Hexane : EtOAc = 4 : 1); HRMS (ES^+, $[M + Na]^+$) found 307.1672. $C_{16}H_{25}O_3FNa$ requires 307.1685; υ_{max} (thin film)/cm^{-1} 2968.6, 2921.1, 1746.8, 1719.6, 1650.0, 1445.6, 1368.1, 1317.8, 1235.8, 1156.0, 1077.9, 1036.4 and 832.6; δ_H (500 MHz, C^2HCl_3) 1.21 (3 H, t, *J* 7.15, CH_2CH_3), 1.52 and 1.53 (6 H, CH_3C=CF and a terminal CH_3 overlap), 1.61 (3 H, s, CH_3), 1.98 (4 H, br, CH_2CH_2), 2.45 (2 H, dt, $J_{H\text{-}F}$ 22.0, *J* 7.5, $CFCH_2CH_2$), 2.67 (2 H, t, *J* 7.5, $CFCH_2CH_2$), 3.38 (2 H, s, $COCH_2CO_2$), 4.12 (2 H, q, *J* 7.0, CH_2CH_3) and 5.01 (1 H, b, $CHCH_2CH_2$); δ_C (125 MHz, C^2HCl_3) 14.06 (CH_2CH_3), 15.41 (d, $J_{C\text{-}F}$ 6.5, CH_3C=CF), 17.59 (CH_3), 22.57 (d, $J_{C\text{-}F}$ 30.0, $CFCH_2CH_2$), 25.64 (CH_3), 26.16 and 29.65 (d, $J_{C\text{-}F}$ 7.5, CH_2CH_2), 39.62 ($CFCH_2CH_2$), 49.36 ($COCH_2CO_2$), 61.38 (CH_2CH_3), 112.41 (d, $J_{C\text{-}F}$ 17.5, CH_3C=CF), 123.55 (CH), 131.73 (quaternary C), 151.50 (d, $J_{C\text{-}F}$ 239.0, CH_3C=CF) and 167.00 and 201.59 (C=O); δ_F (250 MHz, proton decoupled, C^2HCl_3) –114.72; *m/z* (ES^+) 307.2 (100%, $[M + Na]^+$).

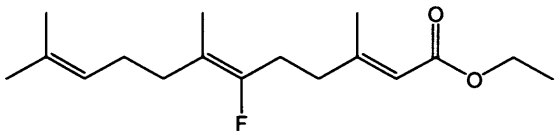
(1*Z*,5*Z*)-1-(Ethoxycarbonyl)-5-fluoro-6,10-dimethylundeca-1,5,9-trien-2-yl diethyl phosphate (235)



To a stirred suspension of NaH (0.07 g, 2.79 mmol) in anhydrous diethyl ether (50 cm^3) at 0 °C was added slowly a solution of β-keto ester **234** in diethyl ether. The mixture was then stirred at 0 °C for 30 min until bubbling stopped. Diethyl chlorophosphate (0.51 cm^3, 3.49 mmol) was added slowly to the resulting clear colourless solution. Stirring was continued at 0 °C for additional 15 min and the reaction quenched by adding satd. NH_4Cl (20 cm^3). The mixture was diluted with water (20 cm^3), and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 15 cm^3). The combined ethereal extracts were washed with water (2 × 15 cm^3) and brine (15 cm^3), dried over $MgSO_4$ then filtered and concentrated under reduced pressure. Purification of the crude product by flash column chromatography on silica gel with hexane and ethyl acetate (2 : 1) as eluent gave the title compound as a pale yellow oil (0.94g, 97%). TLC R_f 0.14 (Hexane : EtOAc = 2 : 1); HRMS (ES^+, $[M + Na]^+$) found 421.2144. $C_{20}H_{35}O_6FP$ requires 421.2155; υ_{max}(thin film)/cm^{-1} 2979.7, 2925.4, 2353.0, 1727.5, 1663.9, 1443.4, 1368.1, 1280.1, 1207.5, 1147.1, 1035.2, 991.5 and 800.6; δ_H (500 MHz, C^2HCl_3) δ 1.19 (3 H, t, *J* = 7.15, $CO_2CH_2CH_3$), 1.30 (6 H, dt, *J* 7.0, *J* 1.0, 2 × $POCH_2CH_3$), 1.51 (3 H, d, *J* 2.5, CH_3C=CF), 1.53 (3 H, s, CH_3C=CH), 1.61 (3 H, s, CH_3CCH), 1.96–2.00 (4 H, m, $(CH_3)_2C$=CHCH_2CH_2), 2.44–2.54 (4 H, m, CFCH_2CH_2), 4.07 (2 H, q, *J* 7.0, $CO_2CH_2CH_3$), 4.20 (4 H, quintet, *J* 7.0, 2 × $POCH_2CH_3$), 5.02 (1 H, b, $(CH_3)_2C$=C*H*CH_2) and 5.30 (1 H, s, C=C*H*CO_2); δ_C (125 MHz, C^2HCl_3) 14.19 ($CO_2CH_2CH_3$), 15.43 (d, $J_{C\text{-}F}$ 6.5, CH_3C=CF), 16.01 ($POCH_2CH_3$), 16.07

($POCH_2CH_3$), 17.58 ($CH_3C=CH$), 25.62 ($CH_3C=CH$), 25.94 (d, $J_{C\text{-}F}$ 30.0, $CFCH_2CH_2$), 26.22 (d, $J_{C\text{-}F}$ 1.5, $(CH_3)_2C=CHCH_2CH_2$), 29.70 (d, $J_{C\text{-}F}$ 7.5, $(CH_3)_2C=CHCH_2CH_2$), 32.47 ($CFCH_2CH_2$), 59.88 ($CO_2CH_2CH_3$), 64.77 and 64.83 (2 × $POCH_2CH_3$), 106.07 (d, $J_{C\text{-}P}$ 7.5, $COCHCO_2$), 113.16 ($CH_3C=CFCH_2$), 123.92 ($(CH_3)_2C=CHCH_2$), 131.71 (quaternary C), 151.05 (d, $J_{C\text{-}F}$ 241.5, $CH_3C=CF$), 160.04 (d, J 7.5, quaternary C) and 163.54 (quaternary C) ; δ_F (250 MHz, proton decoupled, C^2HCl_3) –114.32; δ_P (500 MHz, C^2HCl_3,) –8.66; *m/z* (ES^+) 421.2 (100 %, $[M + H]^+$).

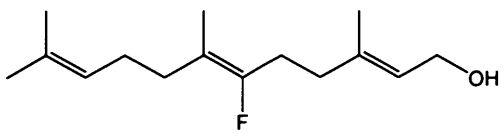
(2*E*,6*Z*)-Ethyl 6-fluoro-3,7,11-trimethyldodeca-2,6,10-trienoate (236)



To a stirred suspension of CuI (0.84 g, 4.43 mmol) in diethyl ether (50 cm^3) at 0 °C was added MeLi (1.6 M in diethyl ether, 5.54 cm^3, 8.86 mmol). A yellow precipitate formed immediately, and the mixture was stirred for 30 min at 0 °C, at which time all the yellow precipitate disappeared, and a nearly colorless solution remained. The colorless solution was then cooled to –78 °C, and enol phosphate **235** (0.93 g, 2.21 mmol) in diethyl ether (5 cm^3) was added slowly over 5 min. The resulting yellow solution was stirred for 3 h at –78 °C. MeI (1.38 cm^3, 22.1 mmol) was added, and the mixture was stirred for an additional 15 min at –78 °C before the yellow orange mixture was poured into an ice-cooled mixture of satd. NH_4Cl and conc. NH_4OH (1 : 1, 10 cm^3). The organic layer was separated, and the aqueous layer was extracted with diethyl ether (2 × 20 cm^3). The combined ethereal extracts were washed with water (2 × 15 cm^3) and brine (15 cm^3), dried over $MgSO_4$ then filtered and concentrated under reduced pressure.

Purification of the crude product by flash column chromatography on silica gel with hexane and ethyl acetate (9 : 1) as eluent gave the title compound **236** as a pale yellow oil (0.56 g, 95%); TLC R_f 0.45 (Hexane : EtOAc = 9 : 1); HRMS (EI^+, M^+) found 282.1988. $C_{17}H_{27}O_2F$ requires 282.1995; υ_{max}(thin film)/cm^{-1} 2965.6, 2925.3, 2855.1, 1717.5, 1649.0, 1447.3, 1381.8, 1272.6, 1223.3, 1149.5, 1049.6 and 855.9; δ_H (500 MHz, C^2HCl_3) 1.28 (3 H, t, *J* 7.0, $CO_2CH_2CH_3$), 1.57 (3 H, d, *J* 2.5, CH_3C=CF), 1.61 (3 H, s, CH_3C=$CHCH_2CH_2$), 1.69 (3 H, s, CH_3C=CH CH_2CH_2), 2.05–2.08 (4 H, m, $(CH_3)_2C$=$CHCH_2CH_2$), 2.19 (3 H, d, *J* 1.0, CH_3C=$CHCO_2$), 2.32 (2 H, t, *J* 7.5, $CFCH_2CH_2$), 2.37–2.44 (2 H, m, $CFCH_2CH_2$), 4.15 (2 H, q, *J* 7.0, $CO_2CH_2CH_3$), 5.11 (1 H, b, $(CH_3)_2C$=$CHCH_2$) and 5.69 (1 H, s, C=$CHCO_2$); δ_C (125 MHz, C^2HCl_3) 14.29 ($CO_2CH_2CH_3$), 15.47 (d, $J_{C\text{-}F}$ 6.5, CH_3C=CF), 17.58 (CH_3C=$CHCH_2$), 18.66 (CH_3C=$CHCO_2$), 25.64 (CH_3C=$CHCH_2$), 26.23 (d, $J_{C\text{-}F}$ 2.5, $(CH_3)_2C$=$CHCH_2CH_2$), 26.87 (d, $J_{C\text{-}F}$ 30.0, $CFCH_2CH_2$), 29.67 (d, $J_{C\text{-}F}$ 7.5, $(CH_3)_2C$=$CHCH_2CH_2$), 37.74 ($CFCH_2CH_2$), 59.48 ($CO_2CH_2CH_3$), 112.21 (d, $J_{C\text{-}F}$ 16.5, CH_3C=$CFCH_2$), 116.24 ($COCHCO_2$), 123.99 ($(CH_3)_2C$=$CHCH_2$), 131.68 (quaternary C), 152.06 (d, $J_{C\text{-}F}$ 240.0, CH_3C=CF), 158.32 and 166.65 (quaternary C); δ_F (250 MHz, proton decoupled, C^2HCl_3) –113.40; *m/z* (EI^+) 282.2 (2%, M^+) and 69.1 (100).

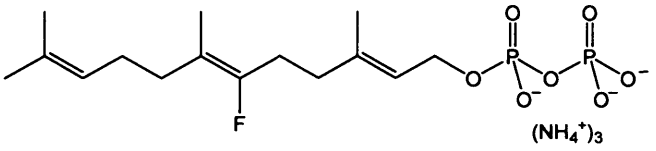
(2*E*,6*Z*)-6-Fluoro-3,7,11-trimethyldodeca-2,6,10-trien-1-ol (237)



This compound was prepared from **236** in a manner identical to that for the alcohol **175**; purification by silica column chromatography using hexane and ethyl acetate (1: 2) as eluent gave alcohol **237** as a colourless oil (0.30 g, 91%); TLC R_f 0.32 (Hexane : EtOAc = 2 : 1); HRMS (EI^+,

M^+) found 240.1897. $C_{15}H_{25}OF$ requires 240.1889; υ_{max} (thin film)/cm^{-1} 3327.8, 2919.0, 1706.3, 1670.5, 1449.1, 1381.5, 1288.4, 1189.6, 1142.0, 1110.8, 1075.8, 1006.2, 924.5 and 829.9; δ_H (500 MHz, C^2HCl_3) 1.46 (1 H, s, OH), 1.49 (3 H, d, *J* 2.5, CH_3CCF), 1.53 (3 H, s, $CH_3C{=}CHCH_2OH$), 1.61 (3 H, s, $CH_3C{=}CHCH_2CH_2$), 1.62 (3 H, s, $CH_3C{=}CHCH_2CH_2$), 1.97 (4 H, m, $(CH_3)_2C{=}CHCH_2CH_2$), 2.12 (2 H, t, *J* 7.5, $CFCH_2CH_2$), 2.22 (2 H, m, $CFCH_2CH_2$), 4.06 (2 H, d, *J* 7.0, CH_2OH), 5.03 (1 H, b, $(CH_3)_2C{=}CHCH_2$) and 5.36 (1 H, dt, *J* 7.0, *J* 1.0, $CCHCH_2OH$); δ_C (125 MHz, C^2HCl_3) 15.47 (d, $J_{C\text{-}F}$ 6.5, $CH_3C{=}CF$), 16.18 ($CH_3C{=}CHCH_2CH_2$), 17.61 ($CH_3C{=}CHCH_2OH$), 25.65 ($CH_3C{=}CHCH_2CH_2$), 26.28 $(CH_3)_2C{=}CHCH_2CH_2$), 27.28 (d, $J_{C\text{-}F}$ 29.0, $CFCH_2CH_2$), 29.65 (d, $J_{C\text{-}F}$ 7.5, $(CH_3)_2C{=}CHCH_2CH_2$), 36.44 ($CFCH_2CH_2$), 59.29 (CH_2OH), 111.48 (d, $J_{C\text{-}F}$ 17.5, $CH_3C{=}CFCH_2$), 124.06 ($CHCH_2OH$), 124.08 [$(CH_3)_2C{=}CHCH_2$], 131.67 and 138.64 (quaternary C) and 152.96 (d, $J_{C\text{-}F}$ 241.5, $CH_3C{=}CF$); δ_F (250 MHz, proton decoupled, C^2HCl_3) –112.73; *m/z* (EI^+) 240.2 (6%, M^+) and 202.2 (100).

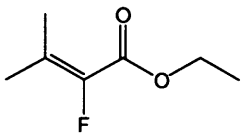
(2*E*,6*Z*)-6-Fluoro-3,7,11-trimethyldodeca-2,6,10-trien-1-yl diphosphate (156)



This compound was prepared in a manner identical to that for the diphosphate **154**, using the alcohol **237** (0.21 g, 0.86 mmol) to give the title compound **156** as a white fluffy solid (188 mg, 48% for two steps); HPLC t_R = 36.45 min; Purity 96.57 % by analytical RP HPLC under 220 nm; HRMS (ES^-, $[M - H]^-$) found 399.1145. $C_{15}H_{26}O_7FP_2$ requires 399.1143; υ_{max} (KBr disc)/cm^{-1} 2929.9, 1707.6, 1494.4, 1456.3, 1200.2, 1124.7, 1088.8, 1041.5, 914.1, 809.9, 720.8 and 595.0; δ_H (500 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 1.45 (3 H, d, *J* 2.0, $CH_3C{=}CFCH_2$), 1.49

(3 H, s, CH_3C=CHCH$_2$CH$_2$), 1.56 (3 H, s, CH_3C=CHCH$_2$O), 1.61 (3 H, s, CH_3C=CHCH$_2$CH$_2$), 1.96 (4 H, m, (CH$_3$)$_2$C=CHCH_2CH_2), 2.10 (2 H, t, *J*7.5, CFCH$_2$CH_2), 2.26 (2 H, td, *J*25.0, *J*7.5, CFCH_2CH$_2$), 4.34 (2 H, t, *J*6.5, CHCH_2O), 5.06 (1 H, br, (CH$_3$)$_2$C=C*H*CH$_2$) and 5.35 (1 H, t, *J* 7.5, C=C*H*CH$_2$O); δ_C (125 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 14.57 (d, $J_{C\text{-}F}$ 6.5, *C*H$_3$C=CF), 15.60 (*C*H$_3$C=CHCH$_2$CH$_2$), 16.95 (*C*H$_3$C=CHCH$_2$CH$_2$), 24.90 (*C*H$_3$C=CHCH$_2$O), 25.45 (CH$_3$)$_2$C=CH*C*H$_2$CH$_2$), 26.63 (d, $J_{C\text{-}F}$ 25.0, CF*C*H$_2$CH$_2$), 28.86 (d, $J_{C\text{-}F}$ 7.5, (CH$_3$)$_2$C=CHCH$_2$*C*H$_2$), 35.94 (CFCH$_2$*C*H$_2$), 62.47 (d, $J_{C\text{-}P}$ 5.0, CH*C*H$_2$O), 112.22 (d, $J_{C\text{-}F}$ 16.5, CH$_3$*C*=CFCH$_2$), 120.56 (d, $J_{C\text{-}P}$ 9.0, *C*HCH$_2$O), 124.14 ((CH$_3$)$_2$C=*C*HCH$_2$), 133.75 and 141.06 (quaternary C) and 153.48 (d, $J_{C\text{-}F}$ 236.5, CH$_3$C=*C*F); δ_F (283 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) –113.69 (t, $J_{H\text{-}F}$ 24.5); δ_P (202 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) –6.95 (d, $J_{P\text{-}P}$ 21.0) and –10.38 (d, $J_{P\text{-}P}$ 21.0); *m/z* (ES^-) 399.1 (100%, $[M - H]^-$).

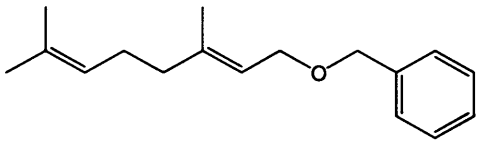
Ethyl 2-fluoro-3-methylbut-2-enoate (240)



To a stirred solution of triethyl-2-fluoro-2-phosphonoacetate (4.20 cm^3, 20.6 mmol) in anhydrous THF (40 cm^3) was added a suspension of NaH (0.55 g, 22.7 mmol) in THF (5 cm^3) was added at 0 °C under an argon atmosphere. After stirring of 30 min at RT, a mixture of acetone (1.82 cm^3, 24.7 mmol) was added, and then the whole reaction mixture was stirred overnight. After quenching with cold saturated NH_4Cl solution (30 cm^3), the result mixture was extracted with diethyl ether (3 × 30 cm^3), and the organic phases were combined, washed with brine (30 cm^3) and dried ($MgSO_4$), filtered and then concentrated under reduced pressure to give the title

compound as a pale yellow oil, Purification of the crude product by flash column chromatography on silica gel with hexane and ethyl acetate (30 : 1) as eluent gave the title compound **240** as a colorless oil (1.34 g, 47%); TLC R_f 0.49 (Hexane : EtOAc = 9 : 1); υ_{max} (thin film)/cm^{-1} 2986.0, 2926.6, 1724.5, 1617.3, 1448.8, 1373.1, 1299.5, 1236.6, 1152.7, 1089.3, 1022.4, 934.0 and 864.6; δ_H (500 MHz, C^2HCl_3) 1.35 (3 H, t, *J* 7.0, CH_2CH_3), 1.87 (3 H, d, $J_{H\text{-}F}$ 4.0, CH_3), 1.61 (3 H, d, $J_{H\text{-}F}$ 3.0, CH_3) and 4.27 (2 H, q, *J* 7.0, CH_2CH_3); δ_C (125 MHz, C^2HCl_3) 14.17 (CH_2CH_3), 18.48 and 18.56 (2 × CH_3), 60.87 (CH_2CH_3), 129.49 (d, *J* 14.0, $(CH_3)_2C$=CF), 142.71 (d, *J* 244.0, $(CH_3)_2C$=CF) and 161.21 (d, *J* 34.0, $CO_2CH_2CH_3$); δ_F (283 MHz, C^2HCl_3) –128.06; *m/z* (CI^+) 164.1 (100%, $[M + NH_4]^+$).

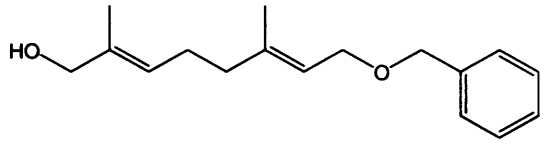
1-{[(*E*)-3,7-Dimethylocta-2,6-dienyloxy]methyl}benzene (245).



This compound was prepared from geraniol in a manner identical to that for the benzyl ether **207**; purification by silica column chromatography using hexane and ethyl acetate (9: 1) as eluent gave the title compound **245** as a colourless oil (5.75 g, 94%); TLC R_f 0.49 (Hexane : EtOAc = 9 : 1); HRMS (EI^+, M^+) found 244.1839. $C_{17}H_{24}O$ requires 244.1827; υ_{max} (thin film)/cm^{-1} 2966.7, 2918.6, 2854.2, 1667.6, 1495.6, 1451.5, 1379.4, 1246.4, 1202.5, 1067.9, 1026.5, 1009.0 and 942.3; δ_H (500 MHz, C^2HCl_3) 1.64 (3 H, s, CH_3), 1.68 (3 H, s, CH_3), 1.72 (3 H, s, CH_3), 2.08 (2 H, t, *J* 7.5, $CHCH_2CH_2C$), 2.14 (2 H, q, *J* 7.5, $CHCH_2CH_2C$), 4.06 (2 H, d, *J* 7.0, $CHCH_2O$), 4.54 (2 H, s, OCH_2Ph), 5.14 (1 H, m, $(CH_3)_2CCHCH_2$), 5.44 (1 H, td, *J* 7.0, *J* 1.0, $CCHCH_2O$) and 7.29 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 16.50 (CH_3), 17.70 (CH_3), 25.71 (CH_3), 26.40 and

39.63 ($CHCH_2CH_2C$), 66.61 ($CHCH_2O$), 71.97 (OCH_2Ph), 120.86 ($CHCH_2O$), 124.04 ($CHCH_2CH_2$), 127.52, 127.84 and 128.35 (Ar-CH) and 131.66, 138.64 and 140.41 (quaternary C); *m/z* (EI^+) 244.2 (2%, M^+) and 91.0 (100).

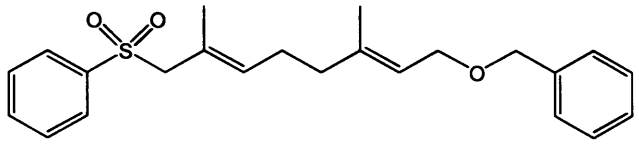
(2*E*,6*E*)-8-(Benzyloxy)-2,6-dimethylocta-2,6-dien-1-ol (246)



To a stirred solution of benzyl ether **245** (5.70 g, 23.4 mmol) in DCM (40 cm^3) was added selenium dioxide (0.26 g, 2.30 mmol), tert-butyl hydroperoxide (70% in H_2O, 55.6 cm^3, 84.2 mmol) and acetic acid (0.13 cm^3, 2.30 mmol) under an argon atmosphere. The whole reaction mixture was then stirred overnight at RT. Water (30 cm^3) was added and the resulting mixture was extracted with diethyl ether (3 × 30 cm^3), and the organic phases were combined, washed with 10% aqueous potassium hydroxide (30 cm^3), brine (30 cm^3) and dried ($MgSO_4$), filtered and then concentrated under reduced pressure to give the title compound as a pale yellow oil. Purification of the crude product by flash column chromatography on silica gel with hexane and ethyl acetate (2 : 1) as eluent gave the title compound as a colorless oil (1.95 g, 32%); TLC R_f 0.26 (Hexane : EtOAc = 2 : 1); υ_{max} (thin film)/cm^{-1} 3391.9, 2919.0, 2856.3, 1667.6, 1451.8, 1364.0, 1202.5, 1065.5, 1010.6, 736.0 and 697.8; δ_H (500 MHz, C^2HCl_3) 1.67 (3 H, s, CH_3), 1.69 (3 H, s, CH_3), 2.11 (2 H, t, *J* 7.5, $CHCH_2CH_2C$), 2.19 (2 H, q, *J* 7.5, $CHCH_2CH_2C$), 4.00 (2 H, s, CCH_2OH), 4.04 (2 H, d, *J* 7.0, $CHCH_2O$), 4.53 (2 H, s, OCH_2Ph), 5.40 (2 H, m, 2 × $C=CHCH_2$) and 7.29 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 13.71 (CH_3), 16.46 (CH_3), 25.81 and 39.15 ($CHCH_2CH_2C$), 66.57 ($CHCH_2O$), 68.95 (CCH_2OH), 72.11 (OCH_2Ph), 121.17 and 125.63 (2 ×

C=C*H*CH_2), 127.57, 127.84 and 128.37 (Ar-CH) and 135.15, 138.52 and 139.94 (quaternary C); *m/z* (CI^+) 278.3 (75%, $[M + NH_4]^+$) and 135.0 (100).

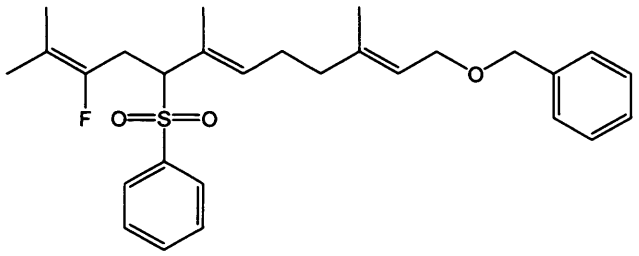
1-[(2*E*,6*E*)-8-(Benzyloxy)-2,6-dimethylocta-2,6-dienylsulfonyl]benzene (242)



A stirred solution of alcohol **246** (1.60 g, 6.15 mmol) and triethylamine (1.71 cm^3, 12.3 mmol) in anhydrous THF (40 cm^3) was cooled to –45 °C and then MsCl (0.62 cm^3, 8.01 mmol) was added. The resulting milky mixture was stirred at –45 °C for 45 min and then a solution of LiBr (2.14 g, 24.6 mmol) in THF (5 mL) was added via a cannula at –45 °C. The suspension was allowed to warm to 0 °C and stirred for addition 1 h. Cold water (40 cm^3) and hexane (40 cm^3) were added. The two layers were separated, and the aqueous layer was extracted with hexane (2 × 30 cm^3). The pooled organic layers were washed with saturated $NaHCO_3$ solution (30 cm^3) and saturated NaCl solution (30 cm^3) and then dried over anhydrous $NaSO_4$. Concentration of the solvent under reduced pressure gave the required bromide **247** as a light yellow oil which was used without further purification. The crude bromide **247** and benzensulfinic acid sodium salt (1.22 g, 7.38 mmol) were dissolved in anhydrous DMF (30 cm^3). The mixture was stirred for 20 h, and then hydrolyzed with water (30 cm^3). The aqueous layer was extracted with diethyl ether (3 × 30 cm^3), and the organic phases were combined, washed with water (30 cm^3) and brine (30 cm^3), dried ($MgSO_4$), filtered and then concentrated under reduced pressure to give the title compound as a pale yellow oil. Purification of the crude product by flash column chromatography on silica gel with hexane and ethyl acetate (2 : 1) as eluent gave the title compound as a colorless oil (1.91 g,

82%); TLC R_f 0.31 (Hexane : EtOAc = 2 : 1); HRMS (ES^+, $[M + NH_4]^+$) found 402.2097. $C_{23}H_{32}O_3NS$ requires 402.2097; υ_{max} (thin film)/cm^{-1} 3061.7, 2918.5, 2855.5, 1666.7, 1585.9, 1448.9, 1386.4, 1310.5, 1255.3, 1134.0, 1086.9, 1026.2, 876.5, 740.8, 691.4 and 616.2; δ_H (500 MHz, C^2HCl_3) 1.61 (3 H, s, CH_3), 1.78 (3 H, s, CH_3), 1.89 (2 H, t, *J* 7.5, $CHCH_2CH_2C$), 2.08 (2 H, q, *J* 7.5, $CHCH_2CH_2C$), 3.73 (2 H, s, CCH_2SO_2), 4.01 (2 H, d, *J* 6.5, $CHCH_2O$), 4.52 (2 H, s, OCH_2Ph), 5.06 (1 H, t, *J* 7.0, $C{=}CHCH_2CH_2$), 5.32 (1 H, td, *J* 6.5, *J* 1.0, $C{=}CHCH_2O$) and 7.29–7.86 (10 H, m, 2 × Ar-H); δ_C (125 MHz, C^2HCl_3) 16.42 (CH_3), 16.71 (CH_3), 26.57 and 38.48 ($CHCH_2CH_2C$), 66.22 (CCH_2SO_2), 66.54 ($CHCH_2O$), 72.20 (OCH_2Ph), 121.31 ($CHCH_2O$), 127.60, 127.81, 128.38, 128.52, 128.90 and 133.51 (Ar-*C*H), 135.64 ($C{=}CHCH_2CH_2$) and 123.61, 138.47, 138.49 and 139.35 (quaternary C); *m/z* (CI^+) 402.3 (40%, $[M + NH_4]^+$) and 135.1 (100).

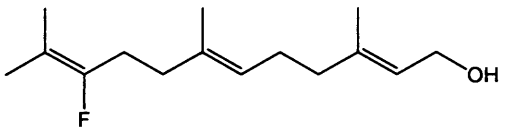
1-[(6*E*,10*E*)-12-(benzyloxy)-3-fluoro-2,6,10-trimethyldodeca-2,6,10-trien-5-ylsulfonyl]benze ne (248)



To the mixture of sulfone **242** (1.57 g, 4.11 mmol) and bromide **241** (0.601 g, 3.60 mmol) in anhydrous THF (25 cm^3) at –78 °C was added *n*-BuLi (2.2 M, 1.87 cm^3, 4.11 mmol) dropwise over 15 min, and the mixture stirred for 2 h at –78 °C. The cooling bath was then removed and the reaction mixture was allowed to warm slowly to 0 °C. Water (20 cm^3) was added and the aqueous layer was extracted with diethyl ether (3 × 20 cm^3), and the organic phases were combined, washed with water (20 cm^3), brine (20 cm^3) and dried ($MgSO_4$), filtered and then

concentrated under reduced pressure to give the title compound as a pale yellow oil. Purification of the crude product by flash column chromatography on silica gel with hexane and ethyl acetate (4 : 1) as eluent gave the title compound as a light yellow oil (1.29 g, 76%); TLC R_f 0.29 (Hexane : EtOAc = 4 : 1); HRMS (ES^+, $[M + NH_4]^+$) found 488.2628. $C_{28}H_{39}O_3NFS$ requires 488.2629; υ_{max}(thin film)/cm^{-1} 2922.3, 2860.0, 1714.5, 1666.9, 1449.5, 1365.9, 1305.8, 1198.9, 1145.1, 1086.5, 737.7, 692.0 and 606.7; δ_H (500 MHz, C^2HCl_3) 1.59 (9 H, m, 3 × CH_3), 1.69 (3 H, s, CH_3), 1.81 (2 H, t, *J* 7.5, $CHCH_2CH_2C$), 1.99 (2 H, m, $CHCH_2CH_2C$), 2.91 (2 H, m, $CFCH_2CH$), 3.79 (1 H, dd, *J* 11.0, *J* 4.0, $CFCH_2CH$), 4.00 (2 H, d, *J* 6.5, $CHCH_2O$), 4.52 (2 H, s, OCH_2Ph), 5.15 (1 H, t, *J* 7.5, $C=CHCH_2CH_2$), 5.31 (1 H, td, *J* 6.5, *J* 1.0, $C=CHCH_2O$) and 7.29–7.83 (10 H, m, 2 × Ar-H); δ_C (125 MHz, C^2HCl_3) 13.79 (CH_3), 15.62 (d, *J* 9.0, CH_3), 16.40 (CH_3), 16.71 (d, *J* 12.5, CH_3), 25.38 (d, $J_{C\text{-}F}$ 29.0, $CFCH_2CH$), 26.49 and 38.36 ($CHCH_2CH_2C$), 66.55 ($CHCH_2O$), 70.98 ($CFCH_2CH$), 72.23 (OCH_2Ph), 110.60 (d, $J_{C\text{-}F}$ 17.5, $C=CFCH_2CH$), 121.21 ($CHCH_2O$), 127.58, 127.82, 128.38, 128.80, 128.81 and 133.51 (Ar-CH), 135.58 ($C=CHCH_2CH_2$), 126.36, 137.94, 138.50 and 139.46 (quaternary C) and 148.42 (d, $J_{C\text{-}F}$ 240.0, $CFCH_2CH$); δ_F (283 MHz, C^2HCl_3) –114.54 (dd, $J_{H\text{-}F}$ 28.0); *m/z* (CI^+) 488.3 (2%, $[M + NH_4]^+$) and 94.1 (100).

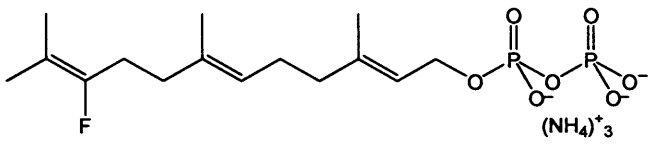
(2*E*,6*E*)-10-Fluoro-3,7,11-trimethyldodeca-2,6,10-trien-1-ol (249)



Tert-butyl alcohol (2.0 cm^3) and benzyl ether **248** (0.37 g, 0.79 mmol) dissolved in anhydrous THF (10 cm^3) were added via a cannula to a lithium bits (0.06 g. 8.57 mmol) with stirring. The

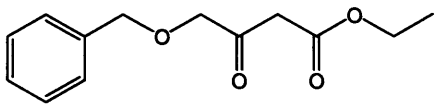
reaction mixture was stirred under nitrogen atmosphere for 5 h, after which another pertion of Li (0.06 g, 8.57 mmol) was added to the reaction mixture together with tert-butyl alcohol (2.0 cm^3) at 0 °C. The reaction mixture was then left to stir overnight. Cold water (10 cm^3) was added and the result mixture was extracted with diethyl ether (3 × 10 cm^3), and the organic phases were combined, washed with brine (10 cm^3) and dried ($MgSO_4$), filtered and then concentrated under reduced pressure to give the title compound as a pale yellow oil. Purification of the crude product by flash column chromatography on $AgNO_3$-impregnated silica gel (5% $AgNO_3$ on silica gel) with hexane and ethyl acetate (3 : 1) as eluent gave the title compound as a colorless oil (0.053 g, 28%); TLC R_f 0.29 (Hexane : EtOAc = 4 : 1); HRMS (EI^+, $[M - H_2O]^+$) found 222.1778. $C_{15}H_{23}F$ requires 222.1784; υ_{max} (thin film)/cm^{-1} 3333.7, 2932.4, 2862.2, 1714.5, 1667.9, 1445.9, 1383.6, 1301.9, 1258.2, 1152.4, 1102.7, 1005.5, 898.1, 798.4 and 737.6; δ_H (500 MHz, C^2HCl_3) 1.44 (1 H, s, OH), 1.49 (3 H, d, $J_{H\text{-}F}$ 2.5, CH_3), 1.55 (6 H, s, 2 × CH_3), 1.61 (3 H, s, C*H*$_3$), 1.96 (2 H, t, *J* 7.5, $CHCH_2CH_2C$), 2.02 (4 H, m, $CHCH_2CH_2C$ and $CFCH_2CH_2$), 2.18 (2 H, dt, $J_{H\text{-}F}$ 23.0, *J* 7.5, $CFCH_2CH_2$), 4.08 (2 H, d, *J* 7.0, $CHCH_2OH$), 5.06 (1 H, td, *J* 7.0, *J* 1.0, $C{=}CHCH_2CH_2$) and 5.34 (1 H, tq, *J* 7.0, *J* 1.0, $C{=}CHCH_2OH$); δ_C (125 MHz, C^2HCl_3) 15.45 (d, $J_{C\text{-}F}$ 10.0, $CH_3C{=}CFCH_2$), 15.91 (CH_3), 16.26 (CH_3), 17.57 (d, $J_{C\text{-}F}$ 6.5, $CH_3C{=}CFCH_2$), 26.32 ($CHCH_2CH_2C$), 27.51 (d, $J_{C\text{-}F}$ 30.0, $CFCH_2CH_2$), 36.58 ($CFCH_2CH_2$), 39.44 ($CHCH_2CH_2C$), 59.38 ($CHCH_2OH$), 107.14 (d, $J_{C\text{-}F}$ 17.5, $C{=}CFCH_2CH_2$), 123.40 ($CHCH_2OH$), 124.53 ($C{=}CHCH_2CH_2$), 134.41 and 139.67 (quaternary C) and 153.00 (d, $J_{C\text{-}F}$ 240.0, $CFCH_2CH_2$); δ_F (283 MHz, C^2HCl_3) –112.75 (t, $J_{H\text{-}F}$ 22.5); *m/z* (EI^+) 222.2 (5%, $[M - H_2O]^+$) and 69.1 (100).

(2*E*,6*E*)-10-Fluoro-3,7,11-trimethyldodeca-2,6,10-trien-1-yl diphosphate tris ammonium salt (157)



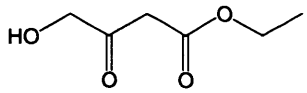
This compound was prepared in a manner identical to that for the diphosphate **154**, using the alcohol **249** (0.041 g, 0.171 mmol) to give the title compound as a white puffy solid (43 mg, 56% for two steps); HPLC t_R = 36.41 min; Purity 96.88 % by analytical RP HPLC under 220 nm; HRMS (ES$^-$, [M – H]$^-$) found 399.1124. $C_{15}H_{26}O_7FP_2$ requires 399.1138; υ_{max} (KBr disc)/cm^{-1} 2818.0, 1716.4, 1670.0, 1456.4, 1201.1, 1120.7, 1090.0, 1025.5, 907.2, 804.7, 722.0, 596.3, 552.5 and 512.9; δ_H (500 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 1.41 (3 H, d, $J_{H\text{-}F}$ 2.5, CH_3C=CFCH$_2$), 1.43 (3 H, d, $J_{H\text{-}F}$ 3.0, CH_3C=CFCH$_2$), 1.48 (3 H, s, CH$_3$), 1.56 (3 H, s, CH$_3$), 1.92 (2 H, t, *J* 7.5, CHCH$_2$CH_2C), 1.98 (4 H, m, CHCH_2CH$_2$C and CFCH$_2$CH_2), 2.20 (2 H, dt, $J_{H\text{-}F}$ 25.0, *J* 7.0, CFCH_2CH$_2$), 4.31 (2 H, t, *J* 6.5, CHCH_2O), 5.07 (1 H, t, *J* 6.5, C=C*H*CH$_2$CH$_2$) and 5.31 (1 H, t, *J* 7.0, C=C*H*CH$_2$O); δ_C (125 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 14.63 (d, $J_{C\text{-}F}$ 10.0, *C*H$_3$C=CFCH$_2$), 15.10 (CH$_3$), 15.64 (CH$_3$), 16.80 (d, $J_{C\text{-}F}$ 6.5, *C*H$_3$C=CFCH$_2$), 25.74 (CH*C*H$_2$CH$_2$C), 26.52 (d, $J_{C\text{-}F}$ 29.0, CF*C*H$_2$CH$_2$), 35.75 (CFCH$_2$*C*H$_2$), 38.79 (CHCH$_2$*C*H$_2$C), 62.51 (d, $J_{C\text{-}P}$ 5.0, CH*C*H$_2$O), 108.63 (d, $J_{C\text{-}F}$ 16.5, *C*=CFCH$_2$CH$_2$), 119.81 (d, $J_{C\text{-}P}$ 9.0, *C*HCH$_2$O), 125.04 (C=*C*HCH$_2$CH$_2$), 135.49 and 142.79 (quaternary C) and 152.94 (d, $J_{C\text{-}F}$ 236.5, *C*FCH$_2$CH$_2$); δ_F (283 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) –113.98 (t, $J_{H\text{-}F}$ 25.5); δ_P (122 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) –7.05 (d, $J_{P\text{-}P}$ 21.0) and –10.35 (d, $J_{P\text{-}P}$ 21.0); *m/z* (ES$^-$) 399.1 (100%, [M – H]$^-$).

Ethyl 4-(benzyloxy)-3-oxobutanoate (252)



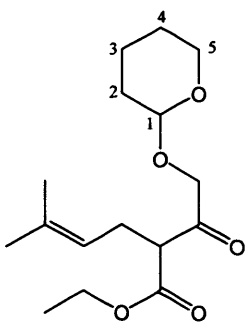
Benzyl alcohol (8.28 cm^3, 80.0 mmol) was added dropwise to a suspension of sodium hydride (3.21 g, 80.0 mmol) in toluene (200 cm^3), slight cooling was required with ice bath to maintain the temperature at RT. After hydrogen evolution had ceased while the thick slurry was allowed to stir for 2 h. Ethyl 4-chloroacetoacetate (5.44 cm^3, 40.0 mmol) was added dropwise over 20 min and the reaction mixture left to stir overnight. Water (100 cm^3) was then added. The result mixture was extracted with diethyl ether (3 × 50 cm^3), and the organic phases were combined, washed with brine (50 cm^3) and dried ($MgSO_4$), filtered and then concentrated under reduced pressure to give the title compound as a pale yellow oil, Purification of the crude product by flash column chromatography on silica gel with hexane and ethyl acetate (4 : 1) as eluent gave the title compound as a pale yellow oil (7.69 g, 82% yield); TLC R_f 0.31 (Hexane : EtOAc = 4 : 1); υ_{max} (thin film)/cm^{-1} 2982.3, 1747.6, 1722.4, 1657.6, 1496.4, 1453.7, 1399.9, 1367.4, 1319.0, 1229.5, 1140.9, 1100.1, 1032.9, 743.3 and 700.3; δ_H (500 MHz, C^2HCl_3) 1.27 (3 H, t, *J* 7.0, CH_2CH_3), 3.56 (2 H, s, $C{=}OCH_2C{=}O$), 4.17 (2 H, s, $CH_2OCH_2C{=}O$), 4.19 (2 H, q, *J* 7.0, CH_2CH_3), 4.61 (2 H, s, $PhCH_2O$) and 7.34 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 14.07 (CH_2CH_3), 46.08 ($C{=}OCH_2C{=}O$), 61.41 (CH_2CH_3), 73.51 ($PhCH_2O$), 74.82 ($CH_2OCH_2C{=}O$), 127.94, 128.12 and 128.56 (Ar-CH), 136.94 (quaternary C), 167.00 ($CO_2CH_2CH_3$) and 201.72 ($C{=}OCH_2O$); *m/z* (CI^+) 254.2 (100%, $[M + NH_4]^+$).

Ethyl 4-hydroxy-3-oxobutanoate (253)



Ethyl 4-benzyloxyacetoacetate **252** (7.70 g, 32.6 mmol) was dissolved in anhydrous ethanol (80 cm^3). Pd-C (3.08 g, 10% w/w) was added, and the solution stirred strongly under a hydrogen atomosphere for 16 h. The solution was filtered through Celite® to remove the catalyst, and the filtrate was concentrated under reduced pressure to give the crude product as a pale yellow oil. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (1 : 2) as eluent gave the title compound as a pale yellow oil (4.52 g, 95 % yield); TLC R_f 0.37 (Hexane : EtOAc = 1 : 2); υ_{max} (thin film)/cm^{-1} 3455.5, 2984.9, 1720.7, 1623.8, 1408.9, 1370.3, 1321.2, 1269.3, 1152.7, 1026.9, 943.9, 858.7 and 810.5; δ_H (500 MHz, C^2HCl_3) 1.29 (3 H, t, *J* 7.0, CH_2CH_3), 3.02 (1 H, br, OH), 3.51 (2 H, s, $C{=}OCH_2C{=}O$), 4.20 (2 H, q, *J* 7.0, CH_2CH_3) and 4.39 (2 H, s, CH_2OH); δ_C (125 MHz, C^2HCl_3) 14.03 (CH_2CH_3), 45.36 ($C{=}OCH_2C{=}O$), 61.84 (CH_2CH_3), 68.54 (CH_2OH); 166.46 ($CO_2CH_2CH_3$) and 202.49 ($C{=}OCH_2OH$); *m/z* (CI^+) 164.1 (100%, $[M + NH_4]^+$).

Ethyl 5-methyl-2-(2-(tetrahydro-2H-pyran-2-yloxy)acetyl)hex-4-enoate (257)

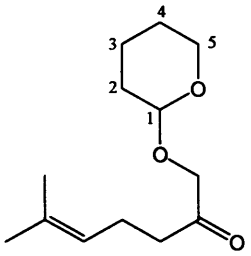


The alcohol **253** (6.30 g, 43.2 mmol) was dissolved in anhydrous DCM (80 cm^3) under a nitrogen

atmosphere. 3,4-dihydro-2*H*-pyran (7.87 cm^3, 86.3 mmol) and ρ-toluenesulfonic acid (0.41g, 2.16 mmol) were added, and the solution was stirred overnight. The solvent was removed under reduced pressure, and the resulting oil was diluted with ethyl acetate (50 cm^3) and water (50 cm^3). The organic layer was washed with water (50 cm^3) and brine (50 cm^3), dried and concentrated to afford the crude THP ether as a amber oil, which was purified by flash column chromatography on silica gel with hexane and ethyl acetate (4 : 1) as eluent to give the partially purified THP ether **254** as a yellow oil (7.25, 73%). This product was used in next step without further purification. To a suspension of NaH (0.37 g, 15.2 mmol) in anhydrous THF (50 cm^3) at 0 °C was added the THP ether **254** (3.50 g, 15.2 mmol) from last step in THF (5 cm^3). The solution was warmed to RT and stirred for 1 h, and then 1-Bromo-3-methylbut-2-ene (2.30 g, 15.2 mmol) was added. The reaction mixture was stirred overnight. Water (50 cm^3) was then added. The result mixture was extracted with ethyl acetate (3 × 30 cm^3), and the organic phases were combined, washed with brine (30 cm^3) and dried ($MgSO_4$), filtered and then concentrated under reduced pressure to give the title compound as a pale yellow oil, Purification of the crude product by flash column chromatography on silica gel with hexane and ethyl acetate (5 : 1) as eluent gave the title compound as a light yellow oil (2.34 g, 53%); TLC R_f 0.33 (Hexane : EtOAc = 5 : 1); HRMS (ES^+, $[M + NH_4]^+$) found 316.2122. $C_{16}H_{30}O_5N$ requires 316.2118; υ_{max} (thin film)/cm^{-1} 2940.1, 1721.2, 1443.2, 1374.3, 1323.6, 1267.3, 1203.0, 1130.1, 1077.2, 1038.6, 968.8, 906.1, 871.5 and 816.6; δ_H (500 MHz, C^2HCl_3) 1.20 (3 H, td, *J* 7.0, *J* 2.0, CH_2CH_3), 1.56 (3 H, s, CH_3), 1.60 (3 H, s, CH_3), 1.47-1.77 (6 H, m, $^2CH_2{}^3CH_2{}^4CH_2$), 2.46–2.53 (2 H, m, C=CHCH_2CH), 3.44 and 3.72 (2 H, m, $CH_2CH_2CH_2{}^5CH_2O$), 3.54 (1 H, dt, *J* 27.0, *J* 7.5, C=OC*H*C=O), 4.07–4.28 (4 H, m, C=OCH_2O and C$H_2$$CH_3$), 4.55 (1 H, dt, *J* 13.0, *J* 3.5, $^1CHCH_2CH_2CH_2$) and 5.06 (1 H, m,

$CH_3C{=}CHCH_2$); δ_C (125 MHz, C^2HCl_3) 14.07 (d, *J* 1.5, CH_2CH_3), 17.72 (CH_3), 18.81 (d, *J* 29.0, $^2CH_2{}^3CH_2{}^4CH_2$), 25.23 ($^2CH_2{}^3CH_2{}^4CH_2$), 25.72 (CH_3), 26.46 (d, *J* 6.5, $C{=}CHCH_2CH$), 30.08 (d, *J* 10.0, $^2CH_2{}^3CH_2{}^4CH_2$), 55.14 (d, *J* 18.0, COCHCO), 61.19 (d, *J* 6.5, CH_2CH_3), 61.92 (d, *J* 41.5, $CH_2CH_2CH_2{}^5CH_2O$), 71.63 (d, *J* 29.0, $C{=}OCH_2O$), 98.46 (d, *J* 59.0, $^1CHCH_2CH_2CH_2$), 119.87 (d, *J* 5.0, $CH_3C{=}CHCH_2$), 134.60 (d, *J* 10.0, $CH_3C{=}CHCH_2$), 169.19 (d, *J* 12.5, $CO_2CH_2CH_3$) and 203.40 (d, *J* 30.0, $C{=}OCH_2O$); *m/z* (CI^+) 316.3 (15%, $[M + NH_4]^+$) and 102.1 (100).

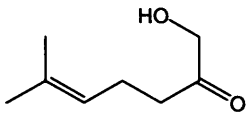
6-Methyl-1-(tetrahydro-2H-pyran-2-yloxy)hept-5-en-2-one (258)



Ester **257** (2.5 g, 8.4 mmol) was dissolved in the mixture of ethanol (80 cm^3) and water (30 cm^3). KOH (3.53 g, 63 mmol) was added, and the solution was stirred for 24 h. The solvent was removed under reduced pressure. The residue was diluted with EtOAc, and washed with water (50 cm^3) and brine (50 cm^3) and dried ($MgSO_4$), filtered and then concentrated under reduced pressure to give the title compound as a pale yellow oil, Purification of the crude product by flash column chromatography on silica gel with hexane and ethyl acetate (5 : 1) as eluent gave the title compound as a light yellow oil product (0.95g, 52%); TLC R_f 0.36 (Hexane : EtOAc = 5 : 1); HRMS (ES^+, $[M + NH_4]^+$) found 244.1906. $C_{13}H_{26}O_3N$ requires 244.1907; υ_{max} (thin film)/cm^{-1} 2941.5, 1720.2, 1442.3, 1380.2, 1263.6, 1203.2, 1127.8, 1074.4, 1037.8, 969.1, 905.9, 871.4 and 815.6; δ_H (400 MHz, C^2HCl_3) 1.54-1.89 (6 H, m, $^2CH_2{}^3CH_2{}^4CH_2$), 1.62 (3 H, s, CH_3), 1.68 (3 H, s, CH_3), 2.28 (2 H, q, *J* 7.5, $C{=}CHCH_2CH_2$), 2.49 (2 H, m, $C{=}CHCH_2CH_2$), 3.51 and 3.81 (2 H,

m, $CH_2CH_2CH_2{}^5CH_2O$), 4.10 (1 H, d, *J* 17.0, $C{=}OC^BH_2O$), 4.24 (1 H, d, *J* 17.0, $C{=}OC^AH_2O$), 4.65 (1 H, t, *J* 3.5, $^1CHCH_2CH_2CH_2$) and 5.08 (1 H, m, $CH_3C{=}CHCH_2$); δ_C (100 MHz, C^2HCl_3) 17.67 (CH_3), 19.14 ($^2CH_2{}^3CH_2{}^4CH_2$), 22.07 and 39.16 ($C{=}CHCH_2CH_2C{=}O$), 25.29 ($^2CH_2{}^3CH_2{}^4CH_2$), 25.70 (CH_3), 30.26 ($^2CH_2{}^3CH_2{}^4CH_2$), 62.28 ($CH_2CH_2CH_2{}^5CH_2O$), 72.06 ($C{=}OCH_2O$), 98.69 ($^1CHCH_2CH_2CH_2$), 122.61 ($CH_3C{=}CHCH_2$), 132.91 ($CH_3C{=}CHCH_2$) and 208.60 ($C{=}OCH_2O$); *m/z* (CI^+) 244.2 (10%, $[M + NH_4]^+$) and 102.1 (100).

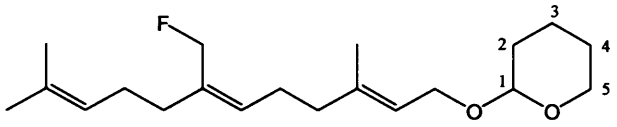
1-Hydroxy-6-methylhept-5-en-2-one (259)



Ketone **258** (0.95 g, 4.2 mmol) was dissolved in ethanol (40 cm^3) under N_2. PPTS (0.11 g, 0.42 mmol) was added, and the solution stirred overnight. The resulting solution was then heated to 50 °C for 1 h, and then the solvent was removed under reduced pressure. The residue was diluted with ethyl acetate (40 cm^3), and washed with water (40 cm^3) and brine (40 cm^3) and dried ($MgSO_4$), filtered and then concentrated under reduced pressure to give the title compound as a pale yellow oil. Purification of the crude product by flash column chromatography on silica gel with hexane and ethyl acetate (4 : 1) as eluent gave the title compound as a light yellow oil product (0.57g, 95%); TLC R_f 0.24 (Hexane : EtOAc = 4 : 1); HRMS (ES^+, $[M + NH_4]^+$) found 160.1330. $C_8H_{18}O_2N$ requires 160.1332; υ_{max} (thin film)/cm^{-1} 3440.6, 2969.2, 2917.1, 1720.4, 1441.8, 1405.8, 1378.2, 1276.3, 1068.1, 996.0 and 829.2; δ_H (500 MHz, C^2HCl_3) 1.62 (3 H, s, CH_3), 1.68 (3 H, s, CH_3), 2.32 (2 H, q, *J* 7.0, $C{=}CHCH_2CH_2$), 2.44 (2 H, t, *J* 5.0, $C{=}CHCH_2CH_2$), 3.17 (1 H, t, *J* 7.0, O*H*), 4.23 (2 H, d, *J* 5.0, CH_2OH) and 5.05 (1 H, m, $CH_3C{=}CHCH_2$); δ_C (125 MHz, C^2HCl_3) 17.63 (CH_3), 22.39 ($CHCH_2CH_2$), 25.62 (CH_3), 38.48 ($CHCH_2CH_2$), 68.26

(CH_2OH), 121.94 ($CH_3C=CHCH_2$), 133.57 ($CH_3C=CHCH_2$) and 209.55 ($C=OCH_2OH$); *m/z* (CI^+) 160.2 (45%, $[M + NH_4]^+$) and 127.1 (100).

2-((2*E*,6*Z*)-7-(Fluoromethyl)-3,11-dimethyldodeca-2,6,10-trienyloxy)-tetrahydro-2H-pyran (261)



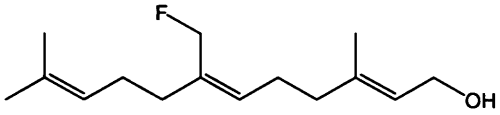
Hydroxyketone **259** (0.57 g, 4.01 mmol) was dissolved in anhydrous DCM (30 cm^3) under N_2. 2,6-Lutidine (1.11 cm^3, 9.56 mmol) was added, and the solution was cooled to 0 °C. Triflic anhydride (0.81 cm3, 4.81 mmol) was added dropwise, and the solution was stirred at 0 °C for 45 min. The solvent was removed under reduced pressure and the residue diluted with ethyl acetate (20 cm^3), washed with 10% $CuSO_4$ (20 cm^3), $KHCO_3$ (20 cm^3) and brine (20 cm^3), dried ($MgSO_4$), filtered and then concentrated under reduced pressure to a red oil. The oil was immediately dissolved in anhydrous THF (30 cm^3) under nitrogen. TBAF (1 M solution in THF, 8.02 cm^3, 8.02 mmol) was added dropwise, and the solution was stirred for 1.5 h. The solution was concentrated under reduced pressure and the residue purified by flash column chromatography on silica gel with hexane and ethyl acetate (19 : 1) as the eluent. Due to the volatility of the fluoro ketone **260**, the solvent was not removed under pressure totally and the product used in next step directly.

A mixture of compound **213** (6.05 g, 21.8 mmol) and triphenylphosphine (8.59 g, 32.8 mmol) in anhydrous acetonitrile (100 cm^3), under a nitrogen atmosphere, was heated under reflux for 14 h. After cooling, the solvent was concentrated under reduced pressure to afford an oily mixture.

Excess triphenyl phosphine was removed by triturating the mixture with anhydrous diethyl ether, and the sticky residue was dried in *vacuo* for 5 h. to afford the intermediate phosphonium salt **214** as a viscous oil. To a stirred solution of crude **214** (1.51 g, 2.80 mmol) in anhydrous THF (40 cm^3) under N_2 at –78 °C, was added lithium hexamethyldisilazide (1 M solution in THF, 2.80 cm^3, 2.80 mmol). The mixture was stirred for 30 min at –78 °C, and then the fluoro ketone **260** above was added dropwise. The whole reaction mixture was allowed to warm to –20 °C over 1 h, and then maintained at –20 °C for 2 h. The reaction was then quenched by addition of diethyl ether (20 cm^3) and water (20 cm^3). The aqueous layer was extracted with diethyl ether (3 × 20 cm^3) and the combined ethereal extracts were washed brine (20 cm^3), dried over $MgSO_4$, filtered and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (30 : 1) as eluent gave THP ether **261** as a light yellow oil (0.39 g, 30% over 2 steps); TLC R_f 0.33 (Hexane : EtOAc = 30 : 1); HRMS (ES^+, $[M + Na]^+$) found 347.2360. $C_{20}H_{33}O_2FNa$ requires 347.2357; υ_{max} (thin film)/cm^{-1} 2937.9, 1739.2, 1667.5, 1443.3, 1380.6, 1261.1, 1200.2, 1134.2, 1117.5, 1075.9, 1024.1, 974.6, 904.5, 869.4 and 813.4; δ_H (500 MHz, C^2HCl_3) 1.43–1.78 (6 H, m, $^2CH_2{}^3CH_2{}^4CH_2$), 1.53 (3 H, s, CH_3), 1.61 (6 H, d, *J* 3.0, 2 × CH_3), 1.98–2.17 (8 H, m, 2 × C=CHCH_2CH_2CCH), 3.42 and 3.80 (2 H, m, $CH_2CH_2CH_2{}^5CH_2O$), 3.93 (1 H, dd, *J* 12.0, *J* 7.0, C=CHC$^BH_2$O), 4.15 (1 H, dd, *J* 12.0, *J* 6.5, C=CHC$^AH_2$O), 4.55 (1 H, t, *J* 3.5, $^1CHCH_2CH_2CH_2$), 4.77 (2 H, d, *J* 48.0, CH_2F), 5.03 (1 H, m, $(CH_3)_2C$=C*H*), 5.30 (1 H, m, C=C*H*CH_2O) and 5.35 (1 H, td, *J* 7.5, *J* 3.0, FCH_2C=C*H*); δ_C (125 MHz, C^2HCl_3) 16.39 (CH_3), 17.69 (CH_3), 19.61, 25.51 and 30.72 ($^2CH_2{}^3CH_2{}^4CH_2$), 25.67 (CH_3), 25.94 (d, *J* 1.25), 26.88, 34.81 and 39.58 (d, *J* 2.5) (2 × C=CHCH_2CH_2), 61.28 ($CH_2CH_2CH_2{}^5CH_2O$), 63.58 (C=CHCH_2O), 79.71 (d, *J* 158.75, CH_2F), 97.87 ($^1CHCH_2CH_2CH_2$), 121.29 (C=CHCH_2O), 123.85

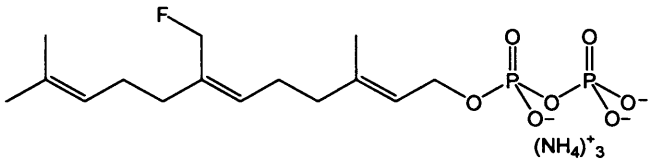
$(CH_3)_2C=CH$), 130.72 (d, *J* 10.0, $FCH_2C=CH$), 131.78 and 139.27 (quaternary C) and 134.65 (d, *J* 14.0, $FCH_2C=CH$); δ_F (283 MHz, C^2HCl_3) –214.38 (t, $J_{H\text{-}F}$ 48.5); *m/z* (CI^+) 342.4 (20%, $[M + NH_4]^+$) and 102.1 (100).

(2*E*,6*Z*)-7-(Fluoromethyl)-3,11-dimethyldodeca-2,6,10-trien-1-ol (262)



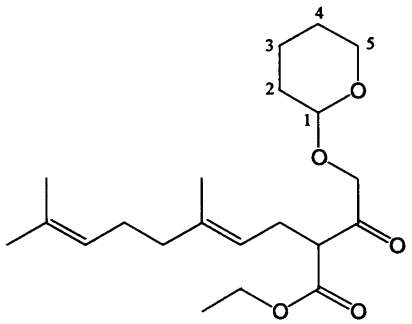
This compound was prepared from **261** in a manner identical to that for the alcohol **211**; purification by silica column chromatography using hexane and ethyl acetate (4: 1) as eluent gave alcohol **262** as a colourless oil (0.11 g, 91%); TLC R_f 0.21 (Hexane : EtOAc = 4 : 1); HRMS (EI^+, M^+) found 240.1889. $C_{15}H_{25}OF$ requires 240.1889; υ_{max}(thin film)/cm^{-1} 3337.4, 2966.8, 2920.9, 1667.4, 1445.1, 1380.2, 1241.6, 1103.5, 978.2, 825.5 and 741.7; δ_H (500 MHz, C^2HCl_3) 1.37 (1 H, b, OH), 1.62 (3 H, s, CH_3), 1.69 (3 H, CH_3), 1.70 (3 H, CH_3), 2.07–2.24 (8 H, m, 2 × $C=CHCH_2CH_2CCH$), 4.16 (2 H, d, *J* 7.0, $CHCH_2OH$), 4.86 (2 H, d, *J* 48.0, CH_2F), 5.11 (1 H, m, $(CH_3)_2C=CH$) and 5.42 (2 H, m, 2 × $C=CHCH_2$); δ_C (125 MHz, C^2HCl_3) 16.25 (CH_3), 17.64 (CH_3), 25.67 (CH_3), 25.96 (d, *J* 2.5), 26.86, 34.82 (d, *J* 1.5) and 39.43 (d, *J* 2.5) (2 × $C=CHCH_2CH_2$), 59.30 ($CHCH_2OH$), 79.72 (d, *J* 160.0, CH_2F), 123.81 $(CH_3)_2C=CH$), 124.06 ($CHCH_2OH$), 130.68 (d, *J* 9.0, $FCH_2C=CH$), 131.85 and 138.78 (quaternary C) and 134.73 (d, *J* 15.0, $FCH_2C=CH$); δ_F (283 MHz, C^2HCl_3) –214.03 (t, $J_{H\text{-}F}$ 48.5); *m/z* (EI^+) 240.2 (1%, M^+) and 69.1 (100).

(2*E*,6*Z*)-7-(Fluoromethyl)-3,11-dimethyldodeca-2,6,10-trien-1-yl diphosphate (158)



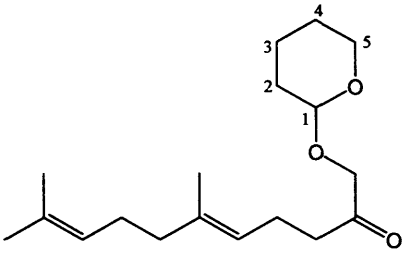
This compound was prepared in a manner identical to that for the diphosphate **154**, using the alcohol **262** (0.10 g, 0.42 mmol) to give the title compound as a white fluffy solid (85.2 mg, 45% for two steps); HPLC t_R = 36.17 min; Purity 98.20 % by analytical RP HPLC; HRMS (ES^-, $[M-H]^-$) found 399.1124. $C_{15}H_{26}O_7FP_2$ requires 399.1138; υ_{max} (KBr disc)/cm^{-1} 2898.3, 1729.0, 1669.0, 1494.4, 1457.6, 1412.6, 1199.9, 1123.0, 1079.8, 1022.1, 909.2, 804.5, 721.0 and 597.1; δ_H (500 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 1.47 (3 H, s, CH_3), 1.55 (3 H, s, CH_3), 1.57 (3 H, s, CH_3), 1.96–2.13 (8 H, m, 2 × $CHCH_2CH_2$), 4.32 (2 H, t, *J* 6.5, $CHCH_2O$), 4.83 (2 H, d, $J_{H\text{-}F}$ 47.0, CH_2F), 5.05 (1 H, b, $(CH_3)_2C{=}CHCH_2CH_2$), 5.32 (1 H, t, *J* 6.5, $CHCH_2O$) and 5.44 (1 H, m, $FCH_2C{=}CHCH_2CH_2$); δ_C (125 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 15.63 (CH_3), 17.98 (CH_3), 24.85 (CH_3), 25.47 (d, $J_{C\text{-}F}$ 2.5), 26.13, 34.10 and 38.85 (d, $J_{C\text{-}F}$ 4.0) (2 × $CHCH_2CH_2$), 62.48 (d, $J_{C\text{-}P}$ 6.5, $CHCH_2O$), 81.01 (d, $J_{C\text{-}F}$ 151.5, CH_2F), 120.27 (d, $J_{C\text{-}P}$ 9.0, $CHCH_2O$), 124.03 [$(CH_3)_2C{=}CHCH_2CH_2$], 132.60 ($FCH_2C{=}CHCH_2CH_2$), 133.77 and 142.03 (quaternary C) and 134.09 (d, *J* 12.5, CCH_2F); δ_F (283 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) –210.48 (t, $J_{H\text{-}F}$ 48.0); δ_P (122 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) –7.54 (d, $J_{P\text{-}P}$ 21.0) and –10.38 (d, $J_{P\text{-}P}$ 21.0); *m/z* (ES^-) 399.1 (100%, $[M-H]^-$).

(*E*)-Ethyl 5,9-dimethyl-2-(2-(tetrahydro-2H-pyran-2-yloxy)acetyl)deca-4,8-dienoate (265)



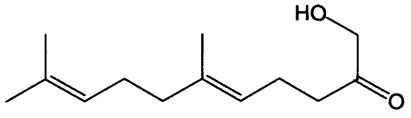
This compound was prepared and purified in a manner similar to that described for the compound **257**, using the THP ether **254** (7.16 g, 31.1 mmol) to give the title compound **265** as a colourless oil (5.23 g, 46%); TLC R_f 0.33 (Hexane : EtOAc = 4 : 1); υ_{max} (thin film)/cm^{-1} 2935.9, 1720.6, 1442.6, 1373.6, 1320.8, 1264.7, 1201.9, 1076.1, 1037.9, 969.1, 905.6, 870.9 and 815.9; δ_H (500 MHz, C^2HCl_3) 1.28 (3 H, td, *J* 7.0, *J* 1.5, CH_2CH_3), 1.61 (3 H, s, CH_3), 1.64 (3 H, s, CH_3), 1.69 (3 H, s, CH_3), 1.56–1.86 (6 H, m, $^2CH_2{}^3CH_2{}^4CH_2$), 1.97–2.07 (4 H, m, C=CHCH_2CH_2), 2.56-2.63 (2 H, m, C=CHCH_2CH), 3.52 and 3.81 (2 H, m, $CH_2CH_2CH_2{}^5CH_2O$), 3.64 (1 H, dt, *J* 25.5, *J* 7.5, C=OC*H*C=O), 4.16–4.38 (4 H, m, C=OCH_2O and CH_2CH$_3$), 4.64 (1 H, dt, *J* 13.0, *J* 3.0, $^1CHCH_2CH_2CH_2$) and 5.06 (2 H, m, 2 × CH_3C=C*H*CH_2); δ_C (125 MHz, C^2HCl_3) 14.12 (CH_2CH_3), 16.11 (CH_3), 17.72 (CH_3), 18.81, 19.04 and 25.25 ($^2CH_2{}^3CH_2{}^4CH_2$), 25.71 (CH_3), 26.39 (d, *J* 7.5, C=CH*C*H$_2$CH) 26.54 and 39.70 (C=CHCH_2CH_2), 55.20 (d, *J* 14.0, C=O*C*HC=O), 61.28 (d, *J* 14.0, 5, *C*H$_2$CH$_3$), 61.93 (d, *J* 41.0, $CH_2CH_2CH_2{}^5CH_2O$), 71.66 (d, *J* 29.0, C=O*C*H$_2$O), 98.44 (d, *J* 59.0, $^1CHCH_2CH_2CH_2$), 119.69 (d, *J* 4.0, CH_3CCHCH_2), 124.02 (d, *J* 2.5, CH_3CCHCH_2), 131.56 (d, *J* 2.0, $CH_3CCHCH_2CH_2$), 138.31 (d, *J* 9.0, CH_3CCHCH_2CH), 169.24 (d, *J* 12.0, $CO_2CH_2CH_3$) and 203.48 (d, *J* 28.0, *C*=OCH$_2$O); *m/z* (CI$^+$) 384.4 (100%, $[M + NH_4]^+$).

(*E*)-6,10-Dimethyl-1-(tetrahydro-2H-pyran-2-yloxy)undeca-5,9-dien-2-one (266)



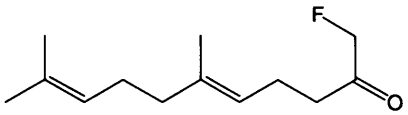
This compound was prepared and purified in a manner similar to that described for the compound **258**, using the compound **265** (4.93 g, 13.5 mmol) to give the title compound **266** as a colourless oil (2.96 g, 75%); TLC R_f 0.18 (Hexane : EtOAc = 9 : 1); υ_{max} (thin film)/cm^{-1} 2924.0, 1720.1, 1442.0, 1380.7, 1353.4, 1263.2, 1203.1, 1127.3, 1073.1, 1037.8, 968.8, 905.6, 871.5 and 816.5; δ_H (500 MHz, C^2HCl_3) 1.61 (3 H, s, CH_3), 1.63 (3 H, s, CH_3), 1.69 (3 H, s, CH_3), 1.56–1.87 (6 H, m, $^2CH_2{}^3CH_2{}^4CH_2$), 1.98 (2 H, t, *J* 7.5, C=CHCH$_2$CH_2CCH), 2.06 (2 H, q, *J* 7.5, C=CHCH_2CH$_2$CCH), 2.30 (2 H, q, *J* 7.5, C=CHCH_2CH$_2$CO), 2.50 (2 H, td, *J* 7.5, *J* 3.0, C=CHCH$_2$CH_2CO), 3.52 and 3.83 (2 H, m, CH$_2$CH$_2$CH$_2{}^5$CH_2O), 4.12 (1 H, d, *J* 17.5, COC^BH_2O), 4.24 (1 H, d, *J* 17.5, C=OC^AH_2O), 4.66 (1 H, t, *J* 3.5, ^{1}C*H*CH$_2$CH$_2$CH$_2$) and 5.07 (2 H, m, 2 × CH$_3$C=C*H*CH$_2$); δ_C (125 MHz, C^2HCl_3) 15.98 (CH_3), 17.67 (CH_3), 19.17, 25.30 and 30.28 ($^2CH_2{}^3CH_2{}^4CH_2$), 22.01 (C=CH*C*H$_2$CH$_2$C=O), 25.67 (CH_3), 26.62 (C=CH*C*H$_2$CH$_2$C=CH), 38.52 (C=CHCH$_2$*C*H$_2$C=O), 39.64 (C=CHCH$_2$*C*H$_2$C=CH), 61.93 (CH$_2$CH$_2$CH$_2{}^5$*C*H$_2$O), 72.09 (C=O*C*H$_2$O), 98.74 (^{1}*C*HCH$_2$CH$_2$CH$_2$), 122.49 and 124.19 (2 × CH$_3$C=*C*HCH$_2$), 131.41 and 136.50 (quaternary *C*) and 208.52 (*C*OCH$_2$O); *m/z* (CI$^+$) 312.4 (100%, $[M + NH_4]^+$).

(*E*)-1-Hydroxy-6,10-dimethylundeca-5,9-dien-2-one (267)



This compound was prepared and purified in a manner identical to that described for the compound **259**, using the THP ether **266** (2.78 g, 9.46 mmol) to give the title compound **267** as a colourless oil (1.53 g, 81%); TLC R_f 0.21 (Hexane : EtOAc = 4 : 1); υ_{max} (thin film)/cm^{-1} 3434.8, 2965.6, 2916.8, 1720.4, 1441.4, 1379.3, 1273.1, 1065.7, 990.4 and 830.7; δ_H (500 MHz, C^2HCl_3) 1.62 (3 H, s, CH_3), 1.63 (3 H, s, CH_3), 1.70 (3 H, s, CH_3), 1.99 (2 H, t, *J* 7.5, C=CHCH$_2$C*H*$_2$C=CH), 2.07 (2 H, q, *J* 7.5, C=CHC*H*$_2$CH$_2$C=CH), 2.35 (2 H, q, *J* 7.5, C=CHC*H*$_2$CH$_2$C=O), 2.47 (2 H, t, *J* 7.5, C=CHCH$_2$C*H*$_2$C=O), 4.25 (2 H, s, C*H*$_2$OH) and 5.08 (2 H, m, 2 × CH_3C=C*H*CH$_2$); δ_C (125 MHz, C^2HCl_3) 15.99 (CH_3), 17.68 (CH_3), 22.36 (C=CH*C*H$_2$CH$_2$C=O), 25.67 (CH_3), 26.55 (C=CH*C*H$_2$CH$_2$C=CH), 38.52 (C=CHCH$_2$*C*H$_2$C=O), 39.60 (C=CHCH$_2$*C*H$_2$C=CH), 68.30 (*C*H$_2$OH), 121.79 and 124.04 (2 × CH_3C=*C*HCH$_2$), 131.56 and 137.24 (quaternary C) and 209.53 (*C*OCH$_2$OH); *m/z* (CI^+) 228.3 (100%, $[M + NH_4]^+$).

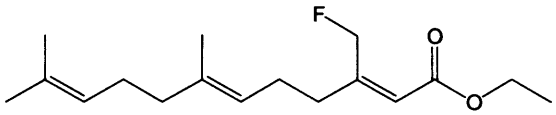
(*E*)-1-Fluoro-6,10-dimethylundeca-5,9-dien-2-one (268)



Hydroxyketone **267** (1.27 g, 6.05 mmol) was dissolved in anhydrous DCM (50 cm^3) under nitrogen. 2,6-Lutidine (1.69 cm^3, 14.5 mmol) was added and the solution was cooled to 0 °C. Triflic anhydride (1.22 cm3, 7.26 mmol) was added dropwise, and the solution was stirred at 0 °C for 45 min. The solvent was removed under reduced pressure and the residue diluted with ethyl acetate (30 cm^3), washed with 10% $CuSO_4$ (30 cm^3), $KHCO_3$ (30 cm^3) and brine (30 cm^3), dried ($MgSO_4$), filtered and then concentrated under reduced pressure to give a red oil. The oil was immediately dissolved in anhydrous THF (50 cm^3) under nitrogen. TBAF (1 M solution in THF, 12.1 cm^3, 12.1 mmol) was added dropwise, and the solution was stirred for 1.5 h. The solution

was concentrated under reduced pressure and the residure purified by flash column chromatography on silica gel with hexane and ethyl acetate (9 : 1) as eluent to give the title compound as a yellow oil (0.88 g, 69%); TLC R_f 0.43 (Hexane : EtOAc = 4 : 1); υ_{max} (thin film)/cm^{-1} 2966.7, 2920.9, 2856.1, 1727.0, 1436.0, 1380.1, 1148.3, 1052.0, 983.9 and 829.7; δ_H (500 MHz, C^2HCl_3) 1.53 (3 H, s, CH_3), 1.55 (3 H, s, CH_3), 1.61 (3 H, s, CH_3), 1.90 (2 H, t, *J* 7.5, C=CHCH$_2$C*H*$_2$C=CH), 1.98 (2 H, q, *J* 7.5, C=CHC*H*$_2$CH$_2$C=CH), 2.24 (2 H, q, *J* 7.5, C=CHC*H*$_2$CH$_2$C=O), 2.50 (2 H, td, *J* 7.5, *J* 2.5, C=CHCH$_2$C*H*$_2$C=O), 4.68 (2 H, d, $J_{H\text{-}F}$ 47.5, C*H*$_2$F) and 5.00 (2 H, m, 2 × CH_3C=C*H*CH_2); δ_C (125 MHz, C^2HCl_3) 15.99 (CH_3), 17.71 (CH_3), 21.45 (C=CH*C*H$_2$CH$_2$C=O), 25.72 (CH_3), 26.56 (C=CH*C*H$_2$CH$_2$C=CH), 38.44 (C=CHCH$_2$*C*H$_2$C=O), 39.63 (C=CHCH$_2$*C*H$_2$C=CH), 84.32 (d, $J_{C\text{-}F}$ 184.0, *C*H$_2$F), 121.96 and 124.09 (2 × CH_3C*C*HCH$_2$), 131.56 and 136.99 (quaternary C) and 206.82 (d, $J_{C\text{-}F}$ 20.0, *C*=OCH$_2$F); δ_F (283 MHz, C^2HCl_3) –227.30 (t, $J_{H\text{-}F}$ 48.0); *m/z* (CI^+) 230.3 (100%, $[M + NH_4]^+$).

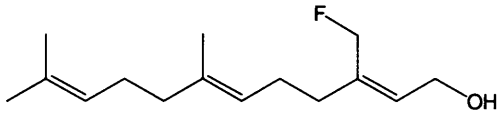
(2*Z*,6*E*)-Ethyl 3-(fluoromethyl)-7,11-dimethyldodeca-2,6,10-trienoate (269)



NaH (0.12 g, 4.92 mmol) was suspended in anhydrous THF (50 cm^3) under nitrogen. Triethyl phosphonoacetate (0.98 cm^3, 4.92 mmol) was added dropwise, and the solution stirred for 1 h. A solution of ketone **268** in anhydrous THF (10 cm^3) was added, and the solution stirred overnight. Water (30 cm^3) and diethyl ether (30 cm^3) were added, and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 25 cm^3). The combined ethereal extracts were washed with water (2 × 20 cm^3) and satd. NaCl (20 cm^3), dried over $MgSO_4$, filtered and then concentrated under reduced pressure. Purification of the crude product by MPLC with hexane and

ethyl acetate (80 : 1) as eluent gave the title compound as a light yellow oil (0.65 g, 64%); TLC R_f 0.19 (Hexane : EtOAc = 60 : 1); υ_{max} (thin film)/cm^{-1} 2975.1, 2922.6, 2857.0, 1717.9, 1660.4, 1446.2, 1380.9, 1320.1, 1238.9, 1163.2, 1125.2, 1043.7 and 867.0; δ_H (500 MHz, C^2HCl_3) 1.21 (3 H, t, *J* 7.0, CH_2CH_3), 1.53 (3 H, s, CH_3), 1.54 (3 H, s, CH_3), 1.61 (3 H, s, CH_3), 1.91 (2 H, t, *J* 7.5, $C=CHCH_2CH_2C=CHCH_2$), 1.97 (2 H, q, *J* 7.5, $C=CHCH_2CH_2C=CHCH_2$), 2.14 (2 H, q, *J* 7.5, $C=CHCH_2CH_2CCH_2F$), 2.29 (2 H, t, *J* 7.5, $C=CHCH_2CH_2CCH_2F$), 4.06 (2 H, q, *J* 7.5, CH_2CH_3), 5.00 (2 H, m, 2 × $CH_3C=CHCH_2$), 5.43 (2 H, dd, $J_{H\text{-}F}$ 48.0, *J* 2.0, CH_2F) and 5.63 (1 H, d, *J* 1.5, $CHCO_2CH_2CH_3$); δ_C (125 MHz, C^2HCl_3) 14.23 (CH_2CH_3), 16.00 (CH_3), 17.67 (CH_3), 25.66 (CH_3), 26.34 ($C=CHCH_2CH_2CCH_2F$), 26.67 ($C=CHCH_2CH_2C=CHCH_2$), 33.61 (d, *J* 7.5, $C=CHCH_2CH_2CCH_2F$), 39.66 ($C=CHCH_2CH_2C=CHCH_2$), 60.10 (CH_2CH_3), 81.43 (d, $J_{C\text{-}F}$ 162.5, CH_2F), 116.02 (d, *J* 5, $CHCO_2CH_2CH_3$), 122.67 and 124.19 (2 × $CH_3C=CHCH_2$), 131.43 and 136.49 (quaternary C), 158.60 (d, J 19.0, $C=CH_2F$) and 165.85 ($CHCO_2CH_2CH_3$); δ_F (283 MHz, C^2HCl_3) –214.06 (t, $J_{H\text{-}F}$ 47.5); *m/z* (CI^+) 300.3 (60%, $[M + NH_4]^+$), 263.3 (100, $[M - F]^+$).

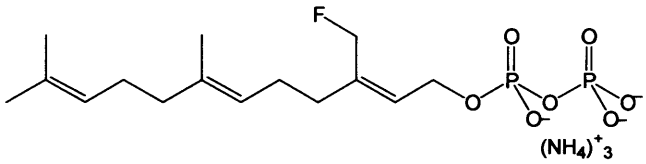
(2*Z*,6*E*)-3-(fluoromethyl)-7,11-dimethyldodeca-2,6,10-trien-1-ol (270)



This compound was prepared from **269** (0.61 g, 2.26 mmol) in a manner similar to that for the alcohol **175**; purification by silica column chromatography using hexane and ethyl acetate (5: 1) as eluent gave alcohol **270** as a colourless oil (0.42 g, 80%); TLC R_f 0.28 (Hexane : EtOAc = 5 : 1); υ_{max} (thin film)/cm^{-1} 3350.5, 2964.9, 2921.9, 2855.1, 1666.6, 1448.6, 1380.7, 1242.6, 1083.4, 999.8, 830.4 and 738.5; δ_H (500 MHz, C^2HCl_3) 1.53 (6 H, s, 2 × CH_3), 1.61 (3 H, s, CH_3), 1.91 (2 H, t, *J* 7.5, $C=CHCH_2CH_2CCHCH_2$), 1.99 (2 H, q, *J* 7.5, $C=CHCH_2CH_2C=CHCH_2$), 2.10 (4 H, m,

C=CHCH_2CH_2CCH$_2$F), 4.13 (2 H, dd, *J* 7.0, *J* 3.5, CH_2OH), 4.82 (2 H, d, $J_{H\text{-}F}$ 47.5, CH_2F), 5.02 (2 H, m, 2 × CH_3C=C*H*CH_2) and 5.63 (1 H, d, *J* 1.5, C*H*CH_2OH); δ_C (125 MHz, C^2HCl_3) 16.03 (CH_3), 17.68 (CH_3), 25.68 (CH_3), 26.46 (C=CHCH_2CH_2CCH$_2$F), 34.57 (d, *J* 1.5, C=CHCH$_2$CH_2CCH$_2$F), 26.69 (C=CHCH_2CH$_2$C=CHCH$_2$), 39.68 (C=CHCH$_2$CH_2C=CHCH$_2$), 58.60 (CH_2OH), 79.70 (d, $J_{C\text{-}F}$ 161.5, CH_2F), 123.28 and 124.26 (2 × CH_3C=C*H*CH_2), 129.13 (d, *J* 7.5, C*H*CH_2OH), 131.41 and 135.90 (quaternary C) and 137.92 (d, *J* 14.0, C=CH_2F); δ_F (283 MHz, C^2HCl_3) –214.06 (t, $J_{H\text{-}F}$ 47.0); *m/z* (CI^+) 258.3 (100%, $[M + NH_4]^+$).

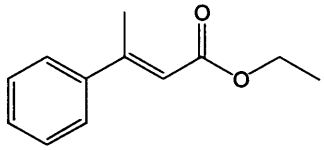
(2*Z*,6*E*)-3-(Fluoromethyl)-7,11-dimethyldodeca-2,6,10-trien-1-yl diphosphate (159)



This compound was prepared in a manner identical to that for the diphosphate **154**, using the alcohol **270** (0.23 g, 0.98 mmol) to give the title compound as a white puffy solid (188 mg, 43% for two steps); HPLC t_R = 36.99 min; Purity 97.61 % by analytical RP HPLC; HRMS (ES^-, $[M - H]^-$) found 399.1140. $C_{15}H_{26}O_7FP_2$ requires 399.1143; υ_{max} (KBr disc)/cm^{-1} 2924.3, 1721.1, 1674.2, 1457.5, 1405.9, 1200.3, 1123.8, 1092.4, 1030.5, 995.0, 913.7, 802.7 and 720.5; δ_H (500 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 1.46 (6 H, d, *J* 4.0, 2 × CH_3), 1.53 (3 H, s, CH_3), 1.86–2.05 (8 H, m, 2 × CHCH_2CH_2), 4.36 (2 H, m, CHCH_2O), 4.87 (2 H, d, $J_{H\text{-}F}$ 47.0, CH_2F), 5.02 (1 H, b, CH_3C=C*H*CH_2), 5.07 (1 H, b, CH_3C=C*H*CH_2) and 5.60 (1 H, m, C*H*CH_2O); δ_C (125 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 15.14 (CH_3), 16.86 (CH_3), 24.75 (CH_3), 25.65, 25.76, 33.73 and 38.69 (2 × CHCH_2CH_2), 61.31 (d, *J* 5.0, CHCH_2O), 80.30 (d, $J_{C\text{-}F}$ 155.0, CH_2F), 123.78 and 124.35 (2 × CH_3C=C*H*CH_2), 126.56 (t, *J* 9.0, C*H*CH_2O), 133.44 and 136.87

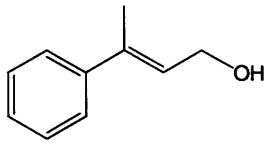
(quaternary C) and 138.64 (d, J 14.0, $C$$CH_2F$); δ_F (283 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) –212.90 (t, $J_{H\text{-}F}$ 47.5); δ_P (122 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) –6.46 (d, $J_{P\text{-}P}$21.0) and –10.45 (d, $J_{P\text{-}P}$21.0); *m/z* (ES^-) 399.1 (100%, $[M - H]^-$).

(*E*)-ethyl 3-phenylbut-2-enoate (273)



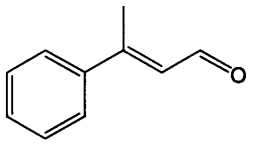
To a stirred solution of sodium hydride (4.40 g, 110 mmol) in anhydrous 1,2-dimethoxyethane (200 cm^3) at room temperature, triethyl phosphonoacetate (21.8 cm^3, 110 mmol) and a solution of acetophenone (11.7 cm^3, 100 mmol) in anhydrous dimethoxyethane were sequentially added, dropwise. After 3 h, water (50 cm^3) was added and the organic layer was separated. The aqueous layer was extracted with diethyl ether (3 × 50 cm^3). The combined organic phases were washed with brine (50 cm^3), dried ($MgSO_4$), filtered and then concentrated under reduced pressure to give the intermediate ester as a pale yellow oil (11.6 g, 61%); TLC R_f 0.39 (Hexane : EtOAc = 9 : 1); HRMS (ES^+, $[M + H]^+$) found 191.1067. $C_{12}H_{15}O_2$ requires 191.1067; υ_{max}(thin film)/cm^{-1} 2980.1, 1713.0, 1628.4, 1576.4, 1494.0, 1446.0, 1365.9, 1343.8, 1272.6, 1171.3, 1044.1, 872.4, 766.9 and 694.9; δ_H (500 MHz, C^2HCl_3) 1.23 (3 H, t, J 7.5, CH_3CH_2O), 2.50 (3 H, d, J 1.5, CH_3CPh), 4.13 (2 H, q, J 7.5, CH_3CH_2O), 6.05 (1 H, q, J 1.5, PhC=C*H*) and 7.27–7.40 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 14.36 (CH_3CH_2O), 17.95 (CH_3C=CH), 59.83 (CH_3CH_2O), 117.22 (PhC=CH), 126.31, 128.49 and 128.97 (Ar-CH), 142.27 and 155.49 (quaternary C) and 166.87 (C=O); *m/z* (ES^+) 191.1 (100%, $[M + H]^+$).

(*E*)-3-Phenylbut-2-en-1-ol (274)



Alcohol **274** was prepared from ester **273** in a manner identical to that for the alcohol **175**; the crude alcohol was purified by silica column chromatography using hexane and ethyl acetate (2: 1) as eluent to give the title compound as a light yellow oil (6.7 g, 96%); R_f 0.27 (Hexane : EtOAc = 2 : 1); HRMS: (EI^+, M^+) found 148.0890. $C_{10}H_{12}O$ requires 148.0888; υ_{max}(thin film)/cm^{-1} 3347.2, 2922.2, 2597.9, 1493.8, 1444.5, 1379.9, 1003.0, 758.1 and 696.1; δ_H(500 MHz, C^2HCl_3) 2.11 (3 H, t, *J* 0.5, C*H*$_3$CPh), 4.40 (2 H, dd, *J* 0.5, *J* 6.5, C*H*$_2$OH), 6.01 (1 H, tq, *J* 1.5, *J* 6.5, PhC=C*H*) and 7.28–7.45 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 16.06 (*C*H$_3$CPh), 59.96 (CH$_2$OH), 126.52 (*C*HCH$_2$OH), 125.81, 127.32 and 128.32 (Ar-CH) and 137.84 and 142.87 (quaternary C); ***m/z*** (EI^+) 148.1 (13%, M^+), 115.1 (100).

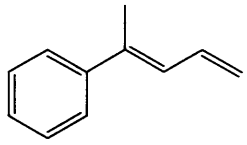
(*E*)-3-Phenylbut-2-enal (276)



This compound was prepared from **274** (6.7 g, 45 mmol) in a manner identical to that for the aldehyde **176**; purification by silica column chromatography using hexane and ethyl acetate (1: 2) as eluent gave aldehyde **276** as a yellow oil (3.7 g, 57%); R_f 0.37 (Hexane : EtOAc = 4 : 1); HRMS: (EI^+, M^+) found: 146.0730. $C_{10}H_{10}O$ requires 146.0732; υ_{max}(thin film)/cm^{-1} 1722.2, 1659.7, 1446.2, 1377.3, 1248.1, 1144.5, 865.5 and 758.6; δ_H (500 MHz, C^2HCl_3) 1.94 (3 H, d, *J* 1.0, CH$_3$CPh), 6.41 (1 H, dq, *J* 7.5, *J* 1.0, PhC=C*H*), 7.10–7.18 (5 H, m, Ar-H) and 10.05 (1 H, d,

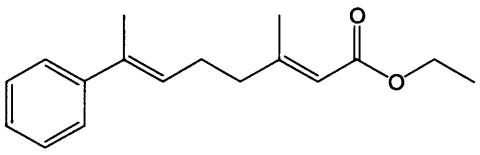
J 7.5, CHO); δ_C (125 MHz, C^2HCl_3) 15.45 (CH_3CPh), 127.37 (PhC=*C*H), 126.16, 128.49 and 129.52 (Ar-CH), 140.77 and 155.78 (quaternary C) and 189.77(CHO). *m/z* (EI^+) 146.1 (46%, M^+), 145.1 (100).

(*E*) 4-Phenyl-buta-1,3-diene (275)



This compound was prepared from **276** in a manner identical to that for **172**; purification by silica column chromatography using hexane and ethyl acetate (2: 1) as eluent gave **275** as a light yellow oil (1.70 g, 78%); R_f 0.57 (Hexane : EtOAc = 19 : 1); HRMS: (EI^+, M^+) found 144.0938. $C_{11}H_{12}$ requires 144.0939; υ_{max}(thin film)/cm^{-1} 3029.5, 2923.2, 1803.9, 1627.6, 1594.2, 1493.3, 1445.8, 1380.1, 1175.1, 1027.4, 985.3, 903.6, 759.8 and 694.8; δ_H (400 MHz, C^2HCl_3) 2.09 (3 H, s, CH_3CPh), 5.10 (1 H, d, *J* 10.0, $CHCHCH_{trans}H_{cis}$), 5.22 (1 H, d, *J* 17.0, $CHCHCH_{trans}H_{cis}$), 6.37 (1 H, d, *J* 11.0, $CHCHCH_2$), 6.68 (1 H, dt, *J* 17.0, *J* 10.5, $CHCHCH_2$) and 7.14–7.26 (5 H, m, Ar-H); δ_C (100 MHz, C^2HCl_3) 16.07 (CH_3CPh), 117.65 ($CHCHCH_2$), 127.76 ($CHCHCH_2$), 125.74, 127.22 and 128.33 (Ar-CH), 133.60 ($CHCHCH_2$) and 136.77 and 143.03 (quaternary C); *m/z* (EI^+) 144.1 (35%, M^+) and 129.1 (100, $[M - CH_3]^+$).

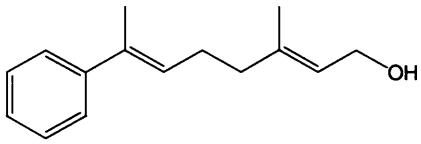
(2*E*,6*E*)-Ethyl 3-methyl-7-phenylocta-2,6-dienoate (277).



This compound was prepared from **275** in a manner identical to that for the ester **177**; purification

by silica column chromatography using hexane and ethyl acetate (2: 1) as eluent gave **277** as a light yellow oil (1.51 g, 59%); R_f 0.27 (Hexane : EtOAc = 19 : 1); HRMS: (ES^+, $[M + NH_4]^+$) found 276.1959. $C_{17}H_{26}O_2N$ requires 276.1958; υ_{max}(thin film)/cm^{-1} 2978.8, 2930.2, 1714.9, 1647.6, 1493.8, 1444.3, 1381.4, 1327.6, 1272.3, 1222.8, 1146.1, 1098.9, 1049.6, 864.4, 757.9 and 696.4; δ_H (500 MHz, C^2HCl_3) 1.20 (3 H, t, *J* 7.0, CH_3CH_2O), 1.96 (3 H, d, *J* 1.0, $CH_3C{=}CHCO_2Et$), 2.13 (3 H, d, *J* 1.0, CH_3CPh), 2.19–2.32 (4 H, m, CH_2CH_2), 4.07 (2 H, q, *J* 7.0, CH_3CH_2O), 5.64 (2 H, m, 2 × C=C*H*) and 7.13–7.30 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 14.37 (CH_3CH_2O), 15.92 (CH_3CCHCO), 18.87 (CH_3CPh), 26.76 and 40.62 (CH_2CH_2), 59.57 (CH_3CH_2O), 115.93 (*C*HCO), 126.66 (PhC=*C*H), 125.68, 126.73 and 128.22 (Ar-CH), 135.78, 144.67 and 159.32 (quaternary C) and 166.86 (C=O); *m/z* (CI^+) 276.2 (100%, $[M + NH_4]^+$).

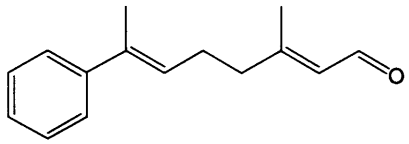
(2*E*,6*E*)-3-Methyl-7-phenylocta-2,6-dien-1-ol (280).



This compound was prepared from **277** in a manner identical to that for the alcohol **175**; purification by silica column chromatography using hexane and ethyl acetate (2: 1) as eluent gave **280** as a light yellow oil (1.2 g, 96%); R_f 0.27 (Hexane : EtOAc = 2 : 1); HRMS: (ES^+, $[M + NH_4]^+$) found 234.1851. $C_{15}H_{24}ON$ requires 234.1852; υ_{max}(thin film)/cm^{-1} 3363.0, 2923.4, 1493.3, 1444.0, 999.4, 756.3 and 695.8; δ_H (500 MHz, C^2HCl_3) 1.65 (3 H, s, $CH_3C{=}CHCH_2OH$), 1.96 (3 H, d, *J* 0.9, CH_3CPh), 2.09–2.29 (4 H, m, CH_2CH_2), 4.09 (2 H, d, *J* 10.0, CH_2OH), 5.40 (1 H, tq, *J* 10.0, *J* 1.5, C*H*CH_2OH), 5.68 (1 H, tq, *J* 7.0, *J* 1.5, C*H*CH_2CH_2) and 7.13–7.31 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 15.87 (CH_3CCH_2OH), 16.36 (CH_3CPh), 27.07 and 39.22

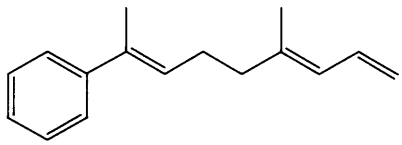
(CH_2CH_2), 59.44 (CH_2OH), 123.70 ($CHCH_2OH$), 127.68 (PhC*C*H), 125.62, 126.59 and 128.20 (Ar-CH), 135.03, 139.44 and 143.85 (quaternary C); *m/z* (CI^+) 234.2 (100%, $[M + NH_4]^+$).

(2*E*,6*E*)-3-methyl-7-phenylocta-2,6-dienal (281).



This compound was prepared from **280** in a manner identical to that for the compound **181**; purification by silica column chromatography using hexane and ethyl acetate (2: 1) as eluent gave **281** as a light yellow oil (0.86 g, 89%); R_f 0.33 (Hexane : EtOAc = 4 : 1); HRMS: (EI^+, M^+) found 214.1350. $C_{15}H_{18}O$ requires 214.1352; υ_{max}(thin film)/cm^{-1} 1672.0, 1493.5, 1443.9, 1193.1, 1124.0, 757.7 and 696.2; δ_H (500 MHz; $C_6{}^2H_6$) 1.62 (3 H, d, *J* 1.1, CH_3C=CHCHO), 1.92 (3 H, d, *J* 1.0, CH_3CPh), 1.86–2.10 (4 H, m, CH_2CH_2), 5.64 (1 H, tq, *J* 7.0, *J* 1.0, C=C*H*CH_2CH_2), 5.95 (1 H, dq, *J* 7.5, *J* 1.0, C*H*CHO), 7.21–7.44 (5 H, m, Ar-H) and 9.98 (1 H, d, *J* 7.5, CHO); δ_C (125 MHz; d^6-benzene) 15.69 (CH_3CPh), 16.72 (CH_3C=CHCHO), 26.33 and 39.75 (CH_2CH_2), 126.32 (PhC=*C*H), 127.50 (*C*HCHO), 125.81, 126.96 and 128.37 (Ar-CH), 136.00, 143.75 and 160.88 (quaternary C) and 189.61 (CHO); *m/z* (CI^+) 232.2 (100%, $[M + NH_4]^+$).

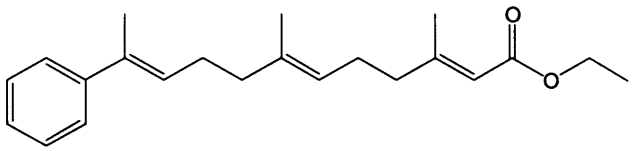
(2*E*,7*E*)-8-Phenyl-4-methyl-nona-1,3,7-triene (278).



This compound was prepared from **281** in a manner identical to that for the **172**; purification by silica column chromatography using hexane and ethyl acetate (2 : 1) as eluent gave **278** as a light

yellow oil (0.68 g, 81%); R_f 0.68 (Hexane : EtOAc = 9 : 1); HRMS: (EI^+, M^+) found 212.1559. $C_{16}H_{20}$ requires 212.1560; υ_{max}(thin film)/cm^{-1} 2919.4, 1649.7, 1597.7, 1493.5, 1443.8, 1379.7, 987.8, 896.6, 756.3 and 695.3; δ_H (500 MHz, C^2HCl_3) 1.73 (3 H, s, CH_3C=CHCH=CH_2), 1.92 (3 H, s, CH_3CPh), 2.11–2.29 (4 H, m, CH_2CH_2), 4.92 (1 H, d, *J* 10.0, CH=CHCH$_{trans}H_{cis}$), 5.02 (1 H, dd, *J* 17.0, *J* 1.5, CH=CH$_{trans}$H$_{cis}$), 5.68 (1 H, t, *J* 7.0, C=C*H*CH_2CH_2), 5.82(1 H, d, *J* 11.0, C*H*CH=CH_2), 6.52 (1 H, dt, *J* 17.0, *J* 10.5, C*H*=CH_2) and 7.12–7.30 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 15.88 (CH_3CPh), 16.76 (CH_3C=CHCH_2CH_2), 27.26 and 39.55 (CH_2CH_2), 114.91 (CH=CH_2), 125.79 (CHCH=CH_2), 127.76 (PhC=CH), 125.66, 126.57 and 128.19 (Ar-CH), 133.37 (CH=CH_2) and 135.03, 139.13 and 143.92 (quaternary C); *m/z* (CI^+) 213.1 (100%, [M + H]$^+$).

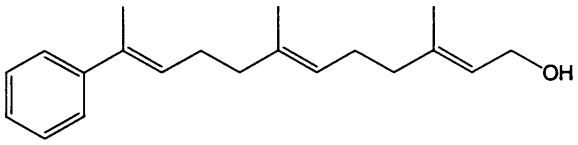
(2*E*,6*E*,10*E*)-Ethyl 3,7-dimethyl-11-phenyldodeca-2,6,10-trienoate (279)



This compound was prepared from **278** in a manner identical to that for the ester **177**; purification by silica column chromatography using hexane and ethyl acetate (2 : 1) as eluent to gave **279** as a light yellow oil (0.56 g, 54%); R_f 0.32 (Hexane : EtOAc = 25 : 1); HRMS (ES^+, [M + H]$^+$) found 327.2320. $C_{22}H_{31}O_2$ requires 327.2319; υ_{max}(thin film)/cm^{-1} 2928.8, 1714.9, 1647.2, 1444.1, 1381.4, 1221.1, 1143.0, 757.1 and 695.9; δ_H (500 MHz, C^2HCl_3) 1.21 (3 H, t, *J* 6.5, CH_3CH_2O), 1.50, 1.58 and 1.96 (9 H, s, 3 × CH_3C), 2.02–2.24 (8 H, m, 2 × CH_2CH_2), 4.07 (2 H, q, *J* 6.5, CH_3CH_2O), 5.08 and 5.67 (2 H, m, 2 × C=C*H*CH_2CH_2), 5.60 (1 H, d, *J* 1.0, C*H*CO_2Et) and 7.13–7.30 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 14.37 (CH_3CH_2O), 15.83, 16.09 and 18.87 (3 ×

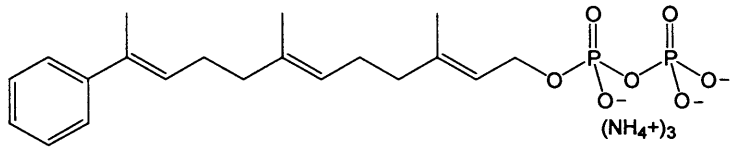
CH_3), 25.99, 27.40, 39.35 and 40.95 (2 × CH_2CH_2), 59.51 (CH_3CH_2O), 115.62 ($CHCO_2Et$), 123.33 and 128.08 (C=CH), 125.59, 126.96 and 128.17 (Ar-CH), 131.56, 135.84, 143.95 and 159.81 (quaternary C) and 166.94 (C=O); *m/z* (CI^+) 344.3 (100%, $[M + NH_4]^+$) and 327.3 (65, $[M + H]^+$).

(2*E*,6*E*,10*E*)-3,7-Dimethyl-11-phenyldodeca-2,6,10-trien-1-ol (282)



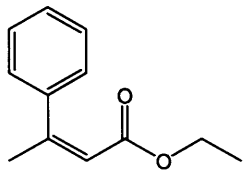
This compound was prepared from **279** in a manner identical to that for the alcohol **175**; purification by silica column chromatography using hexane and ethyl acetate (2 : 1) as eluent gave **282** as a light yellow oil (0.41 g, 86%); R_f 0.31 (Hexane : EtOAc = 2 : 1); HRMS (EI^+, M^+) found 284.2141. $C_{20}H_{28}O$ requires 284.2140; υ_{max}(thin film)/cm^{-1} 3321.5, 2921.0, 1666.9, 1597.7, 1493.7, 1444.1, 1381.1, 1000.4, 846.3, 756.6 and 696.1; δ_H (500 MHz, C^2HCl_3) 1.57 (3 H, s, CH_3CCH), 1.60 (3 H, s, CH_3C=CHCH$_2$OH), 1.96 (3 H, d, *J* 1.0, PhCCH_3), 1.97-2.25 (8 H, m, 2 × CH_2CH_2), 4.04 (2 H, d, *J* 7.0, CH_2OH), 5.08 (1 H, dt, *J* 1.0, *J* 7.0, CH_3C=CH), 5.33 (1 H, m, C=CHCH$_2$OH), 5.68 (1 H, dt, *J* 1.5, *J* 7.0, PhC=CH) and 7.12–7.30 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 15.84, 16.06 and 16.32 (3 × CH_3), 26.29, 27.37, 39.36 and 39.53 (2 × CH_2CH_2), 59.41 (CH_2OH), 123.39 (CHCH$_2$OH), 124.25 (CH_3C=CH) and 128.16 (PhC=CH), 125.61, 126.49 and 128.18 (Ar-CH), 134.63, 135.01, 139.71 and 144.01 (quaternary C); *m/z* (EI+) 284.2 (1%, M^+), 266.2 (2, $[M - H_2O]^+$) and 131.1 (100).

(2*E*,6*E*,10*E*)-3,7-Dimethyl-11-phenyldodeca-2,6,10-trien-1-yl diphosphate tris-ammonium salt (160)



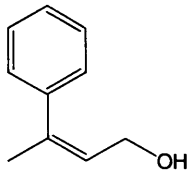
This compound was prepared from alcohol **282** and purified in a manner identical to that for the diphosphate **154** to give the title compound **160** as a white solid (0.12 g, 31%); HPLC t_R 39.28 min, Purity 94.6% by analytical RP HPLC under 220 nm; HRMS (ES^-, $[M - H]^-$) found 443.1410. $C_{20}H_{29}O_7P_2$ requires 443.1389; υ_{max}(KBr disc)/cm^{-1} 2922.8, 2190.6, 1668.4, 1493.0, 1444.1, 1381.1, 1201.3, 1092.4, 1024.6, 906.9, 798.2, 757.3, 722.5 and 696.3; δ_H (500 MHz; ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 1.46 (3 H, s, C*H*$_3$CCH), 1.59 (3 H, s, C*H*$_3$C=CHCH$_2$O), 1.76 (3 H, s, PhCCH$_3$), 1.89-2.10 (8 H, m, 2 × C*H*$_2$C*H*$_2$), 4.37 (2 H, t, *J* 5.5, CHC*H*$_2$O), 5.03 (1 H, t, *J* 6.5, CH$_3$CC*H*), 5.33 (1 H, t, *J* 7.0, C=C*H*CH$_2$O), 5.68 (1 H, t, *J* 6.5, PhC=C*H*) and 6.98–7.16 (5 H, m, Ar-H); δ_C (125 MHz; ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 15.11 (PhC*C*H$_3$), 15.41 (*C*H$_3$CCH), 15.78 (*C*H$_3$C=CHCH$_2$O), 26.02, 26.90, 38.85 and 39.12 (2 × *C*H$_2$*C*H$_2$), 62.37 (d, *J* 5.0, CH$_2$O), 119.68 (d, *J* 7.5, *C*HCH$_2$O), 124.36 (CH$_3$*C*CH), 127.96 (PhC*C*H), 125.31, 126.55 and 128.16 (Ar-CH), and 134.58, 135.49, 142.43 and 143.34 (quaternary C); δ_P (122 MHz; ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) –6.58 (1 P, d, J_{PP} 22.0) and –10.34 (1 P, d, J_{PP} 22.0); *m/z* (ES^-) 443.1 (100%, $[M - H]^-$).

(*Z*)-Ethyl 3-phenylbut-2-enoate (285)



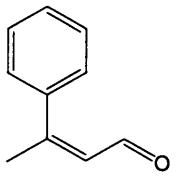
To a stirred solution of the iodide **179** (7.20 g, 30.0 mmol) in anhydrous toluene (100 cm^3) under nitrogen atmosphere was added palladium (II) acetate (0.34 g, 1.50 mmol), triphenylarsine (0.79 g, 3.0 mmol), tripotassium orthophosphate (19.1 g, 90.0 mmol) and phenylboronic acid (5.49 g, 45.0 mmol). The complete reaction mixture was then stirred at 90 °C for 6 h. Water (50 cm^3) and diethyl ether (50 cm^3) were added, and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 30 cm^3). The combined ethereal extracts were washed with water (2 × 30 cm^3) and brine (30 cm^3), dried over $MgSO_4$, filtered and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (9 : 1) as eluent gave **285** as a light yellow oil (2.49 g, 85%); R_f 0.30 (Hexane : EtOAc = 9 : 1); HRMS (ES^+, $[M + H]^+$) found 191.1067. $C_{12}H_{15}O_2$ requires 191.1067; υ_{max} (thin film)/cm^{-1} 2979.6, 1725.4, 1639.5, 1492.5, 1442.6, 1374.8, 1277.2, 1230.2, 1162.1, 1095.6, 1076.6, 1047.3, 867.6, 768.4 and 698.1; δ_H (500 MHz, C^2HCl_3) 1.14 (3 H, t, *J* 7.5, CH_2CH_3), 2.23 (3 H, d, *J* 1.5, CH_3CPh), 4.05 (2 H, q, *J* 7.5, CH_2CH_3), 6.05 (1 H, q, *J* 1.5, PhC=C*H*) and 7.26–7.42 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 14.02 (CH_2CH_3), 27.19 (CH_3CPh), 59.77 (CH_2CH_3), 117.84 (PhC=*C*H), 126.88, 127.77 and 127.94 (Ar-CH), 140.91 and 155.41 (quaternary C) and 165.92 (C=O); *m/z* (CI^+) 208.1 (100%, $[M + NH_4]^+$) and 191.0 (35, $[M + H]^+$).

(*Z*)-3-Phenylbut-2-enol (286)



This compound was prepared from **285** in a manner identical to that for the alcohol **175**; purification by silica column chromatography using hexane and ethyl acetate (2 : 1) as eluent gave **286** as a light yellow oil (4.12 g, 99%); R_f 0.26 (Hexane : EtOAc = 2 : 1); HRMS (CI^+, $[M + NH_4]^+$) found 166.1229. $C_{10}H_{16}ON$ requires 166.1226; υ_{max} (thin film)/cm^{-1} 3331.7, 3055.2, 2969.9, 1656.1, 1600.0, 1493.6, 1434.8, 1376.0, 1246.4, 1065.2, 1002.0, 764.1 and 700.8; δ_H (500 MHz, C^2HCl_3) 1.46 (1 H, b, OH), 2.02 (3 H, d, *J* 1.0, CH_3CPh), 3.99 (2 H, dd, *J* 7.0, *J* 1.0, CH_2OH), 5.64 (1 H, tq, *J* 7.0, *J* 1.5, PhC=C*H*) and 7.09–7.28 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 25.37 (CH_3CPh), 60.31 (CH_2OH), 126.09 (PhC=*C*H), 127.20, 127.78 and 128.17 (Ar-CH) and 140.26 and 140.78 (quaternary C); *m/z* (CI^+) 166.1 (20%, $[M + NH_4]^+$), 148.1 (50, M^+) and 131.0 (100, $[M - OH]^+$),.

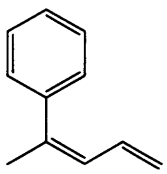
(*Z*)-3-Phenylbut-2-enal (287)



To a stirred solution of oxalyl chloride (2.69 cm^3, 31.3 mmol) in anhydrous CH_2Cl_2 (80 cm^3) at –78 °C under nitrogen atmosphere, was added anhydrous dimethylsulfoxide (4.44 cm^3, 62.6 mmol). The reaction mixture was stirred for 5 min then a solution of alcohol **286** (3.85 g, 26.1 mmol) in CH_2Cl_2 (5.0 cm^3) was added over 5 min and stirring continued for an additional 15 min.

Triethylamine (18.2 cm^3, 130 mmol) was added and the reaction mixture was stirred for 5 min and then allowed to warm to room temperature. Water (50 cm^3) was then added, and the organic layer separated. The aqueous layer was extracted with CH_2Cl_2 (2 × 30 cm^3). The combined organic extracts were washed with water (2 × 30 cm^3) and brine (1 × 30 cm^3), dried over $MgSO_4$, filtered and then concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (2 : 1) as eluent gave **287** as a light yellow oil (3.36 g, 88%); R_f 0.48 (Hexane : EtOAc = 2 : 1); HRMS (ES^+, $[M - H]^+$) found 145.0645. $C_{10}H_9O$ requires 145.0648; υ_{max} (thin film)/cm^{-1} 2357.8, 1668.3, 1614.1, 1433.3, 1388.2, 1136.6, 767.0 and 701.5; δ_H (500 MHz, C^2HCl_3) 1.81 (3 H, s, CH_3CPh), 6.09 (1 H, d, *J* 8.0, PhC=C*H*), 6.95–7.13 (5 H, m, Ar-H) and 9.71 (1 H, dd, *J* 8.0, *J* 3.5, CHO); δ_C (125 MHz, C^2HCl_3) 25.62 (CH_3CPh), 128.27, 128.35 and 128.69 (Ar-CH), 129.40 (PhC*C*H), 138.53 and 160.14 (quaternary C) and 191.63 (CHO); *m/z* (CI^+), 164.1 (95%, $[M + NH_4]^+$), 161.1 (100%) and 146.1 (30, M^+).

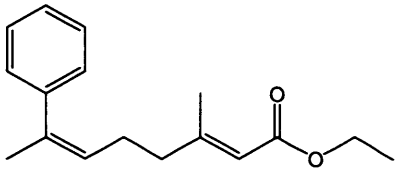
(*Z*)-4-Phenyl-penta-1,3-diene (284)



This compound was prepared from aldehyde **287** in a manner identical to that for the triene **172**; purification by flash chromatography using hexane and ethyl acetate (2 : 1) as eluent gave **284** as a light yellow oil (1.80 g, 76%); R_f 0.63 (Hexane : EtOAc = 9 : 1); HRMS (EI^+, M^+) found 144.0938. $C_{11}H_{12}$ requires 144.0939; υ_{max} (thin film)/cm^{-1} 3080.3, 2960.4, 2856.8, 1805.6, 1636.2, 1601.6, 1492.1, 1433.6, 1414.1, 1375.3, 1024.7, 995.6, 898.4, 766.1 and 700.2; δ_H (400 MHz,

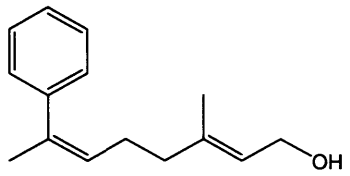
C^2HCl_3) 2.04 (3 H, s, CH_3CPh), 4.86 (1 H, d, *J* 10.0, $CHCH{=}CH_{trans}H_{cis}$), 5.08 (1 H, d, *J* 17.0, $CHCH{=}CH_{trans}H_{cis}$), 6.06 (1 H, d, *J* 11.0, $CHCH{=}CH_2$), 6.32 (1 H, dt, *J* 17.0, *J* 10.5, $CHCH{=}CH_2$) and 7.14–7.28 (5 H, m, Ar-H); δ_C (100 MHz, C^2HCl_3) 25.48 (CH_3CPh), 116.05 ($CHCH{=}CH_2$), 127.94 ($CHCH{=}CH_2$), 127.05, 128.13 and 128.29 (Ar-CH), 134.57 ($CHCHCH_2$) and 139.45 and 141.47 (quaternary C); *m/z* (EI^+) 144.1 (35%, M^+) and 129.1 (100, $[M - CH_3]^+$).

(2*E*,6*Z*)-Ethyl 3-methyl-7-phenylocta-2,6-dienoate (288)



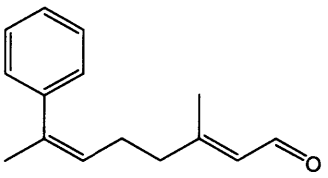
This compound was prepared from diene **284** in a manner identical to that for the ester **177**; the crude product was purified by flash chromatography using hexane and ethyl acetate (2 : 1) as eluent to give **288** as a light yellow oil (1.33 g, 41%); R_f 0.35 (Hexane : EtOAc = 9 : 1); HRMS (ES^+, $[M + H]^+$) found 259.1691. $C_{17}H_{23}O_2$ requires 259.1693; υ_{max} (thin film)/cm^{-1} 2975.8, 2359.6, 1715.0, 1647.3, 1493.4, 1442.0, 1367.2, 1221.8, 1146.1, 1099.6, 1052.2, 865.4, 763.2 and 701.0; δ_H (500 MHz, C^2HCl_3) 1.18 (3 H, t, *J* 7.0, CH_2CH_3), 1.95 (3 H, s, $CH_3C{=}CHCO_2Et$), 1.98 (3 H, d, *J* 1.0, CH_3CPh), 2.07 (4 H, m, CH_2CH_2), 4.07 (2 H, q, *J* 7.0, CH_2CH_3), 5.32 (1 H, m, $CHCH_2CH_2$), 5.52 (1 H, d, *J* 0.5, $CHCO_2$) and 7.07–7.30 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 14.36 (CH_2CH_3), 18.74 (CH_3CPh), 25.65 (CH_3CCHCO), 26.93 and 41.15 (CH_2CH_2), 59.50 (CH_2CH_3), 115.72 ($C{=}CHCO_2Et$), 125.84 ($PhC{=}CH$), 126.64, 127.85 and 128.16 (Ar-CH), 137.46, 141.83 and 159.38 (quaternary C) and 166.85 (C=O); *m/z* (CI^+) 276.2 (100%, $[M + NH_4]^+$) and 259.2 (95, $[M + H]^+$).

(2*E*,6*Z*)-3-Methyl-7-phenylocta-2,6-dien-1-ol (289)



This compound was prepared from ester **288** in a manner identical to that for the alcohol **175**; purification by flash chromatography on silica gel using hexane and ethyl acetate (2 : 1) as eluent gave **289** light yellow oil (0.816 g, 91%); R_f 0.31 (Hexane : EtOAc = 2 : 1); HRMS (ES^+, $[M + NH_4]^+$) found 234.1850. $C_{15}H_{24}ON$ requires 234.1852; υ_{max} (thin film)/cm^{-1} 3335.5, 2965.2, 2914.6, 1667.9, 1492.8, 1436.1, 1376.6, 999.9, 763.1 and 700.1; δ_H (500 MHz, C^2HCl_3) 1.22 (1 H, b, OH), 1.50 (3 H, s, CH_3C=$CHCH_2OH$), 1.95 (3 H, d, J = 1.14, CH_3CPh), 1.97–2.04 (4 H, m, CH_2CH_2), 4.04 (2 H, d, J 7.0, CH_2OH), 5.28 (1 H, dt, J 7.0, J 1.0, C=$CHCH_2OH$), 5.36 (1 H, dt, J 7.0, J 1.5, C=$CHCH_2CH_2$) and 7.10–7.28 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 16.24 (CH_3C=$CHCH_2OH$), 25.60 (CH_3CPh), 27.32 and 39.79 (CH_2CH_2), 59.40 (CH_2OH), 123.45 ($CHCH_2OH$), 126.82 (PhC=CH), 126.51, 127.91 and 128.08 (Ar-CH) and 136.60, 139.45 and 142.05 (quaternary C); *m/z* (CI^+) 234.2 (100%, $[M + NH_4]^+$), 216.2 (80, M^+) and 199.1 (40, $[M - OH]^+$).

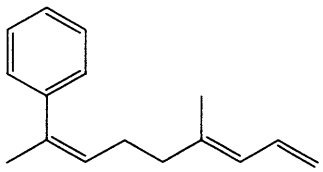
(2*E*,6*Z*)-3-Methyl-7-phenylocta-2,6-dienal (290)



This compound was prepared from alcohol **289** in a manner identical to that for the compound **181**; purification by flash chromatography using hexane and ethyl acetate (2 : 1) as eluent to gave

290 as a light yellow oil (0.86 g, 87%); R_f 0.45 (Hexane : EtOAc = 2 : 1); HRMS (EI^+, M^+) found 214.1352. $C_{15}H_{18}O$ requires 214.1358; υ_{max} (thin film)/cm^{-1} 2912.7, 2851.7, 2360.2, 1672.8, 1630.3, 1492.6, 1439.9, 1379.2, 1193.2, 1124.0, 1025.8, 831.0, 764.1, 702.0 and 668.1; δ_H (500 MHz, C^2HCl_3) 1.49 (3 H, d, *J* 1.0, CH_3C=CHCHO), 1.85–2.08 (4 H, m, CH_2CH_2), 2.03 (3 H, d, *J* 1.0, CH_3CPh), 5.30 (1 H, td, *J* 7.0, *J* 1.0, C*H*CH_2CH_2), 5.85 (1 H, dd, *J* 8.0, *J* 1.0, C*H*CHO), 7.17–7.29 (5 H, m, Ar-H) and 9.91 (1 H, d, *J* 8.0, CHO); δ_C (125 MHz, C^2HCl_3) 16.42 (CH_3CCHCHO), 25.50 (CH_3CPh), 26.66 and 40.42 (CH_2CH_2), 125.95 (PhC=*C*H), 127.57 (*C*HCHO), 126.88, 127.92 and 128.31 (Ar-CH), 137.62, 141.80 and 160.81 (quaternary C) and 189.58 (CHO); ***m/z*** (EI^+) 214.1 (2%, M^+) and 131.1 (100).

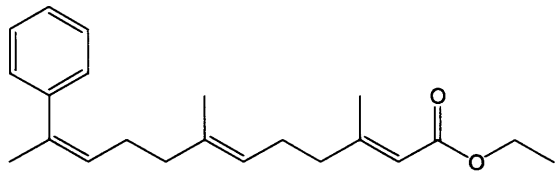
(3*E*,7*Z*)-4-Methyl-8-phenyl-nona-1,3,7-triene (291)



This compound was prepared from aldrhyde **290** in a manner identical to that for the triene **172**; purification by flash chromatography using hexane and ethyl acetate (2 : 1) as eluent gave **291** as a light yellow oil (0.48 g, 86%); R_f 0.62 (Hexane : EtOAc = 9 : 1); HRMS (ES^+, $[M + H]^+$) found 212.1569. $C_{16}H_{20}$ requires 212.1565; υ_{max} (thin film)/cm^{-1} 2964.8, 2358.3, 1649.8, 1598.9, 1493.0, 1436.5, 1378.1, 985.7, 896.9, 761.8 and 700.0; δ_H (500 MHz, C^2HCl_3) 1.70 (3 H, s, CH_3C=CHCH), 2.07 (3 H, s, CH_3CPh), 2.08–2.17 (4 H, m, CH_2CH_2), 5.00 (1 H, dd, *J* 10.0, *J* 1.5, CH=C$H_{cis}H_{trans}$), 5.10 (1 H, dd, *J* 17.0, *J* 2.0, CH=C$H_{cis}H_{trans}$), 5.46 (1 H, dt, *J* 7.0, *J* 1.5, C=C*H*CH_2CH_2), 5.83 (1 H, dd, *J* 11.0, *J* 0.5, C*H*$CH=CH_2$), 6.59 (1 H, dt, *J* 17.0, *J* 10.5, CHC*H*$=CH_2$) and 7.21–7.39 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 16.64 ($CH_3C=CHCH_2$),

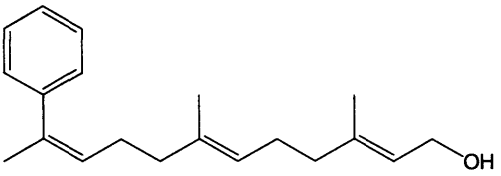
25.63 (CH_3CPh), 27.40 and 40.11 (CH_2CH_2), 114.70 (C=$CHCH_2$), 125.57 ($CHCH=CH_2$), 126.91 (PhC=CH), 126.50, 127.96 and 128.19 (Ar-CH), 133.38 ($CH=CH_2$) and 136.59, 139.14 and 142.09 (quaternary C); *m/z* (EI^+) 212.2 (5%, M^+), 131.1 (100) and 91.1 (40).

(2*E*,6*E*,10*Z*)-Ethyl 3,7-dimethyl-11-phenyldodeca-2,6,10-trienoate (292)



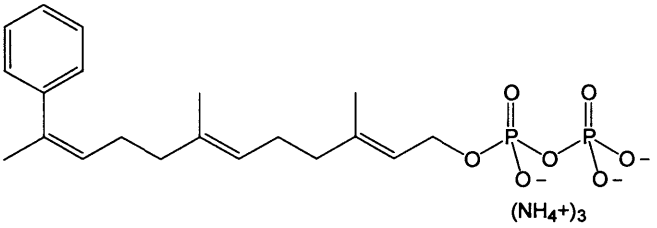
This compound was prepared from **291** in a manner identical to that for the ester **177**; purification by flash chromatography using hexane and ethyl acetate (2 : 1) gave **292** as a light yellow oil (0.29 g, 40%); R_f 0.33 (Hexane : EtOAc = 19 : 1); HRMS (ES^+, $[M + H]^+$) found 327.2318. $C_{22}H_{31}O_2$ requires 327.2319; υ_{max} (thin film)/cm^{-1} 2926.3, 1715.4, 1647.5, 1442.7, 1366.5, 1221.5, 1144.3, 1053.9, 865.3, 762.0 and 700.5; δ_H (500 MHz, C^2HCl_3) 1.20 (3 H, t, *J* 7.0, OCH_2CH_3), 1.43 (3 H, s, $CH_3C=CH$), 1.95 (3 H, d, *J* 1.0, $CH_3C=CH$), 2.08 (3 H, d, *J* 1.0, $CH_3C=CHCO_2Et$), 1.91–2.11 (8 H, m, 2 × CH_2CH_2), 4.06 (2 H, q, *J* 7.0, OCH_2CH_3), 4.97 (1 H, b, $C=CHCH_2CH_2$), 5.35 (1 H, dt, *J* 7.0, *J* 1.0, $C=CHCH_2CH_2$), 5.58 (1 H, s, $CHCO_2Et$) and 7.09–7.30 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 14.37 (OCH_2CH_3), 15.95 ($CH_3C=CH$), 18.85 ($CH_3C=CHCO_2Et$), 25.57 ($CH_3C=CH$), 25.96, 27.52, 39.92 and 40.95 (2 × CH_2CH_2), 59.50 (OCH_2CH_3), 115.59 ($CHCO_2Et$), 123.12 and 127.20 (2 × $C=CHCH_2CH_2$), 126.43, 127.59 and 128.03 (Ar-CH), 135.74, 136.20, 142.14 and 159.87 (quaternary C) and 166.93 (C=O); *m/z* (CI^+) 344.3 (100%, $[M + NH_4]^+$) and 327.3 (50, $[M + H]^+$).

(2*E*,6*E*,10*Z*)-3,7-dimethyl-11-phenyldodeca-2,6,10-trien-1-ol (293)



This compound was prepared from **292** in a manner identical to that for the alcohol **175**; purification by silica column chromatography using hexane and ethyl acetate (2: 1) as eluent gave alcohol **293** as a light yellow oil (0.12 g, 77%); R_f 0.26 (Hexane : EtOAc = 2 : 1); HRMS (ES^+, $[M + NH_4]^+$) found 302.2477. $C_{20}H_{32}ON$ requires 302.2478; υ_{max} (thin film)/cm^{-1} 3344.4, 2919.3, 1666.0, 1597.4, 1493.4, 1443.8, 1381.2, 998.9, 756.6 and 696.0; δ_H (500 MHz, C^2HCl_3) 1.23 (1 H, b, OH), 1.43 (3 H, s, CH_3), 1.60 (3 H, s, CH_3), 1.95 (3 H, d, *J* 1.0, CH_3), 1.91–2.06 (8 H, m, 2 × CH_2CH_2), 4.07 (2 H, d, *J* 7.0, CH_2OH), 4.99 (1 H, td, *J* 7.0, *J* 1.0, C=CH), 5.33 (2 H, m, 2 × C=CH) and 7.10–7.30 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 15.97, 16.31 and 25.58 (3 × CH_3), 26.26, 27.64, 39.54 and 39.97 (2 × CH_2CH_2), 59.42 (CH_2OH), 123.32, 123.98 and 127.33 (3 × C=CH), 126.42, 127.96 and 128.02 (Ar-CH) and 135.01, 136.11, 139.83 and 142.17 (quaternary C); *m/z* (CI^+) 302.3 (100%, $[M + NH_4]^+$) and 284.3 (55, M^+).

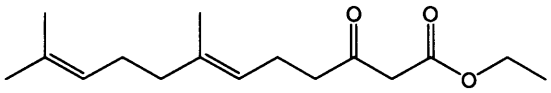
(2*E*,6*E*,10*Z*)-3,7-Dimethyl-11-phenyldodeca-2,6,10-trien-1-yl diphosphate tris- ammonium salt (161)



This compound was prepared from alcohol **293** and purified in a manner identical to that for the diphosphate **154** to give **161** as a white solid (63.8 mg, 36%); HPLC t_R = 38.82 min; Purity

97.53% by analytical RP HPLC under 220 nm; HRMS (ES^-, $[M - H]^-$) found 443.1374, $C_{20}H_{29}O_7P_2$ requires 443.1389; υ_{max} (KBr disc)/cm^{-1} 3292.3, 1494.2, 1457.3, 1409.4, 1202.2, 1122.0, 1089.8, 1024.0, 910.5, 757.0, 723.3 and 697.3; δ_H (500 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 1.34 (3 H, s, CH_3), 1.59 (3 H, s, CH_3), 1.85 (3 H, s, CH_3), 1.88–2.03 (8 H, m, 2 x CH_2CH_2), 4.39 (2 H, b, $CHCH_2OH$), 4.98 (1 H, b, C=CH), 5.33 (2 H, bd, 2 × C=CH) and 7.06–7.18 (5 H, m, Ar-CH); δ_C (125 MHz; ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 15.28, 15.69 and 24.86 (3 × CH_3), 25.87, 227.12, 39.04 and 39.33 (2 × CH_2CH_2), 62.60 (CCH_2OH), 119.55, 124.21 and 127.26 (3 × C=CH), 126.54, 127.75 and 128.03 (Ar-CH) and 135.39, 136.27, 141.76 and 142.57 (quaternary C); δ_P (122 MHz; ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) –6.51 (1 P, d, J_{PP} 21.0) and –10.33 (1 P, d, $J_{P\text{-}P}$ 21.0); *m/z* (ES^-) 443.1 (100%, $[M - H]^-$).

(*E*)-Ethyl 7,11-dimethyl-3-oxododeca-6,10-dienoate (303)

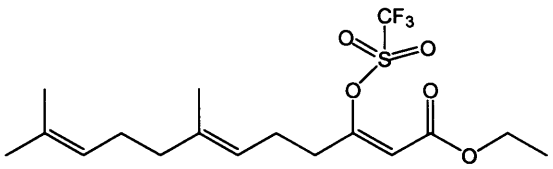


A stirred solution of geraniol (2.60 cm^3 g, 15.0 mmol) and triethylamine (4.20 cm^3, 30.0 mmol) in anhydrous THF (100 cm^3) was cooled to – 45 °C then methanesulfonyl chloride (1.50 cm^3, 19.5 mmol) was added. The resulting milky mixture was stirred at – 45 °C for 45 min. A solution of lithium bromide (5.20 g, 60.0 mmol) in THF (10 cm^3) was then added via a cannula at – 45 °C. The suspension was allowed to warm to 0 °C and stirred for an additional 1 h. Cold water (30 cm^3) and hexane (30 cm^3) were added to quench the reaction. The two layers were separated, and the aqueous layer was extracted with hexane (2 × 20 cm^3). The combined organic layers were washed with saturated $NaHCO_3$ solution (20 cm^3) and then brine (20 cm^3), dried over $NaSO_4$ and filtered. Concentration under reduced pressure gave the intermediate bromide **264** as a light

yellow oil which was used without further purification.

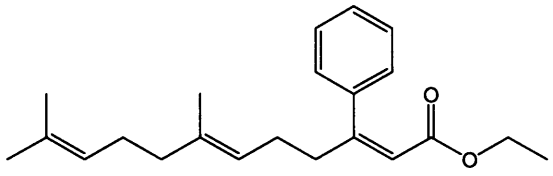
To a stirred suspension of NaH (60% dispersion in mineral oil, 1.20 g, 49.5 mmol) in anhydrous THF (100 cm^3) was added ethyl acetoacetate (5.73 cm^3, 45.0 mmol) dropwise at 0 °C. After 10 min, n-BuLi (2.2 M, 21.5 cm^3, 47.3 mmol) was added slowly over 3 min, during which time the colourless solution gradually turned yellow. This was stirred for additional 10 min at 0 °C, as a solution of the bromide **264** in THF (5 cm^3) was added. The clear solution turned to a cloudy yellow suspension. After stirring for 30 min at 0 °C, hydrochloric acid (3 M, 10.0 cm^3) was added followed by water (30 cm^3) and diethyl ether (30 cm^3) and then the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 20 cm^3). The combined ethereal extracts were washed with water (2 × 20 cm^3) and brine (20 cm^3), dried over $MgSO_4$ then filtered and concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (4 : 1) as eluent gave β-keto ester **303** as a pale yellow oil (3.61g, 92%); R_f 0.45 (Hexane : EtOAc = 2 : 1); HRMS (ES^+, $[M + H]^+$) found 267.1954. $C_{16}H_{27}O_3$ requires 267.1954; υ_{max} (thin film)/cm^{-1} 2968.0, 2918.6, 1746.5, 1717.2, 1648.9, 1445.8, 1409.9, 1367.4, 1313.3, 1235.8, 1177.2, 1035.9 and 839.8; δ_H (500 MHz, C^2HCl_3) 1.30 (3 H, t, *J* 7.0, CH_2CH_3), 1.61 (3 H, s, CH_3), 1.63 (3 H, s, CH_3), 1.69 (3 H, s, CH_3), 1.98 (4 H, m, $(CH_3)_2C{=}CHCH_2CH_2$), 2.30 (2 H, q, *J* 7.5, $CH_2CH_2C{=}O$), 2.59 (2 H, t, *J* 7.5, $CH_2CH_2C{=}O$), 3.45 (2 H, s, $COCH_2CO$), 4.20 (2 H, q, *J*7.0, CH_2CH_3) and 5.09 (2 H, dt, *J* 1.0, *J*7.0, 2 × C=CH); δ_C (125 MHz, C^2HCl_3) 14.13 (CH_2CH_3), 16.01 (CH_3), 17.71 (CH_3), 22.15 ($CH_2CH_2C{=}O$), 25.72 (CH_3), 26.60 and 39.35 [$(CH_3)_2C{=}CHCH_2CH_2$], 43.08 (CH_2CH_2CO), 49.42 ($COCH_2CO$), 61.38 (CH_2CH_3), 122.06 and 122.38 (2 × C=CH), 131.51 and 136.77 (quaternary C), 167.26 (ester C=O) and 202.72 (ketone C=O); *m/z* (CI^+) 284.2 (100%, $[M + NH_4]^+$) and 267.2 (86, $[M + H]^+$).

(1*Z*,5*E*)-1-(Ethoxycarbonyl)-6,10-dimethylundeca-1,5,9-trien-2-yl trifluoromethanesulfonate (304)



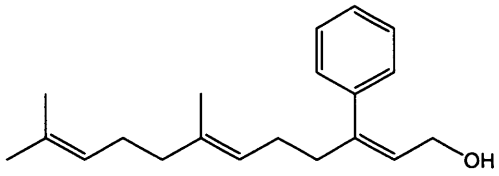
A stirred solution of **303** (546 mg, 2.05 mmol) in anhydrous THF (15 cm^3) under N_2 was cooled to –78 °C and then potassium bis(trimethylsilyl)amide (0.5 M in THF, 4.93 cm^3, 2.46 mmol) was added. The resulting mixture was stirred at –78 °C for 30 min. Trifluoromethanesulfonic anhydride (414 mm^3, 2.46 mmol) was added at –78 °C and the solution was stirred for 16 h whilst slowly warming to room temperature. Diethyl ether (20 cm^3) was added and the solution washed with 10% citric acid solution (2 × 15 cm^3) and water (15 cm^3). The separated organic layer was dried over $MgSO_4$ and filtered. Concentration of the solvent under reduced pressure gave a yellow oil which was purified by flash chromatography on silica gel with hexane and ethyl acetate (4 : 1) as eluent to give **304** as light yellow oil (0.42 g, 51%); R_f 0.41 (Hexane : EtOAc = 4 : 1); υ_{max} (thin film)/cm^{-1} 2975.6, 2905.3, 2855.1, 2353.0, 1731.9, 1676.1, 1427.9, 1209.1, 1141.0, 1037.6, 923.1 and 840.1; δ_H (500 MHz, C^2HCl_3) 1.24 (3 H, t, *J* 7.0, OCH_2CH_3), 1.53 (3 H, s, CH_3), 1.54 (3 H, s, CH_3), 1.61 (3 H, s, CH_3), 1.91 (4 H, m, $(CH_3)_2C{=}CHCH_2CH_2$), 2.20 (2 H, q, *J* 7.5, CH_2CH_2CO), 2.34 (2 H, t, *J* 7.5, CH_2CH_2CO), 4.17 (2 H, q, *J* 7.0, OCH_2CH_3), 4.99 (2 H, t, *J* 7.0, 2 × $C{=}CHCH_2CH_2$) and 5.67 (1 H, s, O-C=CH); δ_C (125 MHz, C^2HCl_3) 14.05 (OCH_2CH_3), 16.05 (CH_3), 17.70 (CH_3), 24.37 (CH_2CH_2CO), 25.68 (CH_3), 26.52 and 39.60 ($(CH_3)_2C{=}CHCH_2CH_2$), 34.60 ($CH_2CH_2C{=}O$), 61.27 (OCH_2CH_3), 112.04 (O-C=CH), 120.61 and 123.93 (2 × C=CH), 131.68 and 138.18 (2 × C=CH), 158.52 ($CH{=}COSO_2CF_3$) and 162.52 (C=O); δ_F (283 MHz, C^2HCl_3) –74.6 (s); *m/z* (CI^+) 398.1 (2%, M^+), 358.0 (80) and 114.0 (100).

(2*Z*,6*E*)-Ethyl 7,11-dimethyl-3-phenyldodeca-2,6,10-trienoate (302)



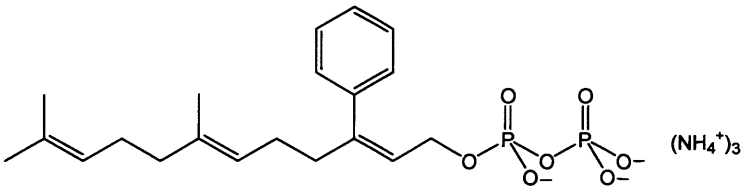
To a stirred solution of **304** (0.36 g, 0.91 mmol) in anhydrous THF (10 cm^3) under N_2, was added palladium (II) acetate (0.02 g, 0.09 mmol), triphenylarsine (0.11 g, 0.36 mmol), silver oxide (0.42 g, 1.81 mmol) and phenylboronic acid (0.17 g, 1.36 mmol) in quick succession, and the complete mixture heated under reflux for 15 h. Water (20 cm^3) and diethyl ether (20 cm^3) were added, and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 15 cm^3). The combined ethereal extracts were washed with water (2 × 10 cm^3) and brine (10 cm^3), dried over $MgSO_4$ filtered and concentrated under reduced pressure. Purification by flash column chromatography on silica gel with hexane and ethyl acetate (9 : 1) as eluent gave **302** as a light yellow oil (0.19 g, 66%); R_f 0.35 (Hexane : EtOAc = 9 : 1); HRMS (ES^+, $[M + H]^+$) found 327.2324. $C_{22}H_{31}O_2$ requires 327.2319; υ_{max} (thin film)/cm^{-1} 2975.7, 2926.3, 2361.9, 1727.2, 1638.2, 1442.5, 1377.2, 1276.4, 1223.8, 1159.0, 1042.6, 865.8 and 698.6; δ_H (500 MHz, C^2HCl_3) 0.99 (3 H, t, *J* 7.0, CH_2CH_3), 1.45 (3 H, s, CH_3), 1.53 (3 H, s, CH_3), 1.60 (3 H, s, CH_3), 1.88 (6 H, m, $(CH_3)_2C$=CHCH_2CH_2 and CH_2CH_2CPh), 2.40 (2 H, dt, *J* 1.0, *J* 8.0, CH_2CH_2CPh), 4.90 (2 H, q, *J* 7.0, CH_2CH_3), 5.01 (2 H, dt, *J* 1.0, *J* 7.0, 2 × C=CH), 5.81 (1 H, s, C=C*H*CO_2Et) and 7.07 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 13.95 (CH_2CH_3), 16.06 (CH_3), 17.69 (CH_3), 25.68 (CH_3), 25.86, 26.65, 39.65 and 40.46 (2 × CH_2CH_2), 59.74 (CH_2CH_3), 117.42 (CHCO), 122.73 and 124.24 (2 × $CHCH_2CH_2$), 127.16, 127.54 and 127.80 (Ar-CH) and 131.41, 136.25, 140.16, 159.30 and 166.07 (quaternary C); *m/z* (CI^+) 344.4 (53%, $[M + NH_4]^+$) and 327.4 (100, $[M + H]^+$).

(2*Z*,6*E*)-7,11-Dimethyl-3-phenyldodeca-2,6,10-trien-1-ol (305)



This compound was prepared from **302** in a manner identical to that for the alcohol **175**; purification by flash chromatography on silica gel using hexane and ethyl acetate (2: 1) as eluent gave alcohol **305** as a light yellow oil (0.11g, 85%); R_f 0.38 (Hexane : EtOAc = 2 : 1); HRMS (EI^+, M^+) found 284.2147. $C_{20}H_{28}O$ requires 284.2140; υ_{max} (thin film)/cm^{-1} 3355.5, 2965.4, 2922.2, 2855.4, 1650.6, 1491.7, 1442.2, 1379.7, 1080.3, 1005.6, 830.9 and 769.8; δ_H (500 MHz, C^2HCl_3) 1.42 (1 H, b, CH_2OH), 1.54 (3 H, s, CH_3), 1.59 (3 H, s, CH_3), 1.71 (3 H, s, CH_3), 1.97 (6 H, m, $(CH_3)_2C{=}CHCH_2CH_2$ and CH_2CH_2CPh), 2.44 (2 H, t, *J* 7.5, CH_2CH_2CPh), 4.07 (2 H, d, *J* 7.0, CH_2OH), 5.11 (2 H, m, 2 × C=CH), 5.72 (1 H, t, *J* 7.0, $C{=}CHCH_2OH$) and 7.15 (5 H, m, Ar-H); δ_C (125 MHz, C^2HCl_3) 16.03 (CH_3), 17.69 (CH_3), 25.69 (CH_3), 26.49, 26.71, 39.00 and 39.68 (2 × CH_2CH_2), 60.28 (CH_2OH), 123.54 and 124.38 (2 × C=CH), 125.68 ($C{=}CHCH_2OH$), 127.07, 128.09 and 128.20 (Ar-CH) and 131.34, 135.54, 140.00 and 144.59 (quaternary C); *m/z* (EI^+) 284.2 (10%, M^+) and 266.2 (100, $[M - H_2O]^+$),.

(2*Z*,6*E*)-7,11-Dimethyl-3-phenyldodeca-2,6,10-trien-1-yl diphosphate tris-ammonium salt (162)



This compound was prepared from alcohol **305** and purified in a manner identical to that for the

diphosphate **154** to give diphosphate **162** as a white solid (53.9 mg, 31%); HPLC t_R 39.02 min; Purity 96.92% by analytical RP HPLC under 220 nm; HRMS (ES^-, $[M - H]^-$) found 443.1375. $C_{20}H_{29}O_7P_2$ requires 443.1389; υ_{max} (KBr disc)/cm^{-1} 2924.0, 1442.5, 1200.4, 1128.9, 1101.1, 1023.2, 971.5, 927.5, 812.5 and 706.3; δ_H (500 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 1.16 (3 H, s, CH_3), 1.23 (3 H, s, CH_3), 1.31 (3 H, s, CH_3), 1.61 (6 H, m, $(CH_3)_2C{=}CHCH_2CH_2$ and CH_2CH_2CPh), 2.24 (2 H, t, *J* 7.5, CH_2CH_2CPh), 4.32 (2 H, t, *J* 6.5, CH_2O), 4.77 (1 H, t, *J* 6.5, C=C*H*), 4.84 (1 H, t, *J* 6.5, C=C*H*), 5.67 (1 H, t, *J* 7.0, $C{=}CHCH_2O$) and 7.05 (5 H, m, Ar-H); δ_C (125 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) 15.49 (CH_3), 17.13 (CH_3), 25.10 (CH_3), 26.29, 26.53, 38.71 and 39.25 (2 × CH_2CH_2), 63.90 (CH_2O), 122.42 ($C{=}CHCH_2O$), 123.66 and 124.34 (2 × C=*C*H), 127.54, 128.33 and 128.39 (Ar-CH) and 131.17, 135.48, 139.41 and 145.93 (quaternary C); δ_P (122 MHz, ^2H_2O at pH 8.5 buffered with $N^2H_4O^2H$) −10.18 (d, J_{PP} 21.0) and −10.82 (d, J_{PP} 21.0); *m/z* (ES^-) 443.1 (100%, $[M - H]^-$).

4.3 Incubation of FPP analogues with AS and characterisation of products with GC-MS

Purified AS (50 μM) was incubated with each FPP analogue (200 mM) in 10 mM Tris, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 15% glycerol (pH 7.5) in a final volume of 500 mm^3 overlayed with pentane (200 mm^3) at 30 °C for 1-7 days. Reactions were terminated by addition of EDTA (100 mM, 100 mm^3) and the products were extracted by vortexing against pentane (3 × 3 cm^3). The pooled extracts were vortexed with 1.5 g of silica then decanted and concentrated under reduced pressure on ice.

The hexane extractable products were analysed by GCMS. This was performed on a system

comprising of a Hewlett Packard 6890 GC fitted with a J&W scientific DB-5MS column (30 m × 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 unless otherwise stated and used helium as the carrier gas. 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).

4.4 Preparative scale incubation of 2-fluoro farnesyl pyrophosphate (155) with AS and characterisation of products

Aristolochene synthase solution (3.60 cm^3, 638 μM) was diluted to 33.5 cm^3 with buffer consisting of 20 mM Tris-HCl, 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$, and 15 % (v/v) glycerol, pH 7.5. The assay solution was gently mixed as **155** (30.0 mg, 0.067 mmol) was added followed by pentane (2 cm^3). After incubation for 48 h at 25 °C, the olefin products were extracted with pentane (3 × 80 cm^3). The pooled pentane extracts were concentrated under a nitrogen stream until about 5 cm^3 of solvent remained. This solution was passed through a short pad of silica gel overlayed with anhydrous $MgSO_4$. The pentane was concentrated under reduced pressure to yield a single product as judged by GCMS analysis in a manner identical to that for the small scale incubation (*vide supra*) (4.0 mg, 27%); GCMS T_r = 26.24 min; HRMS (EI^+) found 222.1785, $C_{15}H_{23}F$ requires 222.1784. Compound displayed conformational isomerism complicating the interpretation of its NMR spectra. Consequently variable temperature NMR experiments were performed (*vide supra*). The ^1H NMR data are reported in Table 4.1. The ^{13}C NMR spectrum could not be observed directly due to the small quantity of material isolated and reduction of line

intensity due to the conformational isomerism but could be indirectly observed and interpreted through the cross peaks observed in the various 2D spectra. δ_C (125 MHz, d^8-toluene, 25 °C) 14.7 (C-14), 19.5 (C-13 and C-15), 25.1 (C-5), 27.5 (C-4), 33.3 (C-9), 33.8 (C-8) 34.1 (C-1) 45.4 (C-10), 108.9 (C-12), 112.0 (C-3), 128.2 (C-7), 137.1 (C-6), 149.6, (C-11), 154.5 and 156.7 (d, C-2); δ_F (283 MHz, d^8-toluene, -60 °C) -89.2 (major conformer) and -91.8 (minor); δ_F (283 MHz, d^8-toluene, 25 °C) –88.8 (major conformer) and –91.3 (minor); *m/z* (EI^+) 222.2 (12%, M^+), 207.2 (19%, $[M-CH_3]^+$), 202.2 (10%, $[M–HF]^+$), 68.1 (100%).

	-60 °C major	-60 °C minor	25 °C	90 °C
CH_2-1	1.90-2.07 (m) and 2.33-2.44 (m)	1.90-2.07 (m) and 2.33-2.44 (m)	1.99-2.14 (m) and 2.35-2.45 (m)	1.88-2.02 (m) and 2.42 CH_2-1 (ddd, *J* 3.5, 14.5, 32.5 Hz)
CH_2-4	1.51-1.58 (m) 2.95 (ddt, *J* 2, 6, 12.5 Hz) 3.00-3.06 (m)	1.51-1.58 (m) 3.03 (br q, *J* 10 Hz)	1.53-1.58 (m) and 2.83-3.02 (br m)	1.52-1.60 (m) and 2.83-2.90 (dq, *J* 1.5, 9.5 Hz)
CH_2-5	2.02-2.14 (m) and 2.25 (ddt, *J* 1.5, 5.5, 12 Hz)	2.02-2.14 (m) and 2.25 (ddt, *J* 1.5, 5.5, 12 Hz)	2.11-2.14 (m) and 2.22-2.30 (br, m)	2.12-2.17 (m) and 2.24-2.26 (m)
CH-6	5.36 (br, d, *J* 12.5 Hz)	5.05 (t, *J* 8 Hz)	5.02-5.10 (br, s) and 5.25-5.32 (br, m)	5.18 (m)
CH_2-8	2.02-2.14 (m)	2.02-2.14 (m)	2.11-2.14 (m)	2.12-2.17 (m)
CH_2-9	1.51-1.58 (m) and 1.90-2.07 (m)	1.51-1.58 (m) and 1.90-2.07 (m)	1.53-1.58 (m) and 1.99-2.14 (m)	1.52-1.60 (m) and 1.88-2.02 (m)
CH-10	2.02-2.14 (m)	2.02-2.14 (m)	2.11-2.14 (m)	2.12-2.17 (m)
CH_2-12	pseudo AB system 4.70, 4.73, 4.76 and 4.78	pseudo AB system 4.70, 4.73, 4.76 and 4.78	4.68 (br, s) and 4.73 (s)	4.64 (s) and 4.70 (s)
CH_3-13	1.67 (s)	1.79 (s)	1.62 (s)	1.62 (s)
CH_3-14	1.60 (s)	1.62 (s)	1.67 (br)	1.66 (s)
CH_3-15	1.26 (d, J_{HF} 3 Hz)	1.28 (d, J_{HF} 3 Hz)	1.32 (d, J_{HF} 3 Hz)	1.36 (d, J_{HF} 2.5 Hz)

Table 4.1. *^1H NMR spectra of* **314** *at -60, 25 and 90 °C.*

4.5 Preparation of wild-type aristolochene synthase

The expression of cDNA for AS and the purification of AS were performed according to published procedure.[64]

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

A 100 mm^3 aliquot of *E. coli* BL21 (DE3) competent cells (from Mr L. H. Tey) was thawed slowly on ice, then 1-2 μg of vector containing a cDNA for AS[64] were added and the mixture was kept on ice for 20 min. The cells were then heat shocked at 42 °C for 2 min in a water-bath and then returned to ice for 2 min. 1 cm^3 of LB medium was added and the solution incubated at 37 °C for 1 h. After this time, the transformed cells were harvested in a centrifuge at 14,000 rpm for 1 min and the supernatant discarded to a volume of 100 mm^3. The cells were re-suspended and the 100 mm^3 cell suspension was aseptically spread on an agar - ampicillin plate. The plate was then incubated at 37 °C overnight.

4.5.2 Overexpression of recombinant AS

A single *E. coli* BL21 (DE3) colony, transformed with the vector containing *Aril,* was grown in 60 cm^3 LB media in the presence of ampicillin at 37 °C overnight. Overnight cultures (6 × 10 cm^3) were used to inoculate in 6 × 500 cm^3 of sterile LB medium in 6 × 2 litres conical flasks, to each of which 50 mg of ampicillin was added followed by incubation at 37 °C for 3 h with shaking (180 rpm). At various intervals, 1 ml of the growing culture was transferred to a 1 ml cuvette and the OD at 600 nm was measured to monitor the growth of bacteria. When the OD at 600 nm was higher than 0.6 the remaining culture was induced by the addition of

Isopropyl-1-thio-β-D-galactopyranoside (IPTG) up to a final concentration of 0.6 - 1 mM. The induced cultures were incubated with shaking (180 rpm) at 37 °C for a further 3 h as it was described for epi-aristolochene synthase.[189, 190] Cells were harvested by centrifugation at 5,100 rpm at 4 °C for 20 min. The supernatant solution was discarded and the pellets were stored at –20 °C until required.

4.5.3 Base extraction and refolding of wild-type aristolochene synthase

The pellet (from above, section 4.5.2) was redissolved in 50 cm^3 cell lysis buffer (5 mM EDTA, 5 mM β-mercaptoethanol, 20 mM Tris, at pH 7.5). The suspension was sonicated for a total of 15 min (30 s on 30 s off) on ice using a Lab Plant (ultrason 250) fitted with the medium probe. The sonicated sample was centrifuged at 5 000 rpm for 10 min and the supernatant solution discarded. The pellets were resuspended in 150 ml of fresh lysis buffer and titrated on ice to pH 11.75 with sodium hydroxide (0.1 M). The solution was stirred on ice for 30 min. The pH was then lowered to pH 8.0 with hydrochloric acid (0.1 M) and β-mercaptoethanol added to a final concentration of 5 mM. The resulting solution was stirred for 30 min on ice and centrifuged at 15000 rpm at 4 °C for 30 min, after which solubilised protein was found in the supernatant solution and stored at 4 °C.[49]

4.5.4 Purification of wild-type AS by ion-exchange chromatography

Anion exchange columns are generally used to purify aristolochene synthase[75, 191]. A Q-Sepharose™ (Amersham Pharmacia Biotech™) High Performance (2.5 × 20 cm) column at 4 °C, was used to purify the protein from the supernatant solution described above (section 4.5.3).

The column was washed with 150 cm^3 of column buffer (20 mM tris, 5 mM EDTA, 5 mM β-mercaptoethanol, pH 7.5). Approximately 50 cm^3 of cell free extract was loaded onto the column followed by another 150 cm^3 of buffer to elute any unbound protein. A 0.1 - 0.6 M NaCl (500 cm^3) gradient was applied to the column and the fractions were collected every 48 s (typical fraction size = 5 cm^3). The column was then washed with 200 cm^3 of 1 M NaCl solution to elute any other product still bound to the resin. The absorbance of the fractions were measured at 280 nm to identify fractions containing protein[192], followed by SDS-PAGE electrophoresis to estimate the purity of the 39 kDa protein.

4.5.5 Dialysis and concentration of AS

To remove the high salt concentration of the protein solution that eluted from the column, the protein solution was dialysed using Spectrum™ Spectra/Por molecular porous dialysis membrane (MW = 3500 cut off), at 4 °C for 3 days in 3 litres of dialysis buffer (10 mM tris, 5 mM β-mercaptoethanol, pH 7.5). The buffer was replaced twice daily.

Following dialysis, the protein was transferred to an Amicon™ ultrafiltration apparatus containing a millipore 44.5 mm ultrafiltration membrane, and concentrated at a pressure of 3 bar at 4 °C, to give a final volume of approximately 3-5 cm^3. Once concentrated, the protein sample was aliquoted (0.5 cm^3), fast frozen in liquid N_2 and stored at –20 °C.

4.5.6 Protein concentration determination

For the determination of wild-type of aristolochene synthase concentrations, the microtannin

assay, was carried out. Tannin solutions A (196 cm^3 HCl 1 M, 4 cm^3 phenol, 20 g tannic acid) and B (0.4 g gum Arabic, 200 cm^3 H_2O) were prewarmed in a water bath at 30 °C. Bovine Serum Albumin (BSA) was used as the standard and the reactions for the calibration curved were prepared as described in Table 4.2.

To 1 cm^3 solutions of BSA standards and samples, 1 cm^3 of prewarmed solution A was added. Immediately after addition of solution A, standards and samples were incubated at 30 °C for 10 minutes. Then, 1 cm^3 of prewarmed solution B was added to each tube of standard and sample and the solutions were kept at room temperature for 10 minutes. The absorbance of the reactions was read at 500 nm.

[BSA] (μg ml^{-1})	**Water (μl)**
0	1000
10	990
20	980
30	970
40	960
50	950
60	940
70	930

Table 4.2 *Calibration curve reaction for the microtannin assay.*

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APPENDIX

Probing the reaction mechanism of aristolochene synthase with 12,13-difluorofarnesyl diphosphate†

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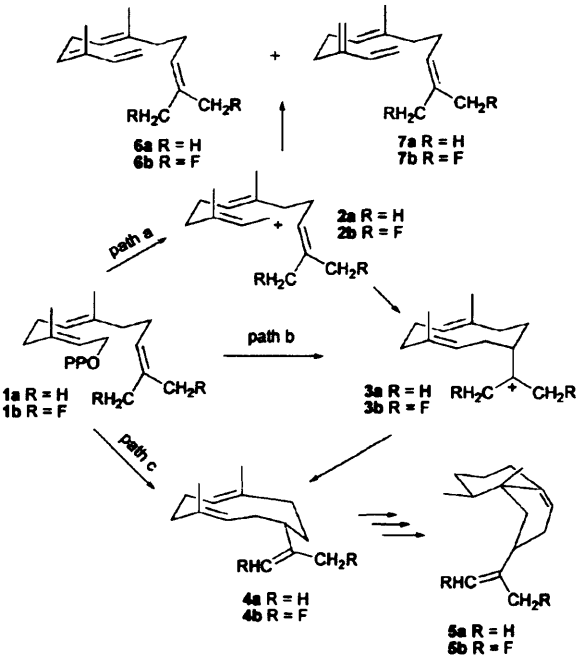
12,13-Difluorofarnesyl diphosphate, prepared using Suzuki–Miyaura chemistry, is a potent inhibitor of aristolochene synthase (AS), indicating that the initial cyclisation during AS catalysis generates germacryl cation in a concerted reaction.

The terpenoid family of natural products consists of many thousand compounds of enormous structural and stereochemical diversity that derive from only a small number of linear isoprenoid precursors. Sesquiterpene synthases, for instance, catalyse the cyclisation of the universal acyclic precursor farnesyl diphosphate (**1a**, FPP) to produce more than 300 different hydrocarbon skeletons, often with high regio- and stereospecificity.[1–4]

The solution of the X-ray crystal structures of 5 sesquiterpene synthases, namely 5-epi-aristolochene synthase from *Nicotiana tabacum*,[5] pentalenene synthase from *Streptomyces* UC5319,[6] trichodiene synthase from *Fusarium sporotrichoides*[7] and aristolochene synthase (AS) from both *Penicillium roqueforti*[8] and *Aspergillus terreus*,[9] revealed that, in spite of the absence of significant sequence similarities, they all adopt the mainly α-helical class I terpene fold. Sesquiterpene synthases therefore serve as high fidelity templates that subtly channel conformation and stereochemistry during the cyclisation reactions.

The mechanisms of the enzyme-catalysed cyclisations of sesquiterpenoids are inherently difficult to study spectrometrically and their elucidation has been largely dependent on the use of substrate mimics and site directed enzyme mutants. Mechanistic studies with substrate analogues suggested that AS converts **1a** into the bicyclic sesquiterpene aristolochene (**5a**) through an initial cyclisation to generate the intermediate (*S*)-germacrene A (**4a**) followed by protonation, 1,2 hydride and methyl shifts and stereospecific deprotonation from C8 (Scheme 1). Surprisingly little is known about the reaction mechanism of the formation of the decalin ring system that could be formed by several mechanistically distinct pathways. The results obtained with site-specific mutants of AS suggested that diphosphate ionisation led to the formation of farnesyl cation (**2a**) which was attacked by the C10–C11 π-bond to generate germacryl cation (**3a**) (path a, Scheme 1).[10,11] Alternatively, the formation of **3a** could take place in a concerted reaction in which farnesyl diphosphate ionisation is accompanied by electrophilic attack of C1 of FPP by the C10, C11 π-system (path b).[9] This mechanism is in agreement with the observation that the reaction of stereospecifically deuterated FPP



Scheme 1 Possible mechanisms for the initial cyclisation during catalysis by aristolochene synthase.

occurred with inversion of configuration at C1.[12] In path c, cyclisation is initiated by deprotonation from C12[13] and concurrent bond formation between C10 and C1. Since it has become increasingly apparent that only a few amino acid substitutions can be sufficient to remodel the active site template provided by terpene synthases, thereby often dramatically altering the reaction products, a detailed mechanistic understanding of the reaction catalysed by these enzymes is central to their further evolution *in vitro* or *in vivo* for the generation of novel products.[14]

Fluoro analogues of prenyl diphosphates have recently been used as powerful reagents to explore the mechanism of the enzyme-catalysed cyclisations to terpenoid products.[15] Fluoro substituents do not greatly affect the binding affinities as a consequence of size and shape but at the same time exert a strong influence on the electronic environment at the site of replacement in that they stabilize cations on the α-carbon by π-donation but exert a destabilizing inductive effect on cations located on the β-carbon. Here we report the synthesis of 12,13-difluorofarnesyl diphosphate (**1b**) and its effects on AS-catalysis.

Due to the electronic effects of fluoro substituents on carbocations described above, incubation of 12,13-difluorofarnesyl

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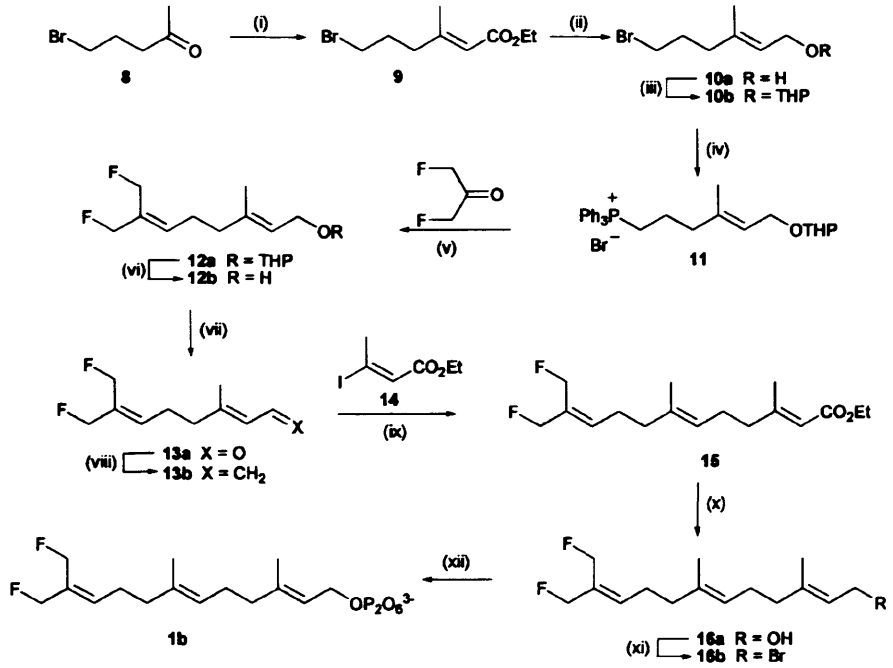

Scheme 2 Synthesis of 12,13-difluorofarnesyl diphosphate **1b**. *Reagents and conditions*: (i) triethyl phosphonoacetate, NaH, THF, 0 °C, 68%; (ii) DIBAL-H, THF, −78 °C, 98%; (iii) 3,4-DHP, CH_2Cl_2, *p*-TsOH, 98%; (iv) PPh_3, CH_3CN, Δ; (v) LiHMDS, THF, −78 °C, 87%; (vi) PPTS, EtOH, 55 °C, 97%; (vii) TPAP, NMO, CH_3CN, 82%; (viii) CH_3PPh_3Br, n-BuLi, THF, 78%; (ix) 9-BBN, THF then **14**, $PdCl_2dppf$, NaOH, $AsPh_3$, THF, 50 °C, 28%; (x) DIBAL-H, THF, −78 °C, 68%; (xi) NEt_3, MsCl, −45 °C then LiBr; (xii) $(Bu_4N)_3HP_2O_7$, CH_3CN then DOWEX ion exchange Bu_4N^+/NH_4^+,10%.

diphosphate with AS would be expected to generate different outcomes for the three cyclisation pathways of Scheme 1. For path a, the destabilizing effect of the two fluoro substituents on the putative carbocation on C11 should lead to the accumulation of the 12,13-difluorofarnesyl cation (**2b**) and the formation of 12,13-difluoro-(*E*,*E*)-α-farnesene (**6b**) and 12,13-difluoro-(*E*)-β-farnesene (**7b**) through deprotonation from C4 or C15. For a reaction where phosphate departure occurs simultaneously with electron flow from the C10, C11 double bond, 12,13-difluorofarnesyl diphosphate would be expected to act as a competitive inhibitor. In pathway c, the acidifying effect of the fluoro substituents on the protons on C12 and C13 should allow the reaction to proceed, ultimately generating 12,13-difluoroaristolochene (**5b**).

12,13-Difluorofarnesyl diphosphate (**1b**) was synthesised from bromoketone **8** (Scheme 2).[16] Compound **8** was initially subjected to a Horner–Emmons reaction with triethyl phosphonoacetate to generate bromoester **9** with good stereoselectivity ($E:Z$ = 11 : 1).[17] Reduction of the ester with DIBAL-H in THF at −78 °C followed by protection of the resulting alcohol with a THP group gave **10b** in near quantitative yield. This compound was converted to the phosphonium bromide salt **11** and then treated with 1,3-difluoroacetone in a Wittig reaction to give **12a** in 87% yield. The THP ether was removed and the alcohol **12b** converted to the 1,3-diene **13b** by successive TPAP oxidation and Wittig reaction. A Suzuki–Miyaura coupling with the crotonyl iodide **14**[18] resulted in the production of ester **15**, which was converted to the diphosphate **1b** by reduction to the alcohol and diphosphorylation.[19] It is worthy of note here that attempts to prepare the ester **15** by the more conventional chain extension chemistry of Weiler and Sum[20] failed since the use of lithium dimethylcuprate resulted in loss of fluorine atoms, most likely by elimination of hydrogen fluoride.

Incubation of **1b** with purified recombinant AS under the standard reaction conditions previously used for the conversion of **1a** to aristolochene[11] did not lead to any hexane extractable products as judged by GC-MS analysis, even after prolonged incubation. Since 12,13-difluorofarnesyl diphosphate clearly did not act as a substrate for AS, the steady-state kinetic parameters of purified recombinant AS were measured in the presence of varying amounts of **1b** by incubation with [1-^{3}H]-FPP and monitoring the formation of tritiated, hexane extractable products.[21] A double reciprocal plot indicated that **1b** was a reversible competitive inhibitor of AS (Fig. 1). The measured K_I of 0.8 ± 0.2 μM was comparable to the Michaelis constant for AS catalysis (K_M = 2.3 μM) reported previously, indicating that the fluoro substituents do not have a negative effect on the affinity of the enzyme for this compound. For comparison, farnesyl thiodiphosphate,[22]‡ a compound known to act as a potent inhibitor of undecaprenyl diphosphate synthase from *E. coli* and *S. aureus*,[22–24] was also tested and found to also act in a competitive fashion, albeit with much reduced affinity (K_I = 10 μM).

The observation that **1b** acts as an inhibitor of AS suggests that the cyclisation of FPP occurs along path b in which farnesyl diphosphate ionisation is accompanied by electrophilic attack of C1 by the C10, C11 π-bond with inversion of configuration at C1 resulting in the formation of germacryl cation. This conclusion is different from that reached previously from experiments with site-specific mutant AS-F112A, in which Phe 112 was replaced with alanine, leading to the production of 36% (*E*)-β-farnesene (**7a**) and 53.5% (*E*,*E*)-α-farnesene (**6a**) in addition to a small amount of

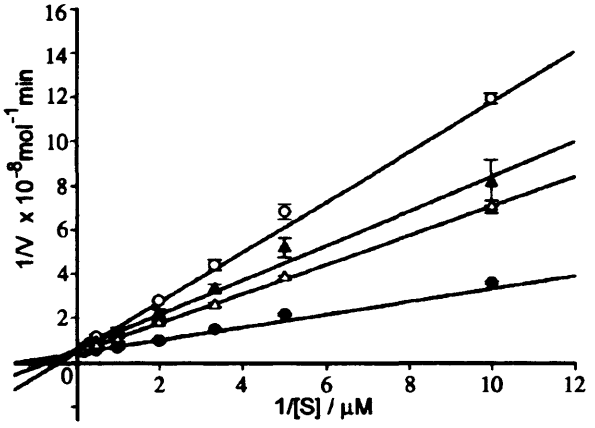

Fig. 1 Double reciprocal plot for inhibition of AS catalysed turnover of **1a** by **1b** at 0 (●), 0.4 μM (△), 0.6 μM (▲) and 1 μM (○) of **1b**.

germacrene A (**4a**).[11] The presence of these linear sesquiterpenoids suggested that farnesyl cation **2a** accumulated during catalysis in the mutant enzyme due to the absence of stabilization through interaction with the aromatic ring of Phe 112 of germacryl cation and the transition state preceding it. The results presented here suggest that in addition to stabilizing carbocation **3a** through its aromatic ring, the presence of Phe 112 (together with other active site residues) may also exert a stereoelectronic effect on the reaction through its steric bulk by aligning the π-orbital of the C10, C11 double bond with the breaking C1–O bond of the diphosphate group. When the size of this side chain is reduced by replacing the benzyl group with the smaller methyl group, this alignment may no longer be optimal, thereby preventing the immediate quenching of the developing positive charge on C1 and leading to the accumulation of farnesyl cation (**2a**) and the production of the linear farnesenes **6a** and **7a**.

According to this argument, **1b** should act as a substrate of the mutant enzyme AS-F112A since the less hindered rotation around the C1–O bond of **1b** in the mutant should lead to the formation of 12,13-difluorofarnesyl cation (**2b**) and hence of the difluorinated farnesenes, **6b** and **7b**. One major and two side products with the molecular ion peaks of *m/z* of 240 expected for difluorinated sesquiterpenoids were detected in the gas chromatogram of the hexane extractable products of incubations of the AS-F112A with **1b** (ESI). While the low activity of AS-F112A even with the unfluorinated substrate (**1a**)[11] allowed for the production of only a small amount of fluorinated products and prevented the detailed molecular characterization of the products, this experiment nevertheless confirmed that AS-F112A could indeed turnover **1b**. This result strongly supported the proposal that AS catalyses the breaking of the ester bond in **1a** and the formation of the cyclic germacryl cation (**3a**), at least in part, by facilitating optimal orbital overlap between the C10–C11 and C1–O bonds in **1a** through the templating effect of bulky active site residues such as Phe 112.

It appears therefore that the interpretation of results from site-specific mutants of terpene cyclases requires some caution, since the change of only a single amino acid in their active site can alter their templating potential. Such local geometrical changes may be inherently difficult to detect since they may not affect the global fold of the proteins but nevertheless lead to the synthesis of novel products through subtle alterations of the reaction pathway. The strong influence on the reactivity exerted by fluoro substituents without significantly affecting size and shape may make these reagents more suitable than mutagenesis experiments to explore the chemistry of terpene synthases. However, only through a combination of experimental approaches will the intricate details of this masterpiece in combinatorial chemistry be deciphered.

We gratefully acknowledge the gift from Prof. Dale Poulter of FSPP, fruitful discussions with Prof. David W. Knight, the support of the EPSRC and the EPSRC National Mass Spectrometry Service, University College, Swansea, and Rob Jenkins, Cardiff University, for some mass spectral analyses.

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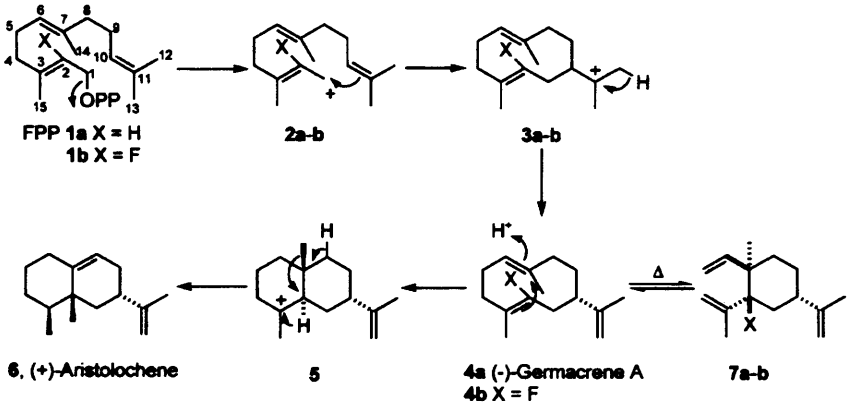
Aristolochene Synthase-Catalyzed Cyclization of 2-Fluorofarnesyl-Diphosphate to 2-Fluorogermacrene A

David J. Miller, Fanglei Yu, and Rudolf K. Allemann*[a]

The mechanism of the conversion of (E,E)-farnesyl diphosphate (FPP, 1a) to aristolochene (6) catalyzed by aristolochene synthase from Penicillium roqueforti *has been proposed to proceed through the neutral intermediate germacrene A (4a). However, much of the experimental evidence is also in agreement with a mechanism in which germacrene A is not an intermediate in the predominant mechanism that leads to the formation of aristolochene, but rather an off-pathway product that is formed in a side reaction. Hence, to elucidate the mechanism of FPP cyclisation the substrate analogue 2-fluoroFPP (1b) was synthesized, and upon incubation with aristolochene synthase was converted to a single pentane extractable product according to GC-MS analysis. On the basis of NMR analyses this product was identified as 2-fluorogermacrene A (4b). Variable temperature ^{1}H NMR spectroscopy indicated the existence of two conformers of 4b that were in slow exchange at −60 °C, while at 90 °C the two isomers gave rise to averaged NMR signals. In the major isomer (~75%) the methyl groups on C3 and C7 were most likely in the down–down orientation as had been observed for other (E,E)-germacranes. This work suggests that after an initial concerted cyclisation of FPP to germacryl cation deprotonation leads to the formation of germacrene A, and provides compelling evidence that germacrene A is indeed an on-pathway product of catalysis by aristolochene synthase.*

Introduction

Terpene synthases are a fascinating class of enzymes due to their almost unique ability to direct the formation and rearrangement of carbocationic species.[1] Sesquiterpene synthases, many of which share a common mainly α-helical fold, transform the shared substrate, farnesyl diphosphate (FPP, **1a**) with often exquisite regio- and stereochemical control into more than three hundred different hydrocarbon scaffolds that form the structural basis of the large family of sesquiterpenoids with many tens of thousands of complex natural products.[2]



Scheme 1. Reaction schemes for the AS catalysed conversion of FPP (**1a**) to aristolochene (**6**) and of 2F-FPP (**1b**) to 2-fluorogermacrene A (**4b**).

Aristolochene synthase (AS) from *Penicillium roqueforti* catalyses the Mg^{2+}-dependent conversion of **1a** to (+)-aristolochene (**6**)—the biochemical precursor of several fungal toxins, which include the potentially lethal PR toxin (Scheme 1).[3] Based on mechanistic studies with labelled substrates and through the analysis of the reaction products obtained with enzyme mutants a reaction mechanism was proposed in which the initial cleavage of the alkyl diphosphate bond leads to farnesyl cation (**2a**) prior to cyclisation. Attack by the C10=C11 double bond to produce germacryl cation (**3a**) followed by proton loss from C12 has been proposed to lead to germacrene A (**4a**). This uncharged intermediate was then postulated to undergo protonation of the C6=C7 double bond and a further cyclisation to form the bicyclic eudesmane cation (**5**). Successive 1,2-hydride shift and methyl migration followed by loss of H_{si} on C8 results in the generation of (+)-aristolochene (**6**).[4]

The intermediacy of germacrene A is tentatively supported by the observation that **4a** is a minor by product of AS catalysis.[5] In addition, results obtained with some AS mutants can be interpreted to support a mechanism that involves germa-

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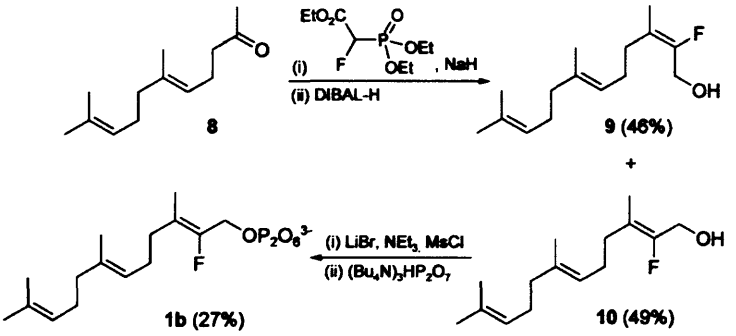
crene A as an intermediate. Replacement of Trp334 with alanine, which based on the 2.5 Å X-ray crystal structure of apo-AS[6] has been postulated to be involved in the stabilization of the positive charge in eudesmane cation, led to the exclusive production of germacrene A,[7] while replacement of Tyr92 with phenylalanine led to increased production of **4a**. While these observations are largely in agreement with the above reaction mechanism, other experiments suggest that germacrene A might not be an intermediate in the predominant mechanism that leads to the formation of aristolochene, but is rather an off-pathway product that is formed in a side reaction. In particular, **4a** does not appear to act as a substrate of AS, as would be expected if germacrene A was an intermediate in the predominant mechanism that leads to the formation of aristolochene.[5] In addition, the active site acid that protonates germacrene A has so far been difficult to identify, even though several candidates have been proposed, which include a proton shuttle from the solvent to Tyr92 in the active site by way of Arg200, Asp203 and Lys206, an unprecedented active site oxonium ion,[8] or the pyrophosphate group.[9]

Rather than analyzing the effects of structural changes of the enzyme on product distribution, substrate analogues can be used to study the reaction mechanism. The turnover of the 6,7-dihydro analogue of FPP by AS generates dihydro germacrene A.[10] However, X-ray crystallographic studies with sesquiterpene cyclases have suggested that the conformation of FPP in the active site is central to the selectivity of the cyclisation reaction. Hence, the reduction of the C6=C7 double bond of FPP might change the reaction mechanism by altering the conformation of both substrate and intermediate. The aim of this work was to address the intermediacy of germacrene A by intercepting the AS-catalysed ring closure to eudesmane cation through the use of a suitable substrate analogue, and to identify the structure of the putative germacrene A analogue. Fluorinated substrates have proved useful in the elucidation of mechanistic details of terpenoid biosynthesis due to the special properties of fluorine substituents which do not greatly affect binding affinities by altering size and shape. At the same time fluorine substituents exert a strong influence on the electronic environment at the site of replacement in that they stabilize cations on the α carbon by π donation, but exert a destabilizing inductive effect on cations located on the β carbon.

We report here the synthesis of 2-fluorofarnesyl diphosphate (**1b**) and the characterization of the reaction products obtained during AS catalysis. The results provide strong support for a reaction mechanism in which an initial cyclisation of FPP to germacryl cation is followed by proton loss from C12 to generate **4a**.[4]

Results and Discussion

We considered that the introduction of a fluorine atom on C2 of FPP should lead to a destabilisation of eudesmane cation during AS catalysis, and hence to the accumulation of 2-fluorogermacrene A (**4b**). We have therefore studied the AS-catalyzed reaction of 2-fluorofarnesyldiphosphate (**1b**), which was prepared by using a modification of a published procedure.[11] Geranyl acetone was treated with triethyl 2-fluoro-2-phosphonoacetate and sodium hydride, followed by DIBAL-H reduction of the resulting ester mixture to give **9** and **10** in a ratio of approximately 1:1 (Scheme 2). Bromination of **10** followed by diphosphorylation[12] and purification by reversed-phase HPLC gave **1b** in 36% yield over these two steps.



Scheme 2. Synthesis of 2-fluorofarnesyl-diphosphate (**1b**).

Enzymatic cyclisation of 2-fluorofarnesyl diphosphate

Preparative scale incubation of **1b** with recombinant AS (pH 7.5, 25 °C, 48 h) followed by GC mass spectral analysis of the pentane-extractable material revealed a single product (Figure 1), which showed the molecular mass expected for a monofluorinated sesquiterpenoid (*m/z* 222; Figure 2).

The ^{1}H NMR spectroscopic analysis of compound **4b** at room temperature provided a rather poorly resolved spectrum, which was similar to the unassigned literature reference spectrum of germacrene A.[13] The ^{19}F NMR spectrum of **4b** indicated two resonances ($\delta_F = -88.8$ and -90.9 ppm) at room temperature. Because of the known flexibility of cyclodecadienes a variable temperature NMR study was carried out in [D_8]toluene between −80 and 90 °C, at 10 °C intervals. The compound proved to be stable over the whole temperature range as evidenced by the reversibility of the temperature-dependent spectral changes. Significant line sharpening was observed both on lowering and increasing the temperature from 25 °C (Figure 3). Compound **4b** existed as two resolvable conformers at temperatures below 0 °C, while at elevated temperatures the position of the sharp resonance signals indicated a fast equilibrium between the two conformers, which resulted in a weighted average of the NMR-resonances. A coalescence temperature of approximately 30 °C was observed.

Variable temperature NMR spectroscopic analysis of 4b

The COSY and HSQC NMR spectra were obtained both at −60 and 90 °C; this made it possible to assign all the ^{1}H resonances of **4b** (Table 1). Together with the room temperature HMBC spectrum these data also allowed us to assign the ^{13}C NMR

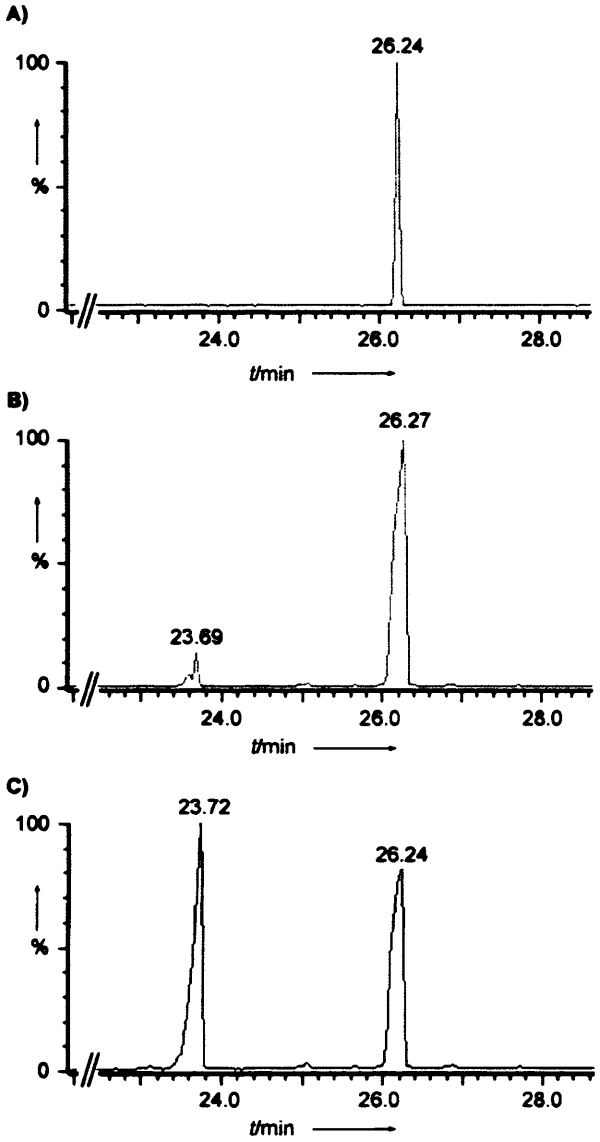


Figure 1. Analysis of the temperature-induced reaction of the pentane extractable products of 2-fluoroFPP (**1b**) utilisation by AS. A) Total ion chromatogram (TIC) from the GC analysis of the compound produced by AS from **1b** at an injection port temperature of 50 °C. B) TIC from the GC analysis of the same compound at an injection port temperature of 250 °C. C) TIC from the GC analysis of the same compound at an injector port temperature of 300 °C.

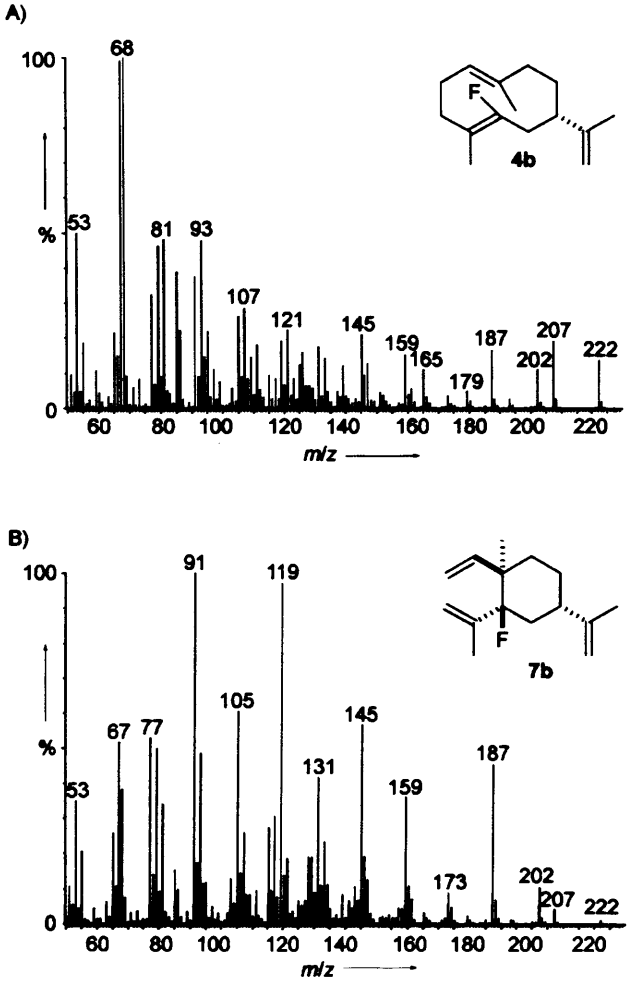


Figure 2. Mass spectral analysis of the two isomers produced by thermal rearrangement in the GC injector. A) EI^+ Mass spectrum of the material eluted at 26.24 min, which is the presumed 2-fluoro-germacrene A derivative **4b**; B) EI^+ mass spectrum of the material eluted at 23.72 min, which is the presumed β-elemene analogue **7b**.

spectrum of this compound. Based on these assignments the spectrum of **4b** at room temperature could also be assigned. The appearance of signals for the olefinic protons on C12 (δ_H = 4.68 and 4.73 ppm at 25 °C), which resolved into an AB system at low temperature, together with the disappearance of the signals for the C12 methyl group and the C1 methylene protons at 4.42 ppm in FPP are consistent with bond formation between C1 and C10. The double bond was assigned to be between C12 and C11 as opposed to C13 and C11 based on the known stereochemistry of germacrene A formation by AS from FPP.[4] While all ^1H NMR signals of **4b** were dependent on the temperature, the existence of two conformers was most obvious from the resonances for the protons on C4, C6 and C14 (Figure 3). For instance, H6 gave rise to two well-resolved signals at 5.05 and 5.36 ppm at −60 °C, while at high temperature only a poorly resolved double doublet was observed at 5.18 ppm. At low temperature the splitting patterns for the respective C6 protons were different in the two conformers; in the predominant conformer a relatively broad doublet was observed while in the minor conformer coupling to both protons on C5 was evident. Similarly, the patterns of the H4 resonances changed with temperature. One of the two protons leads to a complex multiplet at 90 °C; this proton was well resolved at low temperature with clear coupling to the geminal proton, the vicinal protons on C5 and the fluoro substituent.

Conformational isomerism of the germacrene family of natural products, in particular the (*E,E*)-germacranes, has been studied previously both by NMR spectroscopy and computationally.[14] The ten-membered ring can adopt four distinct conformations with up–up, down–down (Figure 3), up–down and down–up orientations of the methyl groups on C3 and C7.[14]

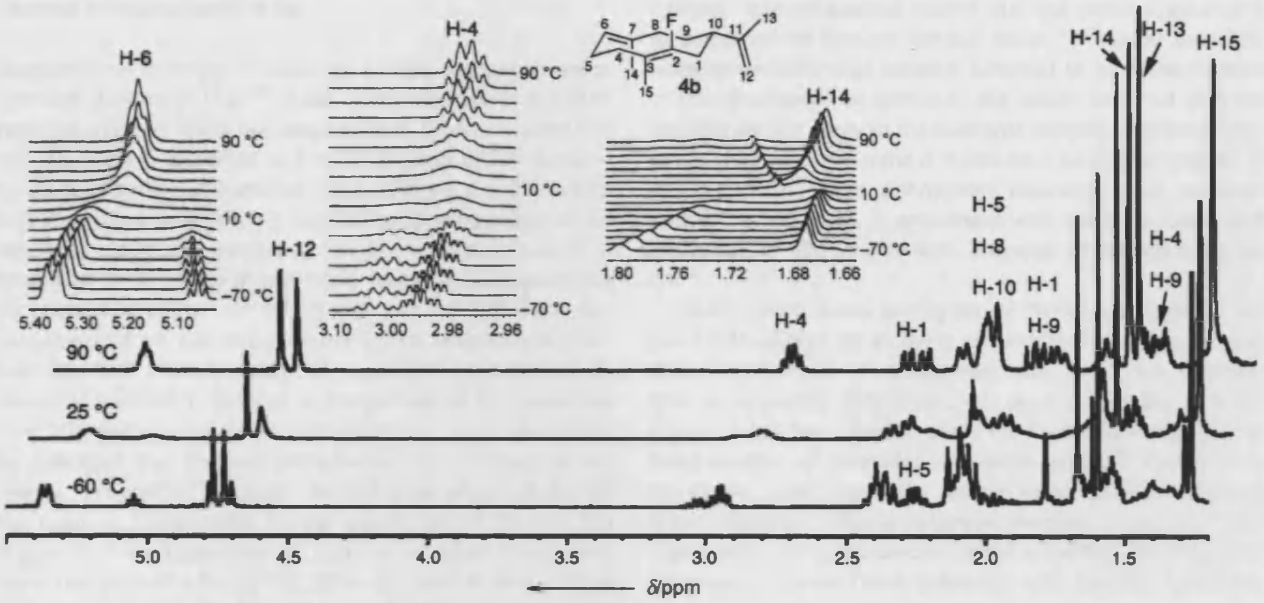


Figure 3. The ^{1}H NMR spectra (500 MHz) of **4b** at −60, 25 and 90 °C with assignments shown. Insets show the temperature dependent change in the resonances for H6, one of the H4 protons and CH_3-14. Clearly two conformational isomers exist in a ratio of approximate 3:1 at the lower temperatures (integrals not shown), and the signals for the two coalesce at approximately 30 °C. The conformation of the down–down isomer of **4b** is also shown.

Table 1. ^{1}H NMR spectra of **4b** at −60, 25 and 90 °C.[a]

	−60 °C major	−60 °C minor	25 °C	90 °C
CH_2-1	1.90–2.07 (m) and 2.33–2.44 (m)	1.90–2.07 (m) and 2.33–2.44 (m)	1.99–2.14 (m) and 2.35–2.45 (m)	1.88–2.02 (m) and 2.42 CH_2-1 (ddd, J = 3.5, 14.5, 32.5 Hz)
CH_2-4	1.51–1.58 (m) 2.95 (ddt, J = 2, 6, 12.5 Hz) 3.00–3.06 (m)	1.51–1.58 (m) 3.03 (brq, J = 10 Hz)	1.53–1.58 (m) and 2.83–3.02 (brm)	1.52–1.60 (m) and 2.83–2.90 (dq, J = 1.5, 9.5 Hz)
CH_2-5	2.02–2.14 (m) and 2.25 (ddt, J = 1.5, 5.5, 12 Hz)	2.02–2.14 (m) and 2.25 (ddt, J = 1.5, 5.5, 12 Hz)	2.11–2.14 (m) and 2.22–2.30 (brm)	2.12–2.17 (m) and 2.24–2.26 (m)
CH-6	5.36 (brd, J = 12.5 Hz)	5.05 (t, J = 8 Hz)	5.02–5.10 (brs) and 5.25–5.32 (brm)	5.18 (m)
CH_2-8	2.02–2.14 (m)	2.02–2.14 (m)	2.11–2.14 (m)	2.12–2.17 (m)
CH_2-9	1.51–1.58 (m) and 1.90–2.07 (m)	1.51–1.58 (m) and 1.90–2.07 (m)	1.53–1.58 (m) and 1.99–2.14 (m)	1.52–1.60 (m) and 1.88–2.02 (m)
CH-10	2.02–2.14 (m)	2.02–2.14 (m)	2.11–2.14 (m)	2.12–2.17 (m)
CH_2-12	pseudo AB system 4.70, 4.73, 4.76, 4.78	pseudo AB system 4.70, 4.73, 4.76, 4.78	4.68 (brs) and 4.73 (s)	4.64 (s) and 4.70 (s)
CH_3-13	1.67 (s)	1.79 (s)	1.62 (s)	1.62 (s)
CH_3-14	1.60 (s)	1.62 (s)	1.67 (br)	1.66 (s)
CH_3-15	1.26 (d, J_{HF} = 3 Hz)	1.28 (d, J_{HF} = 3 Hz)	1.32 (d, J_{HF} = 3 Hz)	1.36 (d, J_{HF} = 2.5 Hz)

[a] All resonances are δ_H (500 MHz, [D_8]toluene) in ppm. Entries are chemical-shift range followed by multiplicity in parentheses and coupling constants. Some proton resonances overlapped; no distinction was made in the chemical-shift range quoted for these protons.

These conformations are interconvertible by rotation of each of the double bonds through the ring and inversion of the C9–C10 unit, the conformation of which is largely determined by the exocyclic subsistent on C10, which favours a pseudo-equatorial orientation. For (+)-hedycaryol ((10*R*)-11-hydroxy-germacrene A) the up–up configuration predominated at all temperatures according to NMR studies (75% at −30 °C).[15] It is reasonable to suggest that the down–down orientation of the methyl groups is the dominant orientation in **4b** since AS produces (−)-germacrene A;[16] hence, the isopropylidene group will predominantly adopt the pseudoequatorial configuration. Analysis of the relative intensities of the resonances for the proton on C6 in the −60 °C ^{1}H NMR spectrum indicates that the ratio between the major and minor conformer of **4b** was 3.22:1; this is similar to what had been observed for (+)-hedycaryol.

Thermal rearrangement of 4b

Germacrene A is known to undergo a Cope rearrangement to generate β-elemene (**7a**).[13] It was partly because of the temperature induced Cope rearrangement in GC-MS experiments that **4a** was first identified as a minor product of FPP turnover by AS.[5] To further characterize compound **4b**, GC-MS analysis was performed with varying injector temperatures but under otherwise identical conditions (Figure 1). Germacrene A is known to undergo significant Cope rearrangement when the injector temperature is set to 200 °C.[5] In contrast, thermal rearrangement of **4b** was not observed at this temperature. However, at 250 °C a small amount of rearranged product was observed and at 300 °C thermal rearrangement of **4b** was dominant (Figure 1); furthermore, high-resolution mass spectrometry indicated that the two compounds had an identical elemental composition ($C_{15}H_{23}F$). As had been observed for **4b** the mass to charge ratio for the parent ion of **7b** was 222 (Figure 2). The fragmentation patterns of both compounds were indicative of a loss of CH_3 (*m/z* −15) and HF (*m/z* −20) as well as loss of both CH_3 and HF (*m/z* −35). The parent molecular ion of **4b** was more intense than that of the presumed fluoro-β-elemene (**7b**); this was most likely as a consequence of facilitated loss of hydrogen fluoride from **7b** to generate a conjugated diene compared to elimination of HF from **4b**.

The presence of the fluoro-substituent in **4b** appears to slow the Cope rearrangement compared to **4a**. The effects of fluorine substituents on six electron electrocylic processes is unclear since steric and electronic effects often compete, which leads to unpredictable effects on the reaction kinetics;[17] hence a rationalization of the reduced reactivity of **4b** is difficult.

Conclusions

In summary, the work described here shows that 2-fluorofarnesyl-diphosphate (**1b**) is a substrate of aristolochene synthase and is converted exclusively to 2-fluorogermacrene A (**4b**). This result provides strong support that germacrene A is generated as an on-path reaction product of the conversion of **1a** to aristolochene (**6**). If germacrene A was produced in a minor, but separate pathway by AS, more than one product would be expected from the incubation with 2-fluoro-FPP. The observation that germacrene A does not act as a substrate for AS is most likely the consequence of its very poor solubility in aqueous solvents.

The effectiveness of the fluoro analogue of germacrene A in blocking the second cyclisation step is most likely a consequence of the inductive effect of this highly electronegative group on the C2=C3 double bond and the destabilising effect on the positive charge on C3 in eudesmane cation. This effect leads to an increase in the height of the energy barrier of the step following **4b** formation. It has been shown previously that vinyl-fluoro groups reduce the rate of solvolysis of fluoro terpenoid methanesulfonates.[18–20] Interestingly, the 2-fluoro substituent of FPP did not prevent the first cyclisation step that leads to 2-fluorogermacrene A in the present case despite evidence from mutational studies that this process occurs in a stepwise fashion through farnesyl cation.[21–23] Either the allylic stabilisation in farnesyl cation is sufficient to push the reaction to the decadiene-ring system or the results obtained with the mutants do not apply to the wild-type enzyme and the cyclisation of FPP to germacrene A is indeed a concerted process in which cyclisation and diphosphate expulsion occur concomitantly. This would be in agreement with the observation that cyclisation of FPP occurs with inversion of configuration on C1.[24]

Clearly, vinyl fluoro analogues of farnesyldiphosphate are powerful reagents for studying the mechanism of enzyme-catalysed cyclisations of sesquiterpenoids, which are reactions that are inherently difficult to study spectrometrically. A similar approach has been used to study the taxadiene synthase-catalysed reaction of geranyl-geranyl-diphosphate.[25] Chemical interception could provide a unique opportunity to interrupt these multistep polyene cylisation reactions to decipher the mechanistic principles used by terpene cyclases to control the chemistry of carbocationic processes with exquisite specificity.

Experimental Section

Expression and purification of AS in *E. coli*: AS was produced in *E. coli* BL21(DE3) cells that harboured a cDNA for AS under the control of the T7 promoter. Cells were grown at 37 °C in Luria–Bertani (LB) medium with ampicillin (0.3 mM) until they reached an A_{600} of 0.5. They were induced with isopropyl-β-D-1-thiogalactopyranoside (0.5 mM), incubated for a further 3 h and harvested by centrifugation at 8000*g* for 10 min. Protein was then extracted from the inclusion bodies and purified by following the protocol described previously.[5] The AS sample was pure as judged by SDS gel electrophoresis.

(2*Z*,6*E*)-2-Fluoro-3,7,11-trimethyldodeca-2,6,10-trien-1-ol (10) and (2*E*,6*E*)-2-fluoro-3,7,11-trimethyldodeca-2,6,10-trien-1-ol (9): Triethyl 2-fluoro-2-phosphonoacetate (0.84 cm^3, 4.13 mmol) and a solution of geranyl acetone (1.02 cm^3, 4.54 mmol) in anhydrous THF (5 cm^3) were sequentially added dropwise to a stirred suspension of sodium hydride (60% dispersion in mineral oil, 0.10 g, 4.13 mol) in anhydrous THF (10 cm^3), at 0 °C under an N_2 atmosphere. The complete reaction mixture was stirred for 14 h under N_2. Water (20 cm^3) and diethyl ether (20 cm^3) were added and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2×15 cm^3). The combined ethereal extracts were washed with water (2×10 cm^3) and saturated NaCl solution (1×10 cm^3), dried over anhydrous $MgSO_4$, filtered and then concentrated under reduced pressure. The resulting ester was a 1:1 mixture of *E* and *Z* isomers and was reduced without further purification. Diisobutylaluminum hydride (1.0 M solution in hexanes, 10.33 cm^3, 10.33 mmol) was added dropwise to a stirred solution of the crude ester in anhydrous THF (30 cm^3) at −78 °C, (over 3 min). The reaction mixture was allowed to stir at −78 °C for 1 h, then 0 °C for 1 h and was then quenched with saturated sodium potassium tartrate solution (30 cm^3). The resulting mixture was then extracted with diethyl ether (3×20 cm^3). The combined organic extracts were dried over anhydrous $MgSO_4$, filtered and then concentrated under reduced pressure. Purification of the residue by flash-column chromatography on silica gel with hexane and ethyl acetate (4:1) as eluent gave the *Z* isomer (0.48 g, 49%) as a colourless oil followed by the *E* isomer (0.46 g, 46%) as a colourless oil.

The *Z* isomer (**10**): R_f 0.28 (4:1 hexane-ethyl acetate); HRMS (EI^+, M^+): found 240.1890, $C_{15}H_{25}OF$ requires 240.1889; ν_{max} (thin film): 3358.1, 2920.4, 1703.7, 1444.7, 1380.2, 1266.9, 1148.9, 1105.3, 1012.5 cm^{-1}; δ_H (500 MHz, C^2HCl_3): 1.62 (6H, s, 2×CH_3), 1.70 (3H, d, $^4J_{HH}$=1.0, CH_3), 1.71 (3H, d, $^4J_{HF}$=3.48, CH_3C=CF), 1.79 (brs, 1H; OH), 1.99–2.14 (8H, m, 2×CH_2CH_2), 4.19 (2H, dd, $^3J_{HF}$=23.0, $^3J_{HH}$=6.0, CH_2OH), 5.09 (2H, m, 2×C=CH); δ_C (125 MHz, C^2HCl_3): 13.48 (d, $^3J_{CF}$=9.0, CH_3C=CF), 15.99 (CH_3), 17.67 (CH_3), 25.67 (CH_3), 26.36 (d, $^4J_{CF}$=4.0, CH_2CH_2C=CF), 26.62 (CH_2), 31.71 (d, $^3J_{CF}$=5.00, CH_2CH_2C=CF), 39.67 (CH_2), 57.67 (d, $^2J_{CF}$=31.0, C=CFCH_2OH), 115.74 (d, $^2J_{CF}$=14, CH_3C=$CFCH_2$), 123.00 (C=CH), 124.14 (C=CH), 131.52 (C=CH), 136.52 (C=CH), 152.57 (d, $^1J_{CF}$=242.50, CH_3C=CF); δ_F (283 MHz, C^2HCl_3): −119.5 (t, $^3J_{HF}$=23.0); *m/z* (EI^+) 240.2 (2%, $[M]^+$), 69.1 (100%), 81.1 (20%).

The *E* isomer (**9**): R_f 0.20 (4:1 hexane-ethyl acetate); HRMS (EI^+, M^+): found 240.1897, $C_{15}H_{25}OF$ requires 240.1889; ν_{max} (thin film): 3349.0, 2931.5, 2361.3, 1703.1, 1667.2, 1450.6, 1380.5, 1270.8, 1238.0, 1158.9, 1105.5, 1012.8, 910.9 cm^{-1}; δ_H (500 MHz, C^2HCl_3): 1.62 (6H, s, 2×CH_3), 1.70 (3H, d, $^4J_{HF}$=2.5, CH_3C=CF), 1.70 (3H, s, CH_3), 1.82 (1H, b, OH), 1.98–2.15 (8H, m, 2×CH_2CH_2), 4.23 (2H, dd, $^3J_{HF}$=22.5, $^3J_{HH}$=5.5, CH_2OH), 5.11 (2H, m, 2×C=CH); δ_C (125 MHz, C^2HCl_3): 15.37 (d, ^3J_C=5.0, CH_3C=CF), 16.0 (CH_3), 17.7 (CH_3), 25.7 (CH_3), 25.9 (d, $^4J_{CF}$=1.25, CH_2CH_2C=CF), 26.7 (CH_2), 29.8 (d, $^3J_{CF}$=6.25, CH_2CH_2C=CF), 39.7 (CH_2), 58.0 (d, $^2J_{CF}$=32.5, CH_2OH), 116.0 (d, $^2J_{CF}$=16.25, CH_3CCFCH_2), 123.5 (C=CH), 124.3 (C=CH), 131.4 (C=CH), 135.7 (C=CH), 151.79 (d, $^1J_{CF}$=240, CH_3C=CF); δ_F (283 MHz, C^2HCl_3): 121.13 (t, $^3J_{HF}$=22.5); *m/z* (EI^+) 240.2 (4%, $[M]^+$), 69.1 (100%), 81.1 (35%).

Tris-ammonium-(2*Z*,6*E*)-2-fluoro-3,7,11-trimethyldodeca-2,6,10-trien-1-yl diphosphate (1b): A stirred solution of alcohol **10** (0.22 g, 0.92 mmol) and triethylamine (0.26 cm^3, 1.84 mmol) in anhydrous THF (10 cm^3) was cooled to −45 °C; MsCl (0.09 cm^3, 1.20 mmol) was then added. The resulting milky mixture was stirred at −45 °C for 45 min, then a solution of LiBr (0.35 g, 3.68 mmol) in THF (5 cm^3) was added at −45 °C, by using a needle. The suspension was allowed to warm to 0 °C and stirred for 1 h, after which time the reaction was judged complete by TLC analysis. Cold water (10 cm^3) and hexane (10 cm^3) were added. The two layers were separated, and the aqueous layer was extracted with hexane (2×10 cm^3). The pooled organic layers were washed with saturated $NaHCO_3$ solution (10 cm^3) and saturated NaCl solution (10 cm^3), and then dried over anhydrous $NaSO_4$ and filtered. Concentration of the solvent under reduced pressure gave the required bromide as a light yellow oil that was used without further purification. Freshly recrystallized tris(tetra-*n*-butylammonium) hydrogen diphosphate (1.80 g, 2.00 mmol), which was prepared by the method of Poulter, was added to a stirred solution of the bromide in anhydrous acetonitrile (10 cm^3) under N_2.[12] The complete reaction mixture was stirred for 2 h. Solvent was then removed under reduced pressure and the resulting residue was dissolved in 1:49 (*v/v*) isopropyl alcohol and 25 mM ammonium hydrogencarbonate solution (2 cm^3; ion-exchange buffer). The pale yellow solution was slowly passed through a column that contained 30 equiv of DOWEX 50W-X8 (100–200 mesh) cation-exchange resin that had been pre-equilibrated with two-column volumes of ion-exchange buffer. The column was eluted with two-column volumes of ion-exchange buffer at a flow rate of one-column volume per 15 min. The clear, light yellow eluent was freeze dried to yield a fluffy yellow solid, which was purified by reverse phase HPLC (150× 21.2 mm Phenomenex Luna C-18 column, eluted under isocratic conditions with 10% B for 20 min, a linear gradient to 60% B over 25 min, then a linear gradient to 100% B over 5 min and finally with 100% B for 10 min; solvent B: CH_3CN; solvent A: 25 mM NH_4HCO_3 in water, flow rate 5.0 $cm^3 min^{-1}$, detection at 220 nm) to give title compound as a white solid (0.15 g, 36%); HPLC t_R 36.99 min; purity 96.33% by analytical HPLC; HRMS (EI^-, $[M-H]^-$): found 399.1135, $C_{15}H_{26}O_7FP_2$ requires 399.1143; ν_{max} (KBr disc): 2963.4, 1701.8, 1494.4, 1455.4, 1411.8, 1204.0, 1164.6, 1122.1, 1091.9, 1034.7, 908.7, 825.9, 723.3, 595.4, 552.7 cm^{-1}; δ_H (500 MHz, ^2H_2O): 1.47 (3H, s, CH_3), 1.48 (3H, s, CH_3), 1.54 (3H, s, CH_3), 1.59 (3H, d, $^4J_{HF}$=2.5, CH_3C=CF), 1.86–2.01 (8H, m, $2CH_2CH_2$), 4.42 (2H, dd, $^3J_{HF}$=24.0, $^3J_{HP}$=6.0, C=CFCH_2OP), 5.02 (1H, t, $^3J_{HH}$=7.0, C=CH), 5.06 (1H, m, C=CH); δ_C (125 MHz, ^2H_2O): 4.82 (d, $^3J_{CF}$=5.0, CH_3C=CF), 15.33 (CH_3), 17.06 (CH_3), 24.98 (CH_3), 25.33 (CH_2), 26.04 (CH_2), 29.38 (d, $^3J_{CF}$=6.0, CH_2CH_2C=CF), 39.04 (CH_2), 60.54 (dd, $^2J_{CF}$=31.25, $^2J_{CP}$=5.00, C=CFCH_2OP), 119.33 (d, $^2J_{CF}$=14.0, CH_3C=$CFCH_2$), 123.92 (C=CH), 124.44 (C=CH), 132.82 (C=CH), 136.62 (C=CH), 149.25 (dd, $^1J_{CF}$=237.5, $^4J_{CP}$=9.0, CH_3C=$CFCH_2OP$); δ_F (283 MHz, ^2H_2O): −120.23 (t, $^3J_{HF}$=24.0); δ_P (121 MHz, ^2H_2O): −6.29 (d, $^3J_{PP}$=21.0), 10.50 (d, $^3J_{PP}$=1.0); MS (ES^-) *m/z* 399.2 (15%, $[M-H^+]^-$), 74.9 (100%,), 198.8 (40%).

Incubation of 2-fluoro farnesyl pyrophosphate (1b) with AS: Aristolochene synthase solution (3.60 cm^3, 638 μM) was diluted with buffer consisting of 20 mM tris-HCl, 5 mM β-mercaptoethanol, 5 mM $MgCl_2$ and 15% (*v/v*) glycerol, pH 7.5 (to 33.5 cm^3). The assay solution was gently mixed as **1b** (30.0 mg, 0.067 mmol) and pentane (2 cm^3) were added sequentially. After incubation for 48 h at 25 °C, the olefin products were extracted with pentane (3×80 cm^3). The pooled pentane extracts were concentrated under a nitrogen stream until about 5 cm^3 of solvent remained. This solution was passed through a short pad of silica gel overlayed with anhydrous $MgSO_4$. The pentane was concentrated under reduced pressure to yield a single product as judged by GC-MS analysis (4.0 mg, 27%); GC-MS t_R 26.24 min. GC-MS was performed by using a Hewlett Packard 6890 GC fitted with a J&W scientific DB-5MS column (30 m×0.25 mm internal diameter) and a Micromass GCT Premiere that detected in the range *m/z* 50–800 in EI^+ mode by 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, this was raised 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).

HRMS (EI^+): found 222.1785, $C_{15}H_{23}F$ requires 222.1784. Compound **4b** displayed conformational isomerism which complicated the interpretation of its NMR spectra. Consequently, variable temperature NMR experiments were performed. The ^1H NMR data are reported in Table 1. The ^{13}C NMR spectrum could not be observed directly due to the small quantity of material isolated and reduction of line intensity due to the conformational isomerism, but could be indirectly observed and interpreted through the cross peaks observed in the various 2D spectra. δ_C (125 MHz, [D_8]toluene, 25 °C): 14.7 (C-14), 19.5 (C-13 and C-15), 25.1 (C-5), 27.5 (C-4), 33.3 (C-9), 33.8 (C-8), 34.1 (C-1), 45.4 (C-10), 108.9 (C-12), 112.0 (C-3), 128.2 (C-7), 137.1 (C-6), 149.6 (C-11), 154.5, 156.7 (d, C-2); δ_F (283 MHz, [D_8]toluene, −60 °C) −89.2 (major conformer) and −91.8 (minor); δ_F (283 MHz, [D_8]toluene, 25 °C) −88.8 (major conformer) and −91.3 (minor); *m/z* (EI^+) 222.2 (12%, $[M]^+$), 207.2 (19%, $[M-CH_3]^+$), 202.2 (10%, $[M-HF]^+$), 187.2 (18%, $[M-HF-CH_3]^+$), 179.1 (5%), 165.1 (11%), 159.1 (18%), 145.1 (22%), 131.0 (20%), 121.1 (21%), 107.1 (29%), 93.1 (50%), 79.1 (48%), 68.1 (100%), 65.0 (21%), 53.0 (50%).

Thermal rearrangement of 4b: The pentane extractable products obtained from incubation of **1b** with AS were diluted to approximately 1 mg mL^{-1} in hexane and 2 mm^3 of this solution was injected onto the GC-MS column by using the conditions described above, except the injector temperature was varied from 50 to

300 °C in 50 °C steps for each run. The peak at t_R 26.24 min remained unchanged. At temperatures 250–300 °C a second peak appeared with t_R 23.72 min (Figure 1); HRMS (EI^+): found 222.1792, $C_{15}H_{23}F$ requires 222.1784; *m/z* (EI^+) 222.2 (2%, $[M]^+$), 207.2 (5%, $[M-CH_3]^+$), 202.2 (10%, $[M-HF]^+$), 187.2 (50%, $[M-HF-CH_3]^+$), 173.1 (10%), 159.1 (38%), 145.1 (60%), 133.1 (25%), 131.1 (40%), 119.1 (98%), 105.1 (60%), 91.1 (100%), 77.0 (55%), 67.1 (50%), 53.0 (35%).

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Keywords: enzyme catalysis · fluorine · natural products · structure–activity relationships · terpenoids

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Competitive inhibition of aristolochene synthase by phenyl-substituted farnesyl diphosphates: evidence of active site plasticity†

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Analogues of farnesyl diphosphate (FPP, **1**) containing phenyl substituents in place of methyl groups have been prepared in syntheses that feature use of a Suzuki–Miyaura reaction as a key step. These analogues were found not to act as substrates of the sesquiterpene cyclase aristolochene synthase from *Penicillium roqueforti* (AS). However, they were potent competitive inhibitors of AS with K_I-values ranging from 0.8 to 1.2 μM. These results indicate that the diphosphate group contributes the largest part to the binding of the substrate to AS and that the active sites of terpene synthases are sufficiently flexible to accommodate even substrate analogues with large substituents suggesting a potential way for the generation of non-natural terpenoids. Molecular mechanics simulations of the enzyme bound inhibitors suggested that small changes in orientations of active site residues and subtle alterations of the conformation of the backbones of the inhibitors are sufficient to accommodate the phenyl-farnesyl-diphosphates.

Introduction

Terpenoids are the largest group of natural products with immense diversity in their structure and function.[1] There has been significant interest in terpenoids as antifungal, antibacterial and anticancer agents for the treatment of human disease with multibillion-dollar sales worldwide. Despite their enormous structural variety, all terpenes are derived from simple linear precursors such as geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranyl geranyl diphosphate (GGPP). Cyclisation of GPP to monoterpenes, FPP to sesquiterpenes and GGPP to diterpenes is accomplished by terpene synthases. These enzymes, many of which share the mainly α-helical class I terpene fold, serve as high fidelity templates that subtly channel conformation and stereochemistry during the cyclisation reactions and are key to the generation of the wide diversity in structure and stereochemistry found in terpenoids (Fig. 1). They bind their respective substrates together with the obligatory Mg^{2+}-cofactor, catalyse the loss of the diphosphate group and chaperone the reaction intermediates along complex reaction pathways; often with exquisite specificity. Terpene synthases catalyse highly regio- and stereospecific cyclisations, hydride

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† Electronic supplementary information (ESI) available: General experimental procedures and protocols for diphosphorylation of target alcohols. Enzyme preparation, purification and kinetic characterisation of inhibitors as well as plots of [*I*] *versus* apparent K_M for FPP turnover by AS in the presence of **19**, **29**, and **36**. See DOI: 10.1039/b713301b

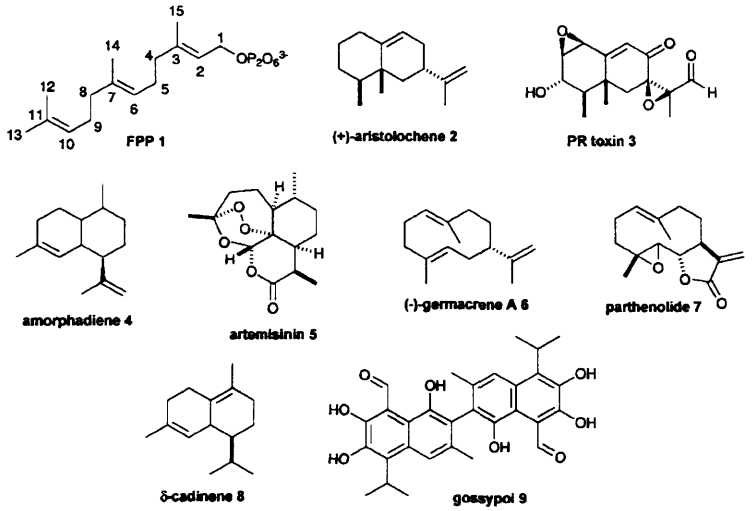
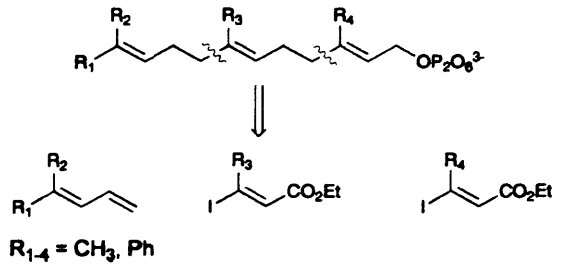


Fig. 1 Structure of FPP (**1**) and some examples of the complexity of natural products derived from it. The parent sesquiterpenes such as **2**, **4**, **6** and **8** may be further transformed by downstream metabolic processes into a diverse set of sesquiterpenoids such as PR toxin (**3**), artemisinin (**5**), parthenolide (**7**), and gossypol (**9**).

and methyl transfers as well as deprotonation reactions while at the same time excluding solvent from the active site to prevent premature quenching by water of the extremely reactive cationic reaction intermediates. The majority of the characterised terpene synthases form only one or a few products; however, there are some enzymes that form a variety of products from a single substrate. The δ-selinene and γ-humulene synathases from *Abies grandis* (the grand fir) produce 34 and 52 sesquiterpenes, respectively.[2,3]

Clearly the exact arrangement of the amino acid residues in the active site of individual terpene cyclases within a common protein fold is crucial for the outcome of the reaction. Site directed replacement of amino acids in and around the active site of several terpene synthases has indicated that the product distribution can often be altered.[4–13] While these experiments indicated the high adaptability of these enzymes, the products generated by the mutant enzymes were always known natural terpenoids. However, the plasticity of terpene synthases suggests that they have the potential for the generation of novel "unnatural" terpenes from unnatural prenyl-diphosphate analogues. While biosynthetic restrictions imply that all prenyl-diphosphates carry methyl substituents only, a synthetic approach to analogues of the substituents of terpene cyclases currently allows the introduction of a wide variety of substituents.

To test whether the active sites of terpene synthases can accommodate alternate substrates we report here syntheses of the FPP analogues containing phenyl substituents in place of the methyl groups on C3 and C11 (Scheme 1). While (2*E*,6*E*,10*E*)-3,7-dimethyl-11-phenyldodeca-2,6,10-trien-1-yl diphosphate (**19**), (2*E*,6*E*,10*Z*)-3,7-dimethyl-11-phenyldodeca-2,6,10-trien-1-yl diphosphate (**29**) and (2*Z*,6*E*)-7,11-dimethyl-3-phenyldodeca-2,6,10-trien-1-yl diphosphate (**36**) were not converted to products by AS from *Penicillium roqueforti*, all three FPP analogues acted as potent competitive inhibitors indicating that they bound to the active site of this sesquiterpene cyclase in a fashion similar to the natural substrate, thereby highlighting the enormous plasticity of these enzymes and their potential for the production of novel terpenoid analogues that might have superior properties for many applications including the treatment of human disease.



Scheme 1

Results

In order to explore the plasticity of terpene cyclases and to investigate their potential for the synthesis of non-natural terpenoid analogues from modified prenyl diphosphates, we have synthesised three FPP analogues in which the methyl groups on C3 and C11 were replaced by phenyl substituents. The general structure of these analogues is shown in Scheme 1 along with the retrosynthetic disconnection used here. Previous syntheses of analogues of farnesol concentrated on modification of the C15 methyl group[14–18] and to some extent the C14[19] with little attention to the C12 and C13 methyl groups of FPP. These syntheses have largely used the elegant and robust chain extension methodology of Weiler and Sum as a key step.[20] For the syntheses of the compounds required here, a synthesis allowing changes to all the methyl groups of FPP was required and it seemed that a disconnection that exploits the oligomeric nature of FPP would be both appropriate and efficient. Hence two series of monomers were envisaged with one group being composed of 1,3-dienes representing the C-terminal end of the farnesyl group and the other set comprising various *E*-crotonyl iodides. The key forward reaction to connect these two monomers would involve transformation of the terminal alkenyl group with a hindered borane such as 9-BBN followed by Suzuki–Miyaura coupling with the appropriate iodide.[21–25]

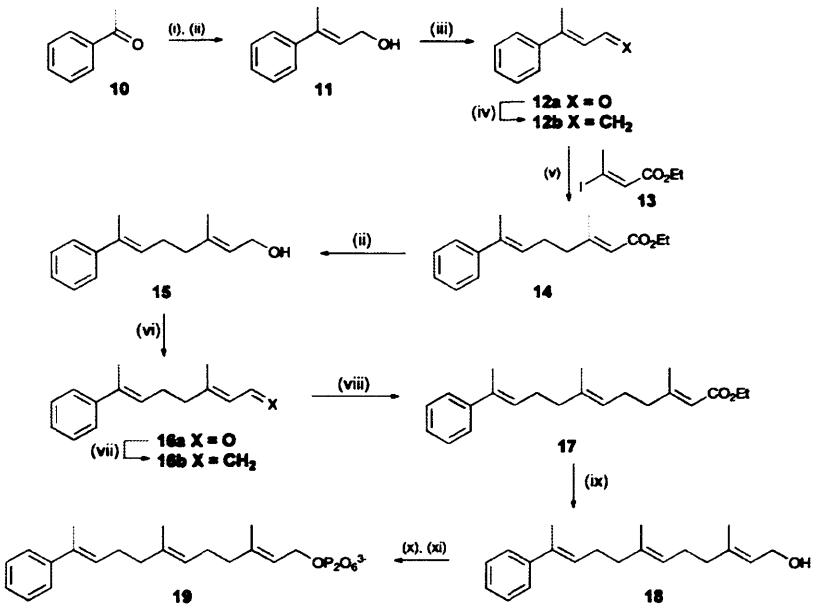
Synthesis of *E*- and *Z*-11-phenyl farnesyl diphosphate analogues

Both of these compounds were prepared using novel Suzuki–Miyaura coupling methodology. The synthesis of the *E*-11-phenyl FPP analogue **19** is shown in Scheme 2. The C-terminal 1,3-diene, compound **12b**, was prepared from acetophenone **10** in four steps. Horner–Emmons modification using triethyl phosphonoacetate and sodium hydride followed by DIBAL-H reduction yielded the *E*-cinnamyl alcohol **11**, then oxidation using PCC and then a Wittig reaction using methyltriphenylphosphonium bromide gave the required *E*-phenyl diene **12b**. This compound was then hydroborated using crystalline 9-BBN and immediately coupled to iodide **13** using $PdCl_2$dppf as catalyst, triphenylarsine as co-ligand and aqueous sodium hydroxide as base.[24] The *E*-phenyl-geranyl ester product **14** was isolated in 59% yield under these conditions. Ester **14** was then homologated to a new 1,3-diene derivative **16b** in a very similar manner to the preparation of **12b** and a second Suzuki coupling to iodide **13** under identical conditions gave the farnesyl ester analogue **17** in 54% yield for the coupling step. Ester **17** was then transformed into the diphosphate **19** by DIBAL-H reduction, bromination of the resulting alcohol and diphosphorylation using the methodology of Poulter *et al.*[26]

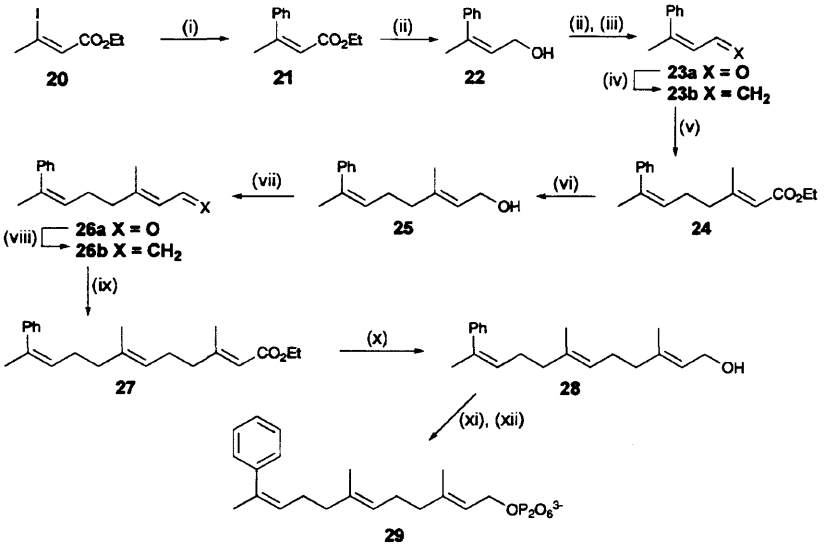
Preparation of the *Z*-11-phenyl FPP analogue was achieved in much the same fashion (Scheme 3). Preparation of the initial *Z*-1,3-diene **23b** was a little more complex because Horner–Emmons or Wittig modification of acetophenone **10** will only yield a small amount of the *Z*-ester **21**. It was therefore made by Suzuki–Miyaura coupling of phenylboronic acid to *Z*-crotonyl iodide **20**, which was an intermediate in the preparation of **13**.[27] Hence treatment of **20** with phenylboronic acid in the presence of $Pd(OAc)_2$, triphenylarsine and K_3PO_4 in toluene at 90 °C yielded ester **21** in 44% yield after chromatography.[28] This ester was then transformed into the 1,3-diene **23b** and then to the *Z*-11-phenyl FPP analogue **29** using almost identical chemistry as used for the preparation of **19**. The two crucial Suzuki–Miyaura coupling steps took place in approximately 40% yield in each case.

Synthesis of 3-phenyl farnesyl diphosphate 36

For the synthesis of **36** the chain extension methodology of Weiler and Sum was found most effective (Scheme 4).[20] Hence geraniol **30** was transformed into geranyl bromide and then treated with



Scheme 2 Synthesis of the *E*-11-phenylfarnesyl diphosphate derivative **19**. *Reagents and conditions:* (i) triethylphosphonoacetate, NaH, DME, 61%; (ii) DIBAL-H, THF, −78 °C, 96%; (iii) PCC, CH_2Cl_2, 56%; (iv) CH_3PPh_3Br, *n*-BuLi, THF, 94%; (v) 9-BBN, THF then **13**, $PdCl_2dppf$, NaOH, $AsPh_3$, THF, 50 °C, 59%; (vi) TPAP, NMO, CH_3CN, 89%; (vii) CH_3PPh_3Br, *n*-BuLi, THF, 81%; (viii) 9-BBN, THF then **13**, $PdCl_2dppf$, NaOH, $AsPh_3$, THF, 50 °C, 54%; (ix) DIBAL-H, THF, −78 °C, 86%; (x) NEt_3, MsCl, −45 °C then LiBr; (xi) $(Bu_4N)_3HP_2O_7$, CH_3CN then cation exchange Bu_4N^+/NH_4^+, 31%.

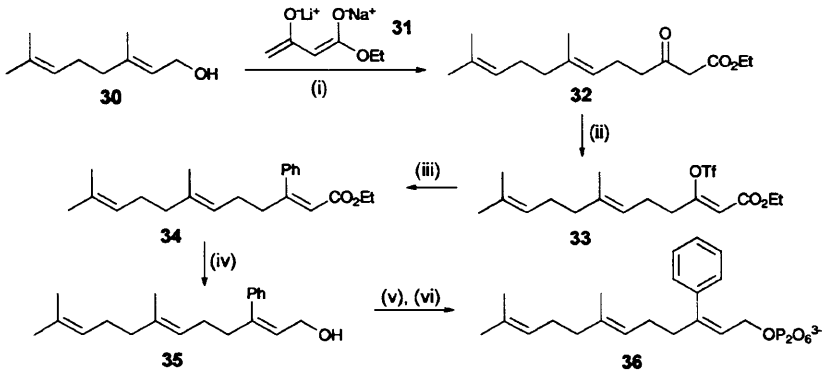


Scheme 3 Synthesis of *Z*-11-phenylfarnesyl diphosphate **29**. *Reagents and conditions:* (i) $PhB(OH)_2$, $Pd(OAc)_2$, $AsPh_3$, K_3PO_4, toluene, 90 °C, 44%; (ii) DIBAL-H, THF, −78 °C, 99%; (iii) oxalyl chloride, DMSO, CH_2Cl_2 −78 °C then NEt_3, 88%; (iv) CH_3PPh_3Br, *n*-BuLi, THF, 76%; (v) 9-BBN, THF then **13**, $PdCl_2dppf$, NaOH, $AsPh_3$, THF, 50 °C, 41%; (vi) DIBAL-H, THF. −78 °C, 91%; (vii) TPAP, NMO, CH_3CN, 87%; (viii) CH_3PPh_3Br, *n*-BuLi, THF, 86%; (ix) 9-BBN, THF then **13**, $PdCl_2dppf$, NaOH, $AsPh_3$, THF, 50 °C, 40%; (x) DIBAL-H, THF, −78 °C, 77%; (xi) NEt_3, MsCl, −45 °C then LiBr; (xii) $(Bu_4N)_3HP_2O_7$, CH_3CN then cation exchange Bu_4N^+/NH_4^+, 36%.

the dienolate **31** derived from ethyl acetoacetate[20] giving the β-ketoester **32** in 92% yield. This was then treated with triflic anhydride and KHMDS at −78 °C in THF to give the *Z*-enol triflate compound **33** in 51% yield.[16] This compound underwent a Suzuki–Miyaura cross-coupling reaction with phenylboronic acid to give **34** in 66% yield. Derivatisation of this compound to the diphosphate analogue **36** was achieved in the usual manner.[26]

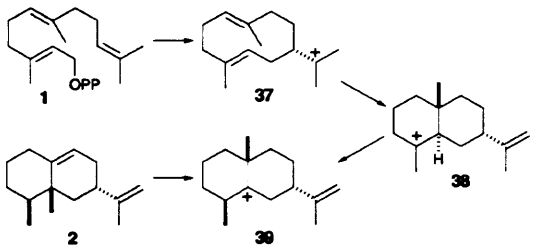
Studies of the kinetics of AS-catalysis in the presence of 19, 29 and 36

To test whether the active site of terpene synthases could accommodate the size increases resulting from the replacement of a methyl with a phenyl group, the farnesyl diphosphate analogues **19**, **29** and **36** were tested as substrates for and as inhibitors of



Scheme 4 Synthesis of 3-phenylfarnesyl diphosphate **36**. *Reagents and conditions:* (i) NEt_3, MsCl, THF, −45 °C then LiBr, then **31**, 92%; (ii) KHMDS, $(CF_3SO_2)_2O$, THF, −78 °C, 51%; (iii) $PhB(OH)_2$, $AsPh_3$, $Pd(OAc)_2$, Ag_2O, THF, Δ, 66%; (iv) DIBAL-H, THF, −78 °C, 85%; (v) NEt_3, MsCl, −45 °C then LiBr; (vi) $(Bu_4N)_3HP_2O_7$, CH_3CN then cation exchange Bu_4N^+/NH_4^+, 31%.

aristolochene synthase. AS is a Mg^{2+} dependent sesquiterpene cyclase that catalyses the conversion of FPP to (+)-aristolochene (**2**), which in the fungus is further transformed to produce a family of toxins that includes PR toxin (**3**) (Fig. 1).[29–31] The catalytic mechanism for the formation of **2** in the active site of AS has been studied extensively by classical substrate labelling studies as well as by analysing the reaction products generated from substrate analogues and by enzyme mutants.[9–11,32,33] The reaction proceeds through the intermediate eudesmane cation (**38**), the positive charge of which is stabilised through interaction with the indole ring of Trp 334 of AS. Hydride and methyl shifts followed by site-specific deprotonation generate the bicyclic product (Scheme 5).



Scheme 5 Proposed catalytic mechanism of AS from *P. roqueforti*.

The phenyl-FPPs **19**, **29** and **36** were incubated at a concentration of 200 μM with 100 nM aristolochene synthase at pH 7.5 in the presence of the essential cofactor Mg^{2+} at 25 °C. The reaction rate for the conversion of 5 μM FPP was proportional to the concentration of AS at this concentration of enzyme.[5,11] Incubations were overlayed with pentane in order to extract potential products and to minimise product inhibition that is often observed with these enzymes due to the hydrophobic nature of the terpenoid products. The organic phase was concentrated carefully and the reaction products analysed by GC-MS. While the production of aristolochene from FPP was manifest by the strong GC signal and by its mass spectrum after only 16 hours, even prolonged incubation with **19**, **29** and **36** of up to 7 days did not indicate the formation of any products as judged by GC-MS analysis suggesting that these compounds were not substrates of AS.

Each compound was examined as an inhibitor of AS using a radiolabelled assay. Initially, IC_{50} values were determined with FPP concentrations maintained at 1 μM (close to the K_M of FPP).[5,29] The concentrations of the phenyl-FPP analogues were varied between 1 mM and 1 nM. **19**, **29** and **36** acted as inhibitors of AS with IC_{50} values ranging from 1–5 μM. Therefore full K_i determinations were performed in order to examine the mode of inhibition of these compounds. The Michaelis constant of FPP was determined both in the presence and absence of each inhibitor at various concentrations by use of a non-linear fit.‡ Double reciprocal plots for each set of fitted data indicated that the compounds were all reversible competitive inhibitors of AS (Fig. 2) and hence bound to the enzyme's active site in a way similar to that of the natural substrate. The K_i-values were determined as 0.8 ± 0.2, 1.2 ± 0.2 and 1.2 ± 0.1 μM for **19**, **29**, and **36**, respectively (see ESI†). No time dependent inactivation of the enzyme was observed when each inhibitor was preincubated with enzyme prior to the assay indicating that the inhibition was reversible in all three cases.

Molecular mechanics simulations of inhibitor-bound AS

Each of the FPP-analogues **19**, **29** and **36** are characterised by the replacement of a methyl group by the much bulkier phenyl substituent. Despite the high structural similarities observed in sesquiterpene synthases, that suggest that these enzymes have evolved to facilitate subtle conformational changes of their common substrate through the positional reorganisation of only a limited number of active site residues, the observation that the phenyl-substituted farnesyl-diphosphates acted as potent competitive inhibitors of AS was somewhat surprising.

Each inhibitor was therefore docked to the active site of AS using the existing molecular model of **1** bound to AS[34] as a starting point. Energy minimisations of the docked structures were performed using the MMFF94 forcefield.[35] Amino acids within 6.5 Å of the inhibitor molecule were allowed to move while the co-ordinates of all other residues were fixed. Each inhibitor appeared to fit well into the active site of AS and only minor reorganisations of active site residues were necessary to avoid steric clashes through the introduction of the bulky phenyl substituents (Fig. 3). In

‡ Data were fitted using Systat Sigmaplot 10.0, 2007. Sigmaplot for Windows Version 10.0, Build 10.0.0.54, 2006, Systat Software Inc. 1735, Technology Drive, Ste 430, San Jose, CA 95110, USA. Molecular Operating Environment (MOE 2004.03) Chemical Computing Group, Inc., 1255 University St. Suite 1600, Montreal, Quebec, Canada. H3B 3 × 3.

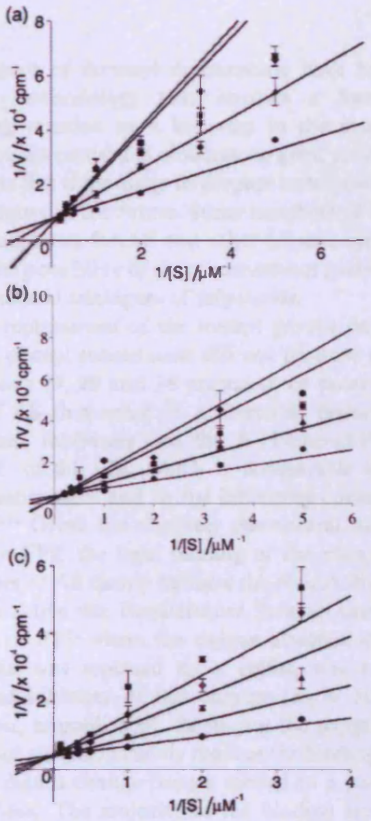


Fig. 2 Double reciprocal plots of initial rates *versus* the concentration of substrate for AS catalysed turnover of FPP in the presence of **19**, **29** and **36** are shown on panels a, b and c for increasing concentrations of inhibitor (0 μM (●), 1 μM (▲), 2 μM (◆) and 3 μM (■)). Intersection of the lines on the *y*-axis indicate that each compound is a competitive inhibitor of AS. All assays were carried out at 37 °C and pH 7.5.

addition, some rearrangements of the inhibitors' prenyl-chains were observed relative to the conformation calculated for FPP. In the *E*-11-phenyl FPP analogue (**19**) an alteration in the orientation of the C7–C11 portion of the prenyl chain and a movement of the diphosphate group towards the Tyr 92 residue of AS was observed. This was accompanied by an approximate 90° rotation of the phenyl ring of Phe 112 and minor rotations of the rings of Phe 178 and Tyr 92. The indole ring of Trp 334 moved slightly away from the bound substrate analogue. The phenyl ring of **19** and that of Phe 178 appear to be in reasonably close proximity in this simulation.

The *Z*-11-phenyl FPP analogue (**29**) showed an entirely different set of movements. In this instance Trp 334 did not move at all. Most interestingly, there appears to be a possible π–π stacking arrangement between the phenyl ring of **29** and Phe 112 aided by a substantial movement of the side chain of Phe 112. In this case the overall fold of the prenyl chain was very similar to that observed for the substrate with a more modest movement of the diphosphate group relative to that observed for **19**. Again Phe 178 and Tyr 92 show small rotations upon binding of the analogue relative to their positions adopted upon binding of FPP.

The simulation of the binding of **36** indicated that the binding of this compound to FPP requires the least amount of active site

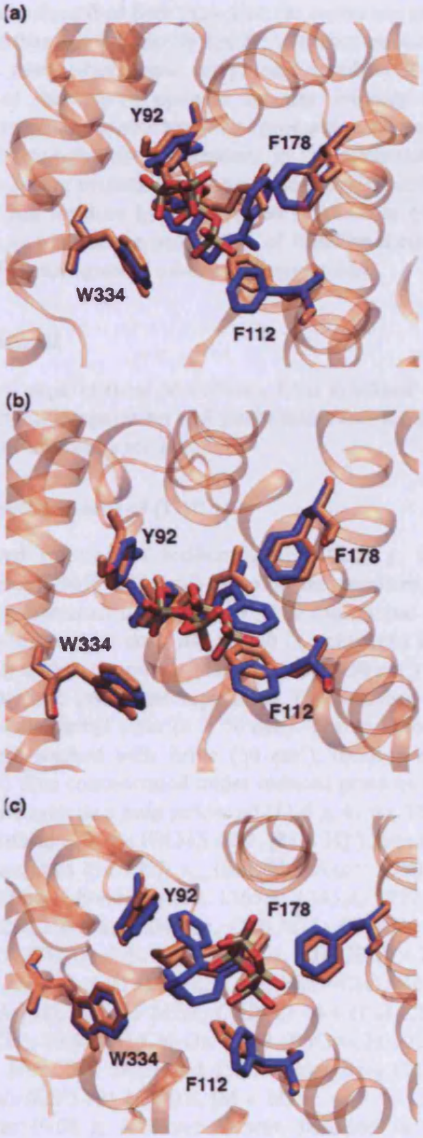


Fig. 3 Sketches from molecular mechanics simulations of the active sites of AS complexed with (2*E*,6*E*,10*E*)-3,7-dimethyl-11-phenyldodeca-2,6,10-trien-1-yl diphosphate (**19**) (a), (2*E*,6*E*,10*Z*)-3,7-dimethyl-11-phenyldodeca-2,6,10-trien-1-yl diphosphate (**29**) (b) and (2*Z*,6*E*)-7,11-dimethyl-3-phenyldodeca-2,6,10-trien-1-yl diphosphate (**36**) (c). The substrate and the inhibitors as well as key amino acid residues are shown in gold for the original structure and in blue for the energy minimised structures of the inhibitor complexes of AS.

movement for the compounds in this study. Trp 334, Phe 178 and Tyr 92 displayed only minor rearrangements; the phenyl ring of Phe 178 was rotated by approximately 45° relative to its position in the substrate complex. The overall fold of the prenyl chain in the inhibitor complex was similar to that observed for the substrate with the exception of the C4–C7 portion of the chain where the apex of the phenyl group of **36** adopts an almost identical position to that of C3 of FPP, thereby forcing the C4–C7 chain downwards (Fig. 3) to accommodate the rest of the bulky phenyl residue in the active site.

Discussion

Three analogues of farnesyl diphosphate have been prepared using novel methodology that involves a Suzuki–Miyaura cross-coupling reaction as a key step in the synthesis. These coupling reactions proceed in moderate to good yield. It should be possible to use this technology to prepare combinatorial libraries of FPP analogues in the future. Some members of such libraries may act as substrates for AS and other terpene cyclases thereby opening up the possibility of short economical routes to complex, synthetic unnatural analogues of terpenoids.

While the replacement of the methyl groups on C11 and C3 of FPP with phenyl substituents did not produce substrates for AS, compounds **19**, **29** and **36** proved to be potent competitive inhibitors of AS that acted in a reversible fashion. The most potent of these inhibitors was the *E*-11-phenyl-FPP analogue **19** with a K_I of 0.8 μM, which is comparable to the K_M of the natural substrate[5,9] and to the inhibitory constant of 12,13-difluoro-FPP.[36] Given the relatively size neutral substitutions in 12,13-difluoro-FPP, the tight binding of the phenyl substituted FPP analogues to AS clearly indicate the remarkable plasticity of this enzyme's active site. Furthermore, farnesyl thiodiphosphate, an analogue of FPP where the oxygen attached directly to the farnesyl chain was replaced by a sulfur, was a much more weakly bound inhibitor of this enzyme (K_I = 10 μM) (Beyer and Allemann, unpublished). Replacing the oxygen with sulfur in the diphosphate group clearly reduces the binding energy more significantly than a change from a methyl to a phenyl group in the prenyl chain. The majority of the binding energy seems to stem from the interaction of the charged diphosphate group to the Mg^{2+} binding site while the interaction of the aliphatic chain with the hydrophobic pocket of the active site contributes a smaller amount.

Analysis of the X-ray crystal structure of AS indicates that the indole ring of Trp 334 is positioned close to C3 of FPP.[7,34] The GC-MS analysis of the sesquiterpenes produced by mutants of AS in which this residue was replaced by non aromatic amino acids indicated that the indole ring was involved in the stabilisation of the positive charge build up on C3 during formation of the eudesmane cation (**38**).[7] The tight binding of **36**, where the methyl group on C3 was replaced with a phenyl substitutent was therefore most interesting. No steric clash between the two aromatic groups appeared to prevent tight binding of the inhibitor. The precise geometry of binding of these compounds in the active site of AS is currently being studied by X-ray crystallography of the inhibitor complexes. However, our molecular modelling studies of AS bound to the **19**, **29** and **36** suggest possible binding modes for these competitive inhibitors. AS appears to be sufficiently flexible to accommodate the extra bulk of the phenyl groups in the active site. The reorganisation of the active site residues, which is accompanied by alteration of the conformation of the prenyl chain, does however lead to loss of catalytic activity. This may be a consequence of changes in the position of the diphosphate group in each simulation thereby preventing the initial diphosphate loss and hence cyclisation of FPP. In addition, the active site conformation of the prenyl chain has been shown to be critical for AS catalysis[6,36] and the rearrangement of the substrate analogue necessary to accommodate the phenyl ring, may lead to an unreactive conformation.

The results described here show that the active site geometry of terpene synthases is sufficiently flexible to accommodate substrate analogues even when these carry large pendant groups. The plasticity of the terpene cyclases appears not only to provide the framework for the combinatorial production of many natural terpenoids through subtle alterations in the composition of the active site during evolution but may also allow modifications of the active site residues by site directed or random mutagenesis *in vitro* or *in vivo* for the production of functional enzymes that convert FPP analogues to unnatural "terpenoids".

Experimental

For general experimental procedures, final synthesis of diphosphates, enzyme preparation and purification and kinetic characterisation of inhibitors see ESI.†

(*E*)-3-Phenylbut-2-en-1-ol (11)[37]

To a stirred solution of sodium hydride (4.40 g, 110 mmol) in anhydrous DME (200 cm^3) at room temperature under N_2, triethyl phosphonoacetate (21.8 cm^3, 110 mmol) and a solution of acetophenone (11.7 cm^3, 100 mmol) in anhydrous DME were sequentially added, dropwise. After 3 h, water (50 cm^3) was added and the organic layer was separated. The aqueous layer was extracted with diethyl ether (3 × 50 cm^3). The combined organic phases were washed with brine (50 cm^3), dried over $MgSO_4$, filtered and then concentrated under reduced pressure to give the intermediate ester as a pale yellow oil (11.6 g, 61%); TLC R_f 0.39 (hexane–EtOAc = 9 : 1); HRMS (ES^+, $[M + H]^+$) found 191.1067, $C_{12}H_{15}O_2$ requires 191.1067; ν_{max}(thin film)/cm^{-1} 2980.1, 1713.0, 1628.4, 1576.4, 1494.0, 1446.0, 1365.9, 1343.8, 1272.6, 1171.3, 1044.1, 872.4, 766.9 and 694.9; δ_H (500 MHz, C^2HCl_3) 1.23 (3 H, t, *J* 7.5, CH_3CH_2O), 2.50 (3 H, d, *J* 1.5, CH_3CPh), 4.13 (2 H, q, *J* 7.5, CH_3CH_2O), 6.05 (1 H, q, *J* 1.5, PhC=CH) and 7.27–7.40 (5 H, m, Ar–H); δ_C (125 MHz, C^2HCl_3) 14.4 (CH_3CH_2O), 18.0 (CH_3C=CH), 59.8 (CH_3CH_2O), 117.2 (PhC=CH), 126.3, 128.4 and 129.0 (Ar–CH), 142.3 and 155.5 (quaternary C) and 166.9 (C=O); *m/z* (ES^+) 191.1 (100%, $[M + H]^+$).

The ester (9.08 g, 47.8 mmol) was dissolved in anhydrous THF (50 cm^3) and cooled to −78 °C (acetone–dry ice bath). To this stirred solution, under N_2, was added diisobutylaluminium hydride (1.0 M solution in hexanes, 152 cm^3, 152 mmol) dropwise over 10 min. This solution was stirred for 2 h at −78 °C then allowed to warm to 0 °C. Saturated potassium sodium tartrate solution (50 cm^3) and diethyl ether (50 cm^3) were added. The mixture was stirred at room temperature for another 30 min, and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 50 cm^3). The combined ethereal extracts were washed with brine (150 cm^3), dried over $MgSO_4$, filtered and then concentrated under reduced pressure. Purification by flash chromatography on silica gel with hexane and ethyl acetate (2 : 1) gave **11** as a light yellow oil (6.7 g, 96%); R_f 0.27 (hexane–EtOAc = 2 : 1); HRMS: (EI^+, M^+) found 148.0890, $C_{10}H_{12}O$ requires 148.0888; ν_{max}(thin film)/cm^{-1} 3347.2, 2922.2, 2597.9, 1493.8, 1444.5, 1379.9, 1003.0, 758.1 and 696.1; δ_H (500 MHz, C^2HCl_3) 2.11 (3 H, s, CH_3CPh), 4.40 (2 H, d, *J* 6.5, CH_2OH), 6.01 (1 H, t, *J* 6.5, PhC=CH) and 7.28–7.45 (5 H, m, Ar–H); δ_C (125 MHz, C^2HCl_3) 16.1 (CH_3CPh), 60.0 (CH_2OH), 126.5

(PhC=*C*H), 125.8, 127.3 and 128.3 (Ar–CH) and 137.8 and 142.9 (quaternary C); *m/z* (EI$^+$) 148.1 (13%, M$^+$) and 115.1 (100).

(*E*)-3-Phenylbut-2-enal (12a)

Pyridinium chlorochromate (12.3 g, 56.0 mmol) was suspended in anhydrous CH_2Cl_2 (50 cm^3) then a solution of **11** (6.7 g, 45 mmol) in CH_2Cl_2 (50 cm^3) was added in one portion to the stirred suspension. After 4 h, dry diethyl ether (100 cm^3) was added and the supernatant liquid was decanted from the resulting black gum. The insoluble residue was washed with diethyl ether (100 cm^3) and became a black granular solid. The organic phases were combined, washed with brine (300 cm^3), dried over $MgSO_4$, filtered and then concentrated under reduced pressure to give **12a** as a yellow oil (3.7 g, 56%); R_f 0.37 (hexane–EtOAc = 4 : 1); HRMS: (EI$^+$, M$^+$) found: 146.0730, $C_{10}H_{10}O$ requires 146.0732; ν_{max}(thin film)/cm^{-1} 1722.2, 1659.7, 1446.2, 1377.3, 1248.1, 1144.5, 865.5 and 758.6; δ_H (500 MHz, C^2HCl_3) 1.94 (3 H, d, *J* 1.0, CH_3CPh), 6.41 (1 H, dq, *J* 7.5, *J* 1.0, PhC=CH), 7.10–7.18 (5 H, m, Ar–H) and 10.05 (1 H, d, *J* 7.5, CHO); δ_C (125 MHz, C^2HCl_3) 15.5 (CH_3CPh), 127.4 (PhC=*C*H), 126.2, 128.5 and 129.5 (Ar–CH), 140.8 and 155.8 (quaternary C) and 189.8 (CHO). *m/z* (EI$^+$) 146.1 (46%, M$^+$) and 145.1 (100).

(*E*)-Penta-2,4-dien-2-ylbenzene (12b)

A stirred suspension of methyltriphenylphosphonium bromide (7.61 g, 21.3 mmol) in anhydrous THF (50 cm^3) was cooled to −78 °C then *n*-BuLi (2.5 M, 8.52 cm^3, 21.3 mmol) was added dropwise under argon. The reaction mixture was allowed to warm to 0 °C giving a clear deep yellow solution. After stirring at 0 °C for 30 min, the aldehyde **12a** (1.83 g, 12.6 mmol) was added dropwise and the complete reaction mixture was stirred for 16 h whilst slowly warming to room temperature. Water (20 cm^3) and diethyl ether (20 cm^3) were added and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 15 cm^3). The combined ethereal extracts were washed with water (2 × 20 cm^3) and brine (20 cm^3), dried over $MgSO_4$, filtered and then concentrated under reduced pressure. Purification by flash chromatography on silica gel with hexane and ethyl acetate (9 : 1) as eluent gave **12b** as a light yellow oil (1.70 g, 94%); R_f 0.57 (hexane–EtOAc = 19 : 1); HRMS: (EI$^+$, M$^+$) found 144.0938, $C_{11}H_{12}$ requires 144.0939; ν_{max}(thin film)/cm^{-1} 3029.5, 2923.2, 1803.9, 1627.6, 1594.2, 1493.3, 1445.8, 1380.1, 1175.1, 1027.4, 985.3, 903.6, 759.8 and 694.8; δ_H (400 MHz, C^2HCl_3) 2.09 (3 H, s, CH_3CPh), 5.10 (1 H, d, *J* 10.0, CHCH=CH$_{trans}H_{cis}$), 5.22 (1 H, d, *J* 17.0, CHCH=CH$_{trans}H_{cis}$), 6.37 (1 H, d, *J* 11.0, C*H*CH=CH$_2$), 6.68 (1 H, dt, *J* 17.0, *J* 10.5, CHC*H*=CH$_2$) and 7.14–7.26 (5 H, m, Ar–H); δ_C (100 MHz, C^2HCl_3) 16.1 (CH_3CPh), 117.7 (CHCH=*C*H$_2$), 127.8 (*C*HCH=CH$_2$), 125.7, 127.2 and 128.3 (Ar–CH), 133.6 (CH*C*H=CH$_2$) and 136.8 and 143.0 (quaternary C); *m/z* (EI$^+$) 144.1 (35%, M$^+$) and 129.1 (100, [M − CH_3]$^+$).

(2*E*,6*E*)-Ethyl 3-methyl-7-phenylocta-2,6-dienoate (14)

A mixture of **12b** (1.45 g, 9.92 mmol) and 9-BBN (3.63 g, 14.9 mmol) dissolved in anhydrous THF (50 cm^3) was stirred at room temperature under N_2 until all the starting material had been consumed as judged by TLC (approx. 2 h). The iodide **13**[27] (2.38 g, 9.92 mmol), triphenylarsine (0.30 g, 0.99 mmol), $PdCl_2$dppf (0.37 g, 0.45 mmol) and aqueous NaOH (6.0 M. 9.92 cm^3, 39.7 mmol) were added in quick succession, the complete solution was then stirred at 50 °C for 15 h. After cooling to room temperature, aqueous hydrogen peroxide solution (30%, 15 cm^3) was carefully added and the solution was stirred for a further 30 min. Water (30 cm^3) and diethyl ether (30 cm^3) were added, and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 25 cm^3). The combined ethereal extracts were washed with water (2 × 20 cm^3) and brine (20 cm^3), dried over $MgSO_4$, filtered and then concentrated under reduced pressure. Purification by flash chromatography on silica gel with hexane and ethyl acetate gave **14** as a light yellow oil (1.51 g, 59%); R_f 0.27 (hexane–EtOAc = 19 : 1); HRMS: (ES$^+$, [M + NH_4]$^+$) found 276.1959, $C_{17}H_{26}NO_2$ requires 276.1958; ν_{max}(thin film)/cm^{-1} 2978.8, 2930.2, 1714.9, 1647.6, 1493.8, 1444.3, 1381.4, 1327.6, 1272.3, 1222.8, 1146.1, 1098.9, 1049.6, 864.4, 757.9 and 696.4; δ_H (500 MHz, C^2HCl_3) 1.20 (3 H, t, *J* 7.0, CH_3CH_2O), 1.96 (3 H, d, *J* 1.0, CH_3C=CHCO$_2$Et), 2.13 (3 H, d, *J* 1.0, CH_3CPh), 2.19–2.32 (4 H, m, CH_2CH_2), 4.07 (2 H, q, *J* 7.0, CH_3CH_2O), 5.64 (2 H, m, 2 × C=CH) and 7.13–7.30 (5 H, m, Ar–H); δ_C (125 MHz, C^2HCl_3) 14.4 (CH_3CH_2O), 15.9 (CH_3C=CHCO), 18.9 (CH_3CPh), 26.8 and 40.6 (CH_2CH_2), 59.6 (CH_3CH_2O), 115.9 (C=*C*HCO), 126.66 (PhC=*C*H), 125.7, 126.73 and 128.2 (Ar–CH), 135.8, 144.7, 159.3 (quaternary C) and 166.9 (C=O); *m/z* (CI$^+$) 276.2 (100%, [M + NH_4]$^+$).

(2*E*,6*E*)-3-Methyl-7-phenylocta-2,6-dien-1-ol (15)

To a stirred solution of **14** (1.46 g, 5.67 mmol) in anhydrous THF (60 cm^3) at −78 °C (acetone–dry ice bath) was added diisobutylaluminium hydride (1.0 M solution in hexanes, 13.6 cm^3, 13.6 mmol) dropwise. The resulting mixture was stirred at −78 °C for 2 h and then allowed to warm to 0 °C at which time the reaction was judged complete by TLC analysis. Saturated potassium sodium tartrate solution (50 cm^3) and diethyl ether (50 cm^3) were added. The mixture was stirred at room temperature for another 30 min, and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 30 cm^3). The combined ethereal extracts were washed with brine (30 cm^3), dried over $MgSO_4$, filtered and then concentrated under reduced pressure. Purification by flash chromatography on silica gel with hexane and ethyl acetate (2 : 1) as eluent gave **15** as a light yellow oil (1.2 g, 96%); R_f 0.27 (hexane–EtOAc = 2 : 1); HRMS: (ES$^+$, [M + NH_4]$^+$) found 234.1851, $C_{15}H_{24}NO$ requires 234.1852; ν_{max}(thin film)/cm^{-1} 3363.0, 2923.4, 1493.3, 1444.0, 999.4, 756.3 and 695.8; δ_H (500 MHz, C^2HCl_3) 1.65 (3 H, s, CH_3C=CHCH$_2$OH), 1.96 (3 H, d, *J* 0.9, CH_3CPh), 2.09–2.29 (4 H, m, CH_2CH_2), 4.09 (2 H, d, *J* 10.0, CH_2OH), 5.40 (1 H, tq, *J* 10.0, *J* 1.5, C*H*CH$_2$OH), 5.68 (1H, tq, *J* 7.0, *J* 1.5, C*H*CH$_2$CH$_2$) and 7.13–7.31 (5 H, m, Ar–H); δ_C (125 MHz, C^2HCl_3) 15.9 (CH_3C=CHCH$_2$OH), 16.4 (CH_3CPh), 27.1 and 39.2 (CH_2CH_2), 59.4 (CH_2OH), 123.7 (*C*HCH$_2$OH), 127.7 (PhC=*C*H), 125.6, 126.6 and 128.2 (Ar–CH) and 135.0, 139.4, 143.9 (quaternary C); *m/z* (CI$^+$) 234.2 (100%, [M + NH_4]$^+$).

(2*E*,6*E*)-3-Methyl-7-phenylocta-2,6-dienal (16a)

A mixture of **15** (0.98 g, 4.52 mmol), *N*-methylmorpholine-*N*-oxide (074 g, 6.33 mmol) and freshly activated powdered 4 Å

molecular sieves (0.35 g) in anhydrous acetonitrile (35 cm^3) was stirred for 10 min whereupon tetra-*n*-propylammonium perruthenate (81 mg, 0.23 mmol) was added. The reaction became warm and was then stirred at room temperature for 16 h. The mixture was filtered through a short pad of Celite® and the solvent was concentrated under reduced pressure. Purification by flash chromatography on silica gel with hexane and ethyl acetate (4 : 1) as eluent gave **16a** as a light yellow oil (0.86 g, 89%); R_f 0.33 (hexane–EtOAc = 4 : 1); HRMS: (EI^+, M^+) found 214.1350, $C_{15}H_{18}O$ requires 214.1352; ν_{max}(thin film)/cm^{-1} 1672.0, 1493.5, 1443.9, 1193.1, 1124.0, 757.7 and 696.2; δ_H (500 MHz; $C_6{}^2H_6$) 1.62 (3 H, d, *J* 1.1, CH_3C=CHCHO), 1.92 (3 H, d, *J* 1.0, CH_3CPh), 1.86–2.10 (4 H, m, CH_2CH_2), 5.64 (1 H, tq, *J* 7.0, *J* 1.0, C=C*H*CH_2CH_2), 5.95 (1 H, dq, *J* 7.5, *J* 1.0, *CH*CHO), 7.21–7.44 (5 H, m, Ar–H) and 9.98 (1 H, d, *J* 7.5, CHO); δ_C (125 MHz; $C_6{}^2H_6$) 15.7 (CH_3CPh), 16.7 (CH_3C=CHCHO), 26.3 and 39.8 (CH_2CH_2), 126.3 (PhC=*C*H), 127.5 (*C*HCHO), 125.8, 127.0 and 128.4 (Ar–CH), 136.0, 143.8 and 160.9 (quaternary C) and 189.6 (CHO); *m/z* (CI^+) 232.2 (100%, $[M + NH_4]^+$).

(2*E*,7*E*)-8-Phenyl-4-methyl-nona-1,3,7-triene (16b)

This compound was prepared from **16a** in a manner identical to that for the **12b**; purification by flash chromatography using hexane and ethyl acetate (9 : 1) as eluent gave **16b** as a light yellow oil (0.68 g, 81%); R_f 0.68 (hexane–EtOAc = 9 : 1); HRMS: (EI^+, M^+) found 212.1559, $C_{16}H_{20}$ requires 212.1560; ν_{max}(thin film)/cm^{-1} 2919.4, 1649.7, 1597.7, 1493.5, 1443.8, 1379.7, 987.8, 896.6, 756.3 and 695.3; δ_H (500 MHz, C^2HCl_3) 1.73 (3 H, s, CH_3C=CHCH=CH_2), 1.92 (3 H, s, CH_3CPh), 2.11–2.29 (4 H, m, CH_2CH_2), 4.92 (1 H, d, *J* 10.0, CH=CH$CH_{trans}H_{cis}$), 5.02 (1 H, dd, *J* 17.0, *J* 1.5, CH=C$H_{trans}H_{cis}$), 5.68 (1 H, t, *J* 7.0, C=C*H*CH_2CH_2), 5.82 (1 H, d, *J* 11.0, *CH*CH=CH_2), 6.52 (1 H, dt, *J* 17.0, *J* 10.5, C*H*=CH_2) and 7.12–7.30 (5 H, m, Ar–H); δ_C (125 MHz, C^2HCl_3) 15.9 (CH_3CPh), 16.8 (CH_3C=CHCH_2CH_2), 27.3 and 39.6 (CH_2CH_2), 114.9 (CH=CH_2), 125.8 (*C*HCH=CH_2), 127.8 (PhC=*C*H), 125.7, 126.6 and 128.2 (Ar–CH), 133.4 (*C*H=CH_2) and 135.0, 139.1 and 143.9 (quaternary C); *m/z* (CI^+) 213.1 (100%, $[M + H]^+$).

(2*E*,6*E*,10*E*)-Ethyl-3,7-dimethyl-11-phenyldodeca-2,6,10-trienoate (17)

This compound was prepared from **16b** in a manner identical to that for the ester **14**; purification by flash chromatography using hexane and ethyl acetate (25 : 1) as eluent gave **17** as a light yellow oil (0.56 g, 54%); R_f 0.32 (hexane–EtOAc = 25 : 1); HRMS (ES^+, $[M + H]^+$) found 327.2320, $C_{22}H_{31}O_2$ requires 327.2319; ν_{max}(thin film)/cm^{-1} 2928.8, 1714.9, 1647.2, 1444.1, 1381.4, 1221.1, 1143.0, 757.1 and 695.9; δ_H (500 MHz, C^2HCl_3) 1.21 (3 H, t, *J* 6.5, CH_3CH_2O), 1.50, 1.58 and 1.96 (3 × 3 H, s, 3 × CH_3C=CH), 2.02–2.24 (8H, m, 2 × CH_2CH_2), 4.07 (2 H, q, *J* 6.5, CH_3CH_2O), 5.08 and 5.67 (2 H, m, 2 × C=C*H*CH_2CH_2), 5.60 (1 H, d, *J* 1.0, *CH*CO_2Et) and 7.13–7.30 (5 H, m, Ar–H); δ_C (125 MHz, C^2HCl_3) 14.4 (CH_3CH_2O), 15.8, 16.1 and 18.9 (3 × CH_3C=CH), 26.0, 27.4, 39.4 and 41.0 (2 × CH_2CH_2), 59.5 (CH_3CH_2O), 115.6 (*C*HCO_2Et), 123.3 and 128.1 (C=*C*H), 125.6, 127.0 and 128.2 (Ar–CH), 131.6, 135.8, 144.0 and 159.8 (quaternary C) and 166.9 (C=O); *m/z* (CI^+) 344.3 (100%, $[M + NH_4]^+$), 327.3 (65, $[M + H]^+$).

(2*E*,6*E*,10*E*)-3,7-Dimethyl-11-phenyldodeca-2,6,10-trien-1-ol (18)

This compound was prepared from **17** in a manner identical to that for the alcohol **15**; purification by flash chromatography using hexane and ethyl acetate (2 : 1) as eluent gave **18** as a light yellow oil (0.41 g, 86%); R_f 0.31 (hexane–EtOAc = 2 : 1); HRMS (EI^+, M^+) found 284.2141, $C_{20}H_{28}O$ requires 284.2140; ν_{max}(thin film)/cm^{-1} 3321.5, 2921.0, 1666.9, 1597.7, 1493.7, 1444.1, 1381.1, 1000.4, 846.3, 756.6 and 696.1; δ_H (500 MHz, C^2HCl_3) 1.57 (3 H, s, CH_3C=CH), 1.60 (3 H, s, CH_3C=CHCH_2OH), 1.96 (3 H, d, *J* 1.0, PhCCH_3), 1.97–2.25 (8 H, m, 2 × CH_2CH_2), 4.04 (2 H, d, *J* 7.0, CH_2OH), 5.08 (1 H, dt, *J* 7.0, *J* 1.0, CH_3C=C*H*), 5.33 (1 H, m, C=C*H*CH_2OH), 5.68 (1 H, dt, *J* 7.0, *J* 1.5, PhC=C*H*) and 7.12–7.30 (5 H, m, Ar–H); δ_C (125 MHz, C^2HCl_3) 15.8, 16.1 and 16.3 (3 × CH_3), 26.3, 27.4, 39.4 and 39.5 (2 × CH_2CH_2), 59.4 (CH_2OH), 123.4 (*C*HCH_2OH), 124.3 (CH_3C=*C*H) and 128.2 (PhC=*C*H), 125.6, 126.5 and 128.2 (Ar–CH) and 134.6, 135.0, 139.7 and 144.0 (quaternary C); *m/z* (EI+) 284.2 (1%, M^+), 131.1 (100), 266.2 (2, $[M - H_2O]^+$).

(*Z*)-Ethyl 3-phenylbut-2-enoate (21)[27,28]

To a stirred solution of the iodide **20** (7.20 g, 30.0 mmol) in anhydrous toluene (100 cm^3) under N_2 was added palladium(II) acetate (0.34 g, 1.50 mmol), triphenylarsine (0.79 g, 3.00 mmol), tripotassium orthophosphate (19.1 g, 90.0 mmol) and phenylboronic acid (5.49 g, 45.0 mmol). The complete reaction mixture was then stirred at 90 °C for 6 h. Water (50 cm^3) and diethyl ether (50 cm^3) were added, and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 30 cm^3). The combined ethereal extracts were washed with water (2 × 30 cm^3) and brine (30 cm^3), dried over $MgSO_4$, filtered and then concentrated under reduced pressure. Purification by flash chromatography on silica gel with hexane and ethyl acetate (9 : 1) as eluent gave **21** as a light yellow oil (2.49 g, 44%); R_f 0.30 (hexane–EtOAc = 9 : 1); HRMS (ES^+, $[M + H]^+$) found 191.1067, $C_{12}H_{15}O_2$ requires 191.1067; ν_{max} (thin film)/cm^{-1} 2979.6, 1725.4, 1639.5, 1492.5, 1442.6, 1374.8, 1277.2, 1230.2, 1162.1, 1095.6, 1076.6, 1047.3, 867.6, 768.4 and 698.1 cm^{-1}; δ_H (500 MHz, C^2HCl_3) 1.14 (3 H, t, *J* 7.5, CH_3CH_2O), 2.23 (3 H, d, *J* 1.5, CH_3CPh), 4.05 (2 H, q, *J* 7.5, CH_3CH_2O), 6.05 (1 H, q, *J* 1.5, PhC=C*H*) and 7.26–7.42 (5 H, m, Ar–H); δ_C (125 MHz, C^2HCl_3) 14.0 (CH_3CH_2O), 27.2 (CH_3CPh), 59.8 (CH_3CH_2O), 117.8 (PhC=*C*H), 126.9, 127.8 and 127.9 (Ar–CH), 140.9 and 155.4 (quaternary C) and 165.9 (C=O); *m/z* (CI^+) 208.1 (100%, $[M + NH_4]^+$) and 191.0 (35%, $[M + H]^+$).

(*Z*)-3-Phenylbut-2-ene-1-ol (22)

This compound was prepared from **21** in a manner identical to that for the alcohol **15**; purification by flash chromatography using hexane and ethyl acetate (2 : 1) as eluent gave **22** as a colourless oil (4.12 g, 99%); R_f 0.26 (hexane–EtOAc = 2 : 1); HRMS (CI^+, $[M + NH_4]^+$) found 166.1229, $C_{10}H_{16}NO$ requires 166.1226; ν_{max} (thin film)/cm^{-1} 3331.7, 3055.2, 2969.9, 1656.1, 1600.0, 1493.6, 1434.8, 1376.0, 1246.4, 1065.2, 1002.0, 764.1 and 700.8; δ_H (500 MHz, C^2HCl_3) 1.46 (1 H, b, OH), 2.02 (3 H, d, *J* 1.0, CH_3CPh), 3.99 (2 H, dd, *J* 7.0, *J* 1.0, CH_2OH), 5.64 (1 H, tq, *J* 7.0, *J* 1.5, PhC=C*H*) and 7.09–7.28 (5 H, m, Ar–H); δ_C (125 MHz, C^2HCl_3) 25.4 (CH_3CPh), 60.3 (CH_2OH), 126.1 (PhC=*C*H), 127.2, 127.8 and 128.2

(Ar–CH), 140.3 and 140.8 (quaternary C); *m/z* (CI$^+$) 166.1 (20%, $[M + NH_4]^+$), 148.1 (50, M^+) and 131.0 (100, $[M - OH]^+$).

(*Z*)-3-Phenylbut-2-ene-1-al (23a)

To a stirred solution of oxalyl chloride (2.69 cm^3, 31.3 mmol) in anhydrous CH_2Cl_2 (80 cm^3) at −78 °C under N_2, was added anhydrous dimethylsulfoxide (4.44 cm^3, 62.6 mmol). The reaction mixture was stirred for 5 min then a solution of **22** (3.85 g, 26.1 mmol) in CH_2Cl_2 was added over 5 min. Stirring was continued at −78 °C for an additional 15 min. Triethylamine (18.2 cm^3, 130 mmol) was added and the reaction mixture was stirred for 5 min and then allowed to warm to room temperature. Water (50 cm^3) was then added, and the organic layer was separated. The aqueous layer was extracted with CH_2Cl_2 (2 × 30 cm^3). The combined organic extracts were washed with water (2 × 30 cm^3) and brine (30 cm^3), dried over $MgSO_4$, filtered and then concentrated under reduced pressure. Purification by flash chromatography on silica gel with hexane and ethyl acetate (2 : 1) as eluent gave **23a** as a light yellow oil (3.36 g, 88%); R_f 0.48 (hexane–EtOAc = 2 : 1); HRMS (ES$^+$, $[M - H]^+$) found 145.0645, $C_{10}H_9O$ requires 145.0648; ν_{max} (thin film)/cm^{-1} 2357.8, 1668.3, 1614.1, 1433.3, 1388.2, 1136.6, 767.0 and 701.5; δ_H (500 MHz, C^2HCl_3) 1.81 (3 H, s, CH_3CPh), 6.09 (1 H, d, *J* 8.0, PhC=C*H*), 6.95–7.13 (5 H, m, Ar–H) and 9.71 (1 H, dd, *J* 8.0, *J* 3.5, CHO); δ_C (125 MHz, C^2HCl_3) 25.6 (CH_3CPh), 128.3, 128.4 and 128.7 (Ar–CH), 129.4 (PhC=*C*H), 138.5 and 160.1 (quaternary C) and 191.6 (CHO); *m/z* (CI$^+$) 164.1 (95%, $[M + NH_4]^+$), 161.1 (100%) and 146.1 (30%, M^+).

(*Z*)-4-Phenyl-penta-1,3-diene (23b)

This compound was prepared from **23a** in a manner identical to that for the diene **12b**; purification by flash chromatography using hexane and ethyl acetate (9 : 1) as eluent gave **23b** as a light yellow oil (1.80 g, 76%); R_f 0.63 (hexane–EtOAc = 9 : 1); HRMS (EI$^+$, M^+) found 144.0938, $C_{11}H_{12}$ requires 144.0939; ν_{max} (thin film)/cm^{-1} 3080.3, 2960.4, 2856.8, 1805.6, 1636.2, 1601.6, 1492.1, 1433.6, 1414.1, 1375.3, 1024.7, 995.6, 898.4, 766.1 and 700.2; δ_H (400 MHz, C^2HCl_3) 2.04 (3 H, s, CH_3CPh), 4.86 (1 H, d, *J* 10.0, CHCH=$CH_{trans}H_{cis}$), 5.08 (1 H, d, *J* 17.0, CHCH=$CH_{trans}H_{cis}$), 6.06 (1 H, d, *J* 11.0, *C*HCH=CH_2), 6.32 (1 H, dt, *J* 17.0, *J* 10.5, CHC*H*=CH_2) and 7.14–7.28 (5 H, m, Ar–H); δ_C (100 MHz, C^2HCl_3) 25.5 (CH_3CPh), 116.1 (CHCH=CH_2), 127.9 (*C*HCH=CH_2), 127.1, 128.1 and 128.3 (Ar–CH), 134.6 (CH*C*H=CH_2) and 139.5 and 141.5 (quaternary C); *m/z* (EI$^+$) 144.1 (35%, M^+), 129.1 (100, $[M - CH_3]^+$).

(2*E*,6*Z*)-Ethyl 3-methyl-7-phenylocta-2,6-dienoate (24)

This compound was prepared from **23b** in a manner identical to that for the ester **14**; the crude product was purified by flash chromatography using hexane and ethyl acetate (9 : 1) as eluent to give **24** as a light yellow oil (1.33 g, 41%); R_f 0.35 (hexane–EtOAc = 9 : 1); HRMS (ES$^+$, $[M + H]^+$) found 259.1691, $C_{17}H_{23}O_2$ requires 259.1693; ν_{max} (thin film)/cm^{-1} 2975.8, 2359.6, 1715.0, 1647.3, 1493.4, 1442.0, 1367.2, 1221.8, 1146.1, 1099.6, 1052.2, 865.4, 763.2 and 701.0; δ_H (500 MHz, C^2HCl_3) 1.18 (3 H, t, *J* 7.0, CH_3CH_2O), 1.95 (3 H, s, CH_3C=CHCO$_2$Et), 1.98 (3 H, d, *J* 1.0, CH_3CPh), 2.07 (4 H, m, CH_2CH_2), 4.07 (2 H, q, *J* 7.0, CH_3CH_2O), 5.32 (1 H, m, C=C*H*CH_2CH_2), 5.52 (1 H, d, *J* 0.5, C=C*H*CO$_2$Et) and 7.07–7.30 (5 H, m, Ar–H); δ_C (125 MHz, C^2HCl_3) 14.4 (CH_3CH_2O), 18.7 (CH_3CPh), 25.7 (CH_3C=CHCO$_2$Et), 26.9 and 41.2 (CH_2CH_2), 59.5 (CH_3CH_2O), 115.7 (C=CHCO$_2$Et), 125.8 (PhC=CH), 126.6, 127.9 and 128.2 (Ar–CH), 137.5, 141.8 and 159.4 (quaternary C) and 166.9 (C=O); *m/z* (CI$^+$) 276.2 (100%, $[M + NH_4]^+$), 259.2 (95, $[M + H]^+$).

(2*E*,6*Z*)-3-Methyl-7-phenylocta-2,6-dien-1-ol (25)

This compound was prepared from **24** in a manner identical to that for the alcohol **15**; purification by flash chromatography on silica gel using hexane and ethyl acetate (2 : 1) as eluent gave **25** light yellow oil (0.816 g, 91%); R_f 0.31 (hexane–EtOAc = 2 : 1); HRMS (ES$^+$, $[M + NH_4]^+$) found 234.1850, $C_{15}H_{24}NO$ requires 234.1852; ν_{max} (thin film)/cm^{-1} 3335.5, 2965.2, 2914.6, 1667.9, 1492.8, 1436.1, 1376.6, 999.9, 763.1 and 700.1; δ_H (500 MHz, C^2HCl_3) 1.22 (1 H, b, OH), 1.50 (3 H, s, CH_3C=CHCH$_2$OH), 1.95 (3 H, d, *J* 1.0, CH_3CPh), 1.97–2.04 (4 H, m, CH_2CH_2), 4.04 (2 H, d, *J* 7.0, CH_2OH), 5.28 (1 H, dt, *J* 7.0, *J* 1.0, C=C*H*CH$_2$OH), 5.36 (1 H, dt, *J* 7.0, *J* 1.5, C=C*H*CH$_2$CH$_2$) and 7.10–7.28 (5 H, m, Ar–H); δ_C (125 MHz, C^2HCl_3) 16.2 (CH_3C=CHCH$_2$OH), 25.6 (CH_3CPh), 27.3 and 39.8 (CH_2CH_2), 59.4 (CH_2OH), 123.5 (C=CHCH$_2$OH), 126.8 (PhC=CH), 126.5, 127.9 and 128.1 (Ar–CH) and 136.6, 139.5 and 142.1 (quaternary C); *m/z* (CI$^+$) 234.2 (100%, $[M + NH_4]^+$), 216.2 (80, M^+), 199.1 (40, $[M - OH]^+$).

(2*E*,6*Z*)-3-Methyl-7-phenylocta-2,6-dienal (26a)

This compound was prepared from **25** in a manner identical to that for the compound **16a**; purification by flash chromatography using hexane and ethyl acetate (2 : 1) as eluent to gave **26a** as a light yellow oil (0.86 g, 87%); R_f 0.45 (hexane–EtOAc = 2 : 1); HRMS (EI$^+$, M^+) found 214.1352, $C_{15}H_{18}O$ requires 214.1358; ν_{max} (thin film)/cm^{-1} 2912.7, 2851.7, 2360.2, 1672.8, 1630.3, 1492.6, 1439.9, 1379.2, 1193.2, 1124.0, 1025.8, 831.0, 764.1, 702.0 and 668.1; δ_H (500 MHz, C^2HCl_3) 1.49 (3 H, d, *J* 1.0, CH_3C=CHCHO), 1.85–2.08 (4 H, m, CH_2CH_2), 2.03 (3 H, d, *J* 1.0, CH_3CPh), 5.30 (1 H, td, *J* 7.0, *J* 1.0, C=C*H*CH$_2$CH$_2$), 5.85 (1 H, dd, *J* 8.0, *J* 1.0, C=C*H*CHO), 7.17–7.29 (5 H, m, Ar–H) and 9.91 (1 H, d, *J* 8.0, CHO); δ_C (125 MHz, C^2HCl_3) 16.4 (CH_3C=CHCHO), 25.5 (CH_3CPh), 26.7 and 40.4 (CH_2CH_2), 126.0 (PhC=CH), 127.6 (*C*H=CHO), 126.9, 127.9 and 128.3 (Ar–CH), 137.6, 141.8 and 160.8 (quaternary C) and 189.6 (CHO); *m/z* (EI$^+$) 214.1 (2%, M^+), 131.1 (100).

(3*E*,7*Z*)-4-Methyl-8-phenyl-nona-1,3,7-triene (26b)

This compound was prepared from **26a** in a manner identical to that for the diene **12b**; purification by flash chromatography using hexane and ethyl acetate (2 : 1) as eluent gave **26b** as a light yellow oil (0.48 g, 86%); R_f 0.62 (hexane–EtOAc = 9 : 1); HRMS (ES$^+$, $[M + H]^+$) found 212.1569, $C_{16}H_{20}$ requires 212.1565; ν_{max}(thin film)/cm^{-1} 2964.8, 2358.3, 1649.8, 1598.9, 1493.0, 1436.5, 1378.1, 985.7, 896.9, 761.8 and 700.0; δ_H (500 MHz, C^2HCl_3) 1.70 (3 H, s, CH_3C=CHCH=CH_2), 2.07 (3 H, s, CH_3CPh), 2.08–2.17 (4 H, m, CH_2CH_2), 5.00 (1 H, dd, *J* 10.0, *J* 1.5, CH=C$H_{cis}H_{trans}$), 5.10 (1 H, dd, *J* 17.0, *J* 2.0, CH=C$H_{cis}H_{trans}$), 5.46 (1 H, td, *J* 7.0, *J* 1.5, C=C*H*CH$_2$CH$_2$), 5.83 (1 H, dd, *J* 11.0, *J* 0.5, C*H*CH=CH_2), 6.59 (1 H, dt, *J* 17.0, *J* 10.5, CHC*H*=CH_2) and 7.21–7.39 (5 H,

m, Ar–H); δ_C (125 MHz, C^2HCl_3) 16.6 ($CH_3C{=}CHCH_2$), 25.6 (CH_3CPh), 27.4 and 40.1 (CH_2CH_2), 114.7 ($CHCH{=}CH_2$), 125.6 ($CHCH{=}CH_2$), 126.9 ($PhC{=}CH$), 126.5, 128.0 and 128.2 (Ar–CH), 133.4 ($CH{=}CH_2$) and 136.6, 139.1 and 142.1 (quaternary C); *m/z* (EI^+) 212.2 (5%, M^+), 131.1 (100) and 91.1 (40).

(2*E*,6*E*,10*Z*)-Ethyl-3,7-dimethyl-11-phenyldodeca-2,6,10-trienoate (27)

This compound was prepared from **26b** in a manner identical to that for the ester **14**; purification by flash chromatography using hexane and ethyl acetate (19 : 1) gave **27** as a light yellow oil (0.29 g, 40%); R_f 0.33 (hexane–EtOAc = 19 : 1); HRMS (ES^+, $[M + H]^+$) found 327.2318, $C_{22}H_{31}O_2$ requires 327.2319; ν_{max} (thin film)/cm^{-1} 2926.3, 1715.4, 1647.5, 1442.7, 1366.5, 1221.5, 1144.3, 1053.9, 865.3, 762.0 and 700.5; δ_H (500 MHz, C^2HCl_3) 1.20 (3 H, t, *J* 7.0, OCH_2CH_3), 1.43 (3 H, s, $CH_3C{=}CH$), 1.95 (3 H, d, *J* 1.0, $CH_3C{=}CH$), 2.08 (3 H, d, *J* 1.0, $CH_3C{=}CHCO_2Et$), 1.91–2.11 (8 H, m, 2 × CH_2CH_2), 4.06 (2 H, q, *J* 7.0, OCH_2CH_3), 4.97 (1 H, b, $C{=}CHCH_2CH_2$), 5.35 (1 H, dt, *J* 7.0, *J* 1.0, $C{=}CHCH_2CH_2$), 5.58 (1 H, s, $C{=}CHCO_2Et$) and 7.09–7.30 (5 H, m, Ar–H); δ_C (125 MHz, C^2HCl_3) 14.4 (OCH_2CH_3), 16.0 ($CH_3C{=}CH$), 18.9 ($CH_3C{=}CHCO_2Et$), 25.6 ($CH_3C{=}CH$), 26.0, 27.5, 39.9 and 41.0 (2 × CH_2CH_2), 59.5 (OCH_2CH_3), 115.6 ($C{=}CHCO_2Et$), 123.1 and 127.2 (2 × $C{=}CHCH_2CH_2$), 126.4, 127.6 and 128.0 (Ar–CH), 135.7, 136.2, 142.1 and 159.9 (quaternary C) and 166.9 (C=O); *m/z* (CI^+) 344.3 (100%, $[M + NH_4]^+$) and 327.3 (50, $[M + H]^+$).

(2*E*,6*E*,10*Z*)-3,7-Dimethyl-11-phenyldodeca-2,6,10-trien-1-ol (28)

This compound was prepared in a manner identical to that for the alcohol **15**; purification by flash chromatography using hexane and ethyl acetate (2 : 1) as eluent gave **28** as a light yellow oil (0.12 g, 77%); R_f 0.26 (hexane–EtOAc = 2 : 1); HRMS (ES^+, $[M + NH_4]^+$) found 302.2477, $C_{20}H_{32}NO$ requires 302.2478; ν_{max} (thin film)/cm^{-1} 3344.4, 2919.3, 1666.0, 1597.4, 1493.4, 1443.8, 1381.2, 998.9, 756.6 and 696.0; δ_H (500 MHz, C^2HCl_3) 1.23 (1 H, b, OH), 1.43 (3 H, s, $CH_3C{=}CH$), 1.60 (3 H, s, $CH_3C{=}CH$), 1.95 (3 H, d, *J* 1.0, $CH_3C{=}CH$), 1.91–2.06 (8 H, m, 2 × CH_2CH_2), 4.07 (2 H, d, *J* 7.0, CH_2OH), 4.99 (1 H, td, *J* 7.0, *J* 1.0, $C{=}CH$), 5.33 (2 H, m, 2 × $C{=}CH$) and 7.10–7.30 (5 H, m, Ar–H); δ_C (125 MHz, C^2HCl_3) 16.0, 16.3 and 25.6 (3 × CH_3), 26.3, 27.6, 39.5 and 40.0 (2 × CH_2CH_2), 59.4 (CH_2OH), 123.3, 124.0 and 127.3 (3 × $C{=}CH$), 126.4, 128.0 and 128.0 (Ar–CH) and 135.0, 136.1, 139.8 and 142.2 (quaternary C); *m/z* (CI^+) 302.3 (100%, $[M + NH_4]^+$), 284.3 (55, M^+).

(*E*)-Ethyl 7,11-dimethyl-3-oxododeca-6,10-dienoate (32)[20,38]

To a stirred solution of geraniol (2.60 cm^3 g, 15.0 mmol) and triethylamine (4.20 cm^3, 30.0 mmol) in anhydrous THF (100 cm^3) at −45 °C under N_2 was added methanesulfonyl chloride (1.50 cm^3, 19.5 mmol). The resulting milky mixture was stirred at −45 °C for 45 min then a solution of lithium bromide (5.20 g, 60.0 mmol) in THF (10 cm^3) was added *via* a cannula at −45 °C. The suspension was allowed to warm to 0 °C and stirred for an additional 1 h before cold water (30 cm^3) and hexane (30 cm^3) were added to quench the reaction. The two layers were separated, and the aqueous layer was extracted with hexane (2 × 20 cm^3). The combined organic layers were washed with saturated $NaHCO_3$ solution (20 cm^3) and then brine (20 cm^3), dried over Na_2SO_4 and filtered. Concentration of the solvent gave the intermediate bromide as a light yellow oil, which was used without further purification.

To a stirred suspension of NaH (60% dispersion in mineral oil, 1.20 g, 49.5 mmol) in anhydrous THF (100 cm^3) was added ethyl acetoacetate (5.73 cm^3, 45.0 mmol) dropwise at 0 °C. After 10 min, *n*-BuLi (2.2 M, 21.5 cm^3, 47.3 mmol) was added slowly over 3 min, during which time the colourless solution gradually turned yellow. This was stirred for an additional 10 min at 0 °C, as a solution of the bromide in THF (5 cm^3) was added. The clear solution turned to a cloudy yellow suspension. After stirring for 30 min at 0 °C, hydrochloric acid (3 M, 10.0 cm^3) was added followed by water (30 cm^3) and diethyl ether (30 cm^3) then the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 20 cm^3). The combined ethereal extracts were washed with water (2 × 20 cm^3) and brine (20 cm^3), dried over $MgSO_4$ then filtered and concentrated under reduced pressure. Purification by flash chromatography on silica gel with hexane and ethyl acetate (4 : 1) as eluent gave **32** as a pale yellow oil (3.61 g, 92%); R_f 0.45 (hexane–EtOAc = 2 : 1); HRMS (ES^+, $[M + H]^+$) found 267.1954, $C_{16}H_{27}O_3$ requires 267.1954; ν_{max} (thin film)/cm^{-1} 2968.0, 2918.6, 1746.5, 1717.2, 1648.9, 1445.8, 1409.9, 1367.4, 1313.3, 1235.8, 1177.2, 1035.9 and 839.8; δ_H (500 MHz, C^2HCl_3) 1.30 (3 H, t, *J* 7.0, CH_3CH_2O), 1.61 (3 H, s, $CH_3C{=}CH$), 1.63 (3 H, s, $CH_3C{=}CH$), 1.69 (3 H, s, $CH_3C{=}CH$), 1.98 (4 H, m, $(CH_3)_2C{=}CHCH_2CH_2$), 2.30 (2 H, q, *J* 7.5, $CH_2CH_2C{=}O$), 2.59 (2 H, t, *J* 7.5, $CH_2CH_2C{=}O$), 3.45 (2 H, s, $COCH_2CO$), 4.20 (2 H, q, *J* 7.0, OCH_2CH_3) and 5.09 (2 H, dt, *J* 1.0, *J* 7.0, 2 × $C{=}CH$); δ_C (125 MHz, C^2HCl_3) 14.1 (OCH_2CH_3), 16.0 ($CH_3C{=}CH$), 17.7 ($CH_3C{=}CH$), 22.2 ($CH_2CH_2C{=}O$), 25.7 ($CH_3C{=}CH$), 26.6 and 39.4 ($(CH_3)_2C{=}CHCH_2CH_2$), 43.1 (CH_2CH_2CO), 49.4 ($COCH_2CO$), 61.4 (OCH_2CH_3), 122.1 and 122.4 (2 × $C{=}CH$), 131.5 and 136.8 (quaternary C), 167.3 (ester C=O) and 202.7 (ketone C=O); *m/z* (CI^+) 284.2 (100%, $[M + NH_4]^+$), 267.2 (86, $[M + H]^+$).

(1*Z*,5*E*)-1-(Ethoxycarbonyl)-6,10-dimethylundeca-1,5,9-trien-2-yl trifluoromethanesulfonate (33)[16]

A stirred solution of **32** (546 mg, 2.05 mmol) in anhydrous THF (15 cm^3) under N_2 was cooled to −78 °C then potassium bis(trimethylsilyl)amide (0.5 M in THF, 4.93 cm^3, 2.46 mmol) was added. The resulting mixture was stirred at −78 °C for 30 min. Trifluoromethanesulfonic anhydride (414 mm^3, 2.46 mmol) was added at −78 °C and the solution stirred for 16 h whilst slowly warming to room temperature. Diethyl ether (20 cm^3) was added and the solution was washed with 10% citric acid solution (2 × 15 cm^3) and water (15 cm^3). The separated organic layer was dried over $MgSO_4$ and filtered. Evaporation of the solvent gave a yellow oil which was purified by flash chromatography on silica gel with hexane and ethyl acetate (4 : 1) as eluent to give **33** as light yellow oil (0.42 g, 51%); R_f 0.41 (hexane–EtOAc = 4 : 1); ν_{max} (thin film)/cm^{-1} 2975.6, 2905.3, 2855.1, 2353.0, 1731.9, 1676.1, 1427.9, 1209.1, 1141.0, 1037.6, 923.1 and 840.1; δ_H (500 MHz, C^2HCl_3) 1.24 (3 H, t, *J* 7.0, OCH_2CH_3), 1.53 (3 H, s, $CH_3C{=}CH$), 1.54 (3 H, s, $CH_3C{=}CH$), 1.61 (3 H, s, $CH_3C{=}CH$), 1.91 (4 H, m, $(CH_3)_2C{=}CHCH_2CH_2$), 2.20 (2 H, t, *J* 7.5, $CH_2CH_2C{=}O$), 2.34 (2 H, t, *J* 7.5, $CH_2CH_2C{=}O$), 4.17 (2 H, q, *J* 7.0, OCH_2CH_3), 4.99 (2 H, t, *J* 7.0, 2 × $C{=}CHCH_2CH_2$) and 5.67 (1 H, s, $O{-}C{=}CH$);

δ_C (125 MHz, C^2HCl_3) 14.1 (OCH_2CH_3), 16.1 (CH_3C=CH), 17.7 (CH_3C=CH), 24.4 (CH_2CH_2CO), 25.7 (CH_3C=CH), 26.5 and 39.6 ($(CH_3)_2C$=$CHCH_2CH_2$), 34.6 (CH_2CH_2C=O), 61.3 (OCH_2CH_3), 112.0 (O–C=CH), 120.6 and 123.9 (2 × C=CH), 131.7 and 138.2 (2 × C=CH), 158.5 (CH=$COSO_2CF_3$) and 162.5 (C=O); δ_F (283 MHz, C^2HCl_3) −74.6 (s). *m/z* (CI^+) 398.1 (2%, M^+), 358.0 (80), 314.1 (18), 267.0 (21), 190 (23) and 114 (100).

(2*Z*,6*E*)-Ethyl-7,11-dimethyl-3-phenyldodeca-2,6,10-trienoate (34)

To a stirred solution of **33** (0.36 g, 0.91 mmol) in anhydrous THF (10 cm^3) under N_2, was added palladium(II) acetate (0.02 g, 0.09 mmol), triphenylarsine (0.11 g, 0.36 mmol), silver oxide (0.42 g, 1.81 mmol) and phenylboronic acid (0.17 g, 1.36 mmol) in quick succession. The complete mixture was then heated under reflux for 15 h. Water (20 cm^3) and diethyl ether (20 cm^3) were added, and the organic layer was separated. The aqueous layer was extracted with diethyl ether (2 × 15 cm^3). The combined ethereal extracts were washed with water (2 × 10 cm^3) and brine (10 cm^3), dried over $MgSO_4$ filtered and concentrated under reduced pressure. Purification by flash chromatography on silica gel with hexane and ethyl acetate (9 : 1) as eluent gave **34** as a light yellow oil (0.19 g, 66%); R_f 0.35 (hexane–EtOAc = 9 : 1); HRMS (ES^+, $[M + H]^+$) found 327.2324, $C_{22}H_{31}O_2$ requires 327.2319; ν_{max} (thin film)/cm^{-1} 2975.7, 2926.3, 2361.9, 1727.2, 1638.2, 1442.5, 1377.2, 1276.4, 1223.8, 1159.0, 1042.6, 865.8 and 698.6; δ_H (500 MHz, C^2HCl_3) 0.99 (3 H, t, *J* 7.0, OCH_2CH_3), 1.45 (3 H, s, CH_3C=CH), 1.53 (3 H, s, CH_3C=CH), 1.60 (3 H, s, CH_3C=CH), 1.88 (6 H, m, $(CH_3)_2C$=$CHCH_2CH_2$ and CH_2CH_2CPh), 2.40 (2 H, dt, *J* 1.0, *J* 8.0, CH_2CH_2CPh), 4.90 (2 H, q, *J* 7.0, OCH_2CH_3), 5.01 (2 H, dt, *J* 1.0, *J* 7.0, 2 × C=CH), 5.81 (1 H, s, C=$CHCO_2Et$) and 7.07 (5 H, m, Ar–CH); δ_C (125 MHz, C^2HCl_3) 14.0 (OCH_2CH_3), 16.1 (CH_3C=CH), 17.7 (CH_3C=CH), 25.7 (CH_3C=CH), 25.9, 26.7, 39.7 and 40.5 (2 × CH_2CH_2), 59.7 (OCH_2CH_3), 117.4 (C=$CHCO_2Et$), 122.7 and 124.2 (2 × C=$CHCH_2CH_2$), 127.2, 127.5 and 127.8 (Ar–CH) and 131.4, 136.3, 140.2, 159.3 and 166.1 (quaternary C); *m/z* (CI^+) 344.4 (53%, $[M + NH_4]^+$) and 327.4 (100, $[M + H]^+$).

(2*Z*,6*E*)-7,11-Dimethyl-3-phenyldodeca-2,6,10-trien-1-ol (35)

This compound was prepared from **34** in a manner identical to that for the alcohol **15**; purification by flash chromatography on silica gel using hexane and ethyl acetate (2 : 1) as eluent gave **35** as a light yellow oil (0.11 g, 85%); R_f 0.38 (hexane–EtOAc = 2 : 1); HRMS (EI^+, M^+) found 284.2147, $C_{20}H_{28}O$ requires 284.2140; ν_{max} (thin film)/cm^{-1} 3355.5, 2965.4, 2922.2, 2855.4, 1650.6, 1491.7, 1442.2, 1379.7, 1080.3, 1005.6, 830.9 and 769.8; δ_H (500 MHz, C^2HCl_3) 1.42 (1 H, b, CH_2OH), 1.54 (3 H, s, CH_3C=CH), 1.59 (3 H, s, CH_3C=CH), 1.71 (3 H, s, CH_3C=CH), 1.97 (6 H, m, $(CH_3)_2C$=$CHCH_2CH_2$ and CH_2CH_2CPh), 2.44 (2 H, t, *J* 7.5, CH_2CH_2CPh), 4.07 (2 H, d, *J* 7.0, CH_2OH), 5.11 (2 H, m, 2 × C=CH), 5.72 (1 H, t, *J* 7.0, C=$CHCH_2OH$) and 7.15 (5 H, m, Ar–CH); δ_C (125 MHz, C^2HCl_3) 16.0 (CH_3), 17.7 (CH_3), 25.7 (CH_3), 26.5, 26.7, 39.0 and 39.7 (2 × CH_2CH_2), 60.3 (CH_2OH), 123.5 and 124.4 (2 × C=CH), 125.7 (C=$CHCH_2OH$), 127.1, 128.1 and 128.2 (Ar–CH) and 131.3, 135.5, 140.0 and 144.6 (quaternary C); *m/z* (EI^+) 284.2 (10%, M^+) and 266.2 (100, $[M - H_2O]^+$).

Molecular mechanics simulations

The X-ray crystal structure of AS from *P. roqueforti* containing a docked FPP molecule (PDB 1FIP) was used as the starting structure.[34] For each docking experiment, the FPP substrate was converted to the relevant substrate analogue **19**, **29** and **36**, respectively, using the molecule builder module of the software package MOE.§ Hydrogen atoms were added to both the protein and FPP automatically using the software. The inhibitor and all amino acid residues containing heavy atoms within 6.5 Å of the inhibitor were selected as the key active site atoms and the MMFF94 molecular mechanics forcefield was applied to these atoms only.[35] The energy of the system was minimised and the resulting coordinates used to generate Fig. 3.

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X-RAY CRYSTALLOGRAPHIC STUDIES OF SUBSTRATE BINDING TO ARISTOLOCHENE SYNTHASE SUGGEST A METAL ION BINDING SEQUENCE FOR CATALYSIS*

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The universal sesquiterpene precursor, farnesyl diphosphate (FPP), is cyclized in a Mg^{2+}-dependent reaction catalyzed by the tetrameric aristolochene synthase from *Aspergillus terreus* to form the bicyclic hydrocarbon aristolochene and a pyrophosphate anion (PP_i) coproduct. The 2.1 Å resolution crystal structure determined from crystals soaked with FPP reveals the binding of intact FPP to monomers A-C, and the binding of PP_i and $Mg^{2+}{}_B$ to monomer D. The 1.89 Å resolution structure of the complex with 2-fluorofarnesyl diphosphate (2F-FPP) reveals 2F-FPP binding to all subunits of the tetramer, with $Mg^{2+}{}_B$ accompanying the binding of this analogue only in monomer D. All monomers adopt open active site conformations in these complexes, but slight structural changes in monomers C and D of each complex reflect the very initial stages of a conformational transition to the closed state. Finally, the 2.4 Å resolution structure of the complex with 12,13-difluorofarnesyl diphosphate (DF-FPP) reveals the binding of intact DF-FPP to monomers A-C in the open conformation, and the binding of PP_i, $Mg^{2+}{}_B$, and $Mg^{2+}{}_C$ to monomer D in a predominantly closed conformation. Taken together, these structures provide 12 independent "snapshots" of substrate or product complexes that suggest a possible sequence for metal ion binding and conformational changes required for catalysis.

Farnesyl diphosphate (FPP)[2], a flexible 15-carbon isoprenoid, is the universal precursor of more than 300 different cyclic sesquiterpenes found in numerous plants, bacteria, and fungi (1, 2). The cyclization of FPP is catalyzed by a sesquiterpene cyclase that utilizes a trinuclear magnesium cluster to trigger the departure of the pyrophosphate (PP_i) leaving group, thereby forming an allylic carbocation that typically reacts with one of the remaining π bonds of the substrate (3-7). The remarkable diversity of sesquiterpene structure and stereochemistry is the consequence of precise control exerted by the cyclase over the conformations of the flexible substrate and carbocation intermediates in the cyclization cascade.

Aristolochene synthase from *A. terreus* is a sesquiterpene cyclase that catalyzes the cyclization of FPP to form aristolochene (Figure 1a), the parent hydrocarbon of a large group of fungal toxins

such as gigantenone, PR-toxin, and bipolaroxin (8). In contrast to aristolochene synthase from *P. roqueforti*, which generates aristolochene predominantly (>90%) but also small amounts of germacrene A and valencene (9, 10), aristolochene synthase from *A. terreus* generates aristolochene exclusively (9). Each cyclase adopts the common α-helical fold of a class I terpenoid cyclase and contains two conserved metal binding motifs: the "aspartate-rich" motif $\mathbf{D^{90}}$DLLE that coordinates to $Mg^{2+}{}_A$ and $Mg^{2+}{}_C$, and the "NSE/DTE" motif $\mathbf{N^{219}}$DIYSYEK**E** that chelates $Mg^{2+}{}_B$ (boldface residues are metal ligands) (11). X-ray crystallographic studies show that the binding of $Mg^{2+}{}_3$-PP_i stabilizes the active site in a closed conformation that is completely sequestered from bulk solvent (Figure 1b) (11). In addition to multiple metal coordination interactions, the PP_i anion accepts hydrogen bonds from conserved residues R175, K226, R314, and Y315 when bound to the closed conformation (Figure 1c). It is likely that the diphosphate group of FPP makes identical metal coordination and hydrogen bond interactions in the Michaelis complex, i.e., the complex between the enzyme and the productively bound substrate that immediately precedes the initiation of the cyclization cascade.

Substrate conformation is a crucial determinant of the biosynthetic outcome of the terpenoid cyclase reaction. The active site of aristolochene synthase from *A. terreus* serves as a high-fidelity template that fixes FPP in a single, productive conformation in the Michaelis complex – otherwise, aberrant cyclization products would result. To study the conformational control of FPP in the active site of aristolochene synthase from *A. terreus*, we now report the structures of crystalline complexes with the intact substrates FPP and 2-fluorofarnesyl diphosphate (2F-FPP) (12), and the inhibitor 12,13-difluorofarnesyl diphosphate (DF-FPP) (13). Differences observed in active site conformations and substrate conformations appear to be linked to differences in metal binding, analysis of which suggests a possible sequence for metal ion binding and conformational changes required for catalysis.

MATERIAL AND METHODS

Enzyme incubations with 2F-FPP and DF-FPP in solution. The syntheses of 2F-FPP and DF-FPP have been reported (12-14) and an additional synthesis of 2F-FPP is outlined in the Supporting Information. Recombinant *A. terreus* aristolochene synthase was expressed in *Escherichia coli* BL21(DE3)pLysS and purified as previously described (11, 15). Analysis of the products generated by enzyme incubation with 2F-FPP was performed using gas chromatography and mass spectroscopy (GC-MS) using a similar procedure as previously described for the reaction with substrate FPP (11).

Briefly, a 4 mL solution of 500 nM aristolochene synthase, 20 mM Hepes (pH 7.6), 10% glycerol, 5 mM $MgCl_2$, and 5 mM β-mercaptoethanol was incubated with 50 – 100 μM 2F-FPP or DF-FPP and overlaid with HPLC-grade *n*-pentane (Fisher P399-1) in a glass centrifuge tube at 30 °C for about 30 h. After the initial 10 h incubation, 500 nM enzyme and 50 μM 2F-FPP or DF-FPP were added twice into the aqueous reaction layer at 6 h intervals. Reaction products were extracted with HPLC-grade *n*-pentane and the organic extract was passed through a 3 cm 230-400 mesh silica gel column. The purified extract was concentrated on an ice-water mixture under reduced pressure until the volume was decreased to about 50 μL. The resulting concentrate was analyzed using a Hewlett-Packard 6890 gas chromatograph coupled to a 5973 mass selective detector and equipped with an HP-5MS capillary column (0.25 mm i.d. × 30 m with 0.25 μm film) (Agilent Technologies). Typically, a 2 μL sample was injected in splitless mode into the GS-MS. The oven temperature was at 35 °C for 1 min, then raised to 230 °C at the rate of 5 °C/min, followed by a 20 °C/min increase

to 280 °C. The temperature was maintained at 280 °C for 20 min.

Crystal structure determinations of enzyme complexes with FPP, 2F-FPP, and DF-FPP. Recombinant *A. terreus* aristolochene synthase was crystallized by the hanging drop vapor diffusion method at 4°C as previously described (11). The structures of complexes with substrate FPP and substrate analogues 2F-FPP and DF-FPP were determined by soaking crystals of the unliganded enzyme for 20-24 hours in a buffer solution containing 100 mM Tris-HCl (pH 8.4), 18% PEG 6000, 0.5 M NaCl, 1 mM $MgCl_2$, and 2 mM substrate or substrate analogue. X-ray diffraction data were collected from crystals of each complex at the Brookhaven National Synchrotron Light Source and processed using HKL (16) or Mosflm (17). Crystals were isomorphous with those of the unliganded enzyme (space group $P2_1$, a = 61.2 Å, b = 147.2 Å, c = 83.7 Å, β = 97.9°, with four molecules in the asymmetric unit (11)). Iterative rounds of refinement and model adjustment for each complex were performed using CNS and O, respectively (18, 19). Individual B-factors were refined in each complex.

The N-termini M1-S12, C-termini V318-D320, and loop segments S231-G239 were disordered in all monomers of each structure. Additionally, loop segments F46-P47 and R160-T161 were disordered in monomer D of the FPP complex, loop segment L286-E287 was disordered in monomer C of the 2F-FPP complex, and loop segments N45-N48, K54-F55, and Y95-S97, were disordered in monomer D of the DF-FPP complex. Disordered residues were excluded from the final model of each complex. The exclusion of 111/1280 residues from each tetramer (8.7% of the scattering matter) likely contributed to the somewhat higher than expected R and R_{free} values recorded in Table 1. Continuous electron density peaks adjacent to 5, 3, and 2 cysteine residues, respectively, were modeled as disulfide-linked BME molecules in the FPP, 2F-FPP, and DF-FPP complexes. Data collection and refinement statistics for each complex are recorded in Table 1. Figures were generated using the program Pymol (20).

RESULTS

Reactivity of fluorinated substrates. Aristolochene synthase from *A. terreus* generates (+)-aristolochene as its sole product from reaction with substrate FPP (9). However, following incubation with 2F-FPP in solution, two products are identified in a 95:5 ratio by GS-MS (Figure 2). The chemical ionization (CI) mass spectrum of the major peak eluting at 25.1 min corresponds to a fluorinated sesquiterpene ($C_{15}H_{23}F$) with m/z = 222 (data not shown). This result is consistent with the generation of 2-fluorogermacrene A, as observed after incubating aristolochene synthase from *P. roqueforti* with 2F-FPP (12). The small peak at 23.37 min has a chemical ionization spectrum indicative of a non-fluorinated sesquiterpene ($C_{15}H_{24}$) with M-1 = 204 (data not shown); this is presumed to be a degradation product. A control experiment conducted in the absence of enzyme indicates that 2F-FPP itself does not react to yield any extractable products based on GC-MS analysis.

Incubation of *A. terreus* aristolochene synthase with DF-FPP for 30 h using the approach described above for 2F-FPP does not generate any pentane-extractable products based on GC-MS analysis. This is consistent with the lack of activity with DF-FPP reported for aristolochene synthase from *P. roqueforti*, against which DF-FPP is a reversible competitive inhibitor with K_i = 0.8 μM (13).

Crystal structure of the complex with FPP. The structure of tetrameric aristolochene synthase determined from crystals soaked in a buffer solution containing FPP reveals that monomers A-D adopt open

active site conformations similar to those of the unliganded enzyme (11). Intact FPP molecules with partially similar folded conformations bind in the active site clefts of monomers A, B, and C (Figures 3a and 3b), while Mg^{2+}_B-PP_i binds in the active site cleft of monomer D (Figure 3c). The r.m.s. deviations of 295 Cα atoms in monomers A and B with the corresponding monomers in the unliganded enzyme are 0.28 Å and 0.26 Å, respectively, indicating that these monomers remain in predominantly open conformations. However, some slight structural changes are observed in monomers C and D that reflect the very initial stages of a transition to the closed conformation: helices C1, D, and D1 and loops A–C1 and D–D1 shift slightly toward their expected positions in the closed conformation of monomer C, and helices C1 and H do likewise in monomer D (for reference, the locations of these structural elements are indicated in Figure 1b). Accordingly, the r.m.s. deviations with the unliganded enzyme are larger for these monomers: 0.59 Å for 295 Cα atoms in monomer C, and 0.51 Å for 290 Cα atoms in monomer D. Even so, the active sites of monomers C and D remain in predominantly open, solvent-exposed conformations.

In each of monomers A, B, and C, the terminal diphosphate group of FPP accepts hydrogen bonds from R314 and Y315, and the terminal isoprenoid group of FPP is nestled between F87 and F153. The conformation of the middle isoprenoid moiety of FPP in monomer B is flipped, however, compared to that observed in monomers A and C, both of which have the predicted relative and absolute orientation of the C3 and C7 methyl groups (Figure 3b). Notably, none of the observed FPP conformations correspond to that required for the formation of germacrene A (the first intermediate in aristolochene biosynthesis), since the electrophilic C1 atom is positioned ~6 Å away from the C10-C11 π bond and is not properly oriented for the C1–C10 bond forming step. The binding of FPP to monomers A, B, and C thus may provide "snapshots" of possible substrate binding conformations in the open active site prior to metal ion binding and active site closure.

The active site cleft of monomer D contains only the Mg^{2+}_B-PP_i complex (Figure 3c). Notably, only monomer D appears to be capable of achieving the closed conformation required for catalysis in complex with Mg^{2+}_3-PP_i (11). While aristolochene synthase from *A. terreus* is a dimer in solution (11), the assembly of two dimers to form the tetramer observed in the crystal structure may hinder the conformational transition from the open, inactive state to the closed, active state, thereby accounting for the attenuation of catalytic activity with increasing protein concentrations (9). Additionally, catalytically required conformational changes may be hindered in monomers A-C due to crystal lattice contacts. In monomer D, the observed single-metal ion complex Mg^{2+}_B-PP_i is presumably a remnant of the FPP cyclization reaction catalyzed by this monomer in the crystal. This structure represents the first observation of product PP_i binding to the predominantly open active site conformation of a wild-type terpenoid cyclase.

Since the orientation of PP_i bound in the presence of Mg^{2+}_B in monomer D differs from the orientations of the FPP diphosphate groups in monomers A-C (Figure 3d), Mg^{2+}_B coordination interactions are presumed to play an important role in orienting the substrate diphosphate group and PP_i anion. Superposition of the Mg^{2+}_B-PP_i complex in the open active site conformation and the Mg^{2+}_3-PP_i complex in the closed active site conformation reveals a similar binding mode for the Mg^{2+}_B-PP_i moiety (Figure 3d). Thus, it appears that Mg^{2+}_B is the key metal ion governing the proper binding orientation of PP_i. Hydrogen bond interactions with R314 and Y315 also orient the binding of PP_i.

Interestingly, although R175, K181, and K226 donate hydrogen bonds to PP_i in the Mg^{2+}_3-PP_i complex in the closed active

site conformation (Figure 1c) (11), these residues do not interact with the diphosphate groups of PP_i or FPP in the open active site conformation. The binding of PP_i to the open conformation of monomer D may provide a "snapshot" of a possible complex just prior to product release at the conclusion of the cyclization cascade.

Crystal structure of the complex with 2F-FPP. Despite the evidence for catalytic activity with 2F-FPP in solution (Figure 2), the crystal structure of the aristolochene synthase tetramer complexed with 2F-FPP reveals the binding of intact 2F-FPP to all four monomers with open active site conformations. As observed in the FPP complex, slight structural changes are observed in monomers C and D that appear to reflect the very initial stages of a transition to the closed conformation. The r.m.s. deviations with the unliganded enzyme are 0.26 Å, 0.29 Å, 0.57 Å, and 0.52 Å for monomers A, B, C, and D, respectively.

Electron density corresponding to the diphosphate group of 2F-FPP is well defined in monomers A and D (Figure 4a) but ambiguous in monomers B and C (data not shown). Only one metal ion, Mg^{2+}_B, is bound in this complex, and it binds only to the enzyme-substrate complex in monomer D. Here, the substrate diphosphate group chelates Mg^{2+}_B and accepts hydrogen bonds from R314, Y315, and K226. The position and orientation of the diphosphate group of 2F-FPP is roughly comparable to that of PP_i in the Mg^{2+}_B-PP_i complex (Figure 4b). Strikingly, comparison of the metal-free and metal-bound conformations of 2F-FPP in monomers A and D, respectively, reveals a significant Mg^{2+}_B-dependent reorientation of the substrate (Figure 4c). Thus, it appears that Mg^{2+}_B is the key metal ion governing the proper binding orientation of the substrate diphosphate group.

In each monomer, the fluorine atom of 2F-FPP accepts a hydrogen bond from K181. As observed for FPP, the terminal isoprenoid group of 2F-FPP is nestled between residues F87 and F153, but the 2F-FPP molecules adopt somewhat varying folded conformations in each monomer. Since none of the observed 2F-FPP conformations in monomers A-D are consistent with that required for C1-C10 bond formation in the first step of catalysis, the observed binding conformations of 2F-FPP in the presence or absence of Mg^{2+}_B suggest that additional metal ion binding and active site closure are necessary to chaperone the substrate into a cyclization-competent conformation. Regardless, the complexes of 2F-FPP with monomers A–D provide additional "snapshots" of possible substrate binding modes to the open active site conformation prior to and subsequent to the binding of Mg^{2+}_B.

Crystal structure of the complex with DF-FPP. The crystal structure of aristolochene synthase complexed with DF-FPP reveals the binding of the intact substrate analogue to the open active site conformations of monomers A, B, and C; the r.m.s. deviations with the unliganded enzyme are 0.30 Å, 0.31 Å, and 0.58 Å, respectively. A representative electron density map of DF-FPP bound to monomer B is shown in Figure 5a. No metal ions accompany DF-FPP binding, and the observed isoprenoid and diphosphate conformations are somewhat variable, suggesting some degree of disorder (Figure 5b). The binding of DF-FPP to monomers A–C provides additional "snapshots" of substrate binding conformations that, in the absence of metal ion binding and full active site closure, are not cyclization-competent.

In contrast, monomer D adopts a predominantly closed conformation and contains a PP_i anion complexed with Mg^{2+}_B and Mg^{2+}_C (Figure 5c). There is no residual electron density in the active site to indicate that an intact DF-FPP molecule is present but disordered. A key conformational change evident in this structure is that the aspartate-rich and NSE metal ion binding motifs on helices D and H, respectively, are brought

closer together in order to accommodate the binding of the binuclear magnesium cluster. Reflecting this significant conformational change, the r.m.s. deviation of 278 Cα atoms with the unliganded structure is 0.83 Å.

Although the incubation of DF-FPP with the *A. terreus* enzyme (data not shown) or the *P. roqueforti* enzyme (13) yields no observable products, it is possible that a slow reaction with this substrate analogue occurs over the time course of the X-ray crystallographic experiment to yield the observed PP_i product. Notably, the Oδ1 and Oδ2 atoms of D90 coordinate to Mg^{2+}_C with bidentate geometry, whereas in the complex of aristolochene synthase with Mg^{2+}_3-PP_i, D90 coordinates to both Mg^{2+}_A and Mg^{2+}_C with *syn-syn* bidentate geometry (i.e., D90 Oδ1 coordinates to Mg^{2+}_A and D90 Oδ2 coordinates to Mg^{2+}_C) (11). Some, but not all, hydrogen bond interactions observed in the Mg^{2+}_3-PP_i cluster (11) are observed in the Mg^{2+}_B-Mg^{2+}_C-PP_i complex: R314 and Y315 donate hydrogen bonds to PP_i but K226 is characterized by poor electron density and appears to be somewhat disordered, and R175 is too far from PP_i (5.7 Å) to be considered a formal hydrogen bond donor.

Importantly, this structure reveals that only 2 Mg^{2+} ions are needed to stabilize the predominantly closed active site conformation. Specifically, the binding of Mg^{2+}_C to the FPP/PP_i-Mg^{2+}_B complex appears to be the key molecular event that triggers the conformational transition of the active site from the open state to the closed state. The binding of Mg^{2+}_A to complete the trinuclear magnesium cluster also completes these conformational transitions such that the r.m.s. deviation of 295 Cα atoms between open and closed conformations is 1.5 Å.

DISCUSSION

The three crystal structures reported herein contain 12 independent structures of *A. terreus* aristolochene synthase monomers that illuminate an unprecedented array of binding interactions: 9 examples of substrate binding in the absence of Mg^{2+} to the open active site conformation; 1 example of substrate binding to the open active site conformation in the presence of one metal ion, Mg^{2+}_B; 1 example of PP_i binding to the open active site conformation in the presence of one metal ion, Mg^{2+}_B; and 1 example of PP_i binding to the predominantly closed active site conformation in the presence of two metal ions, Mg^{2+}_B and Mg^{2+}_C. Along with the structure of PP_i binding to the closed active site conformation in the presence of all three metal ions, Mg^{2+}_A, Mg^{2+}_B, and Mg^{2+}_C (11), these structures suggest a possible sequence for metal ion binding and conformational changes required for catalysis.

Substrate Conformation. The observed conformations of intact FPP, 2F-FPP, and DF-FPP are not cyclization-competent due to the fact that they are bound to the open active site conformation. This is a direct consequence of incomplete metal binding, since a complete trinuclear magnesium cluster is required to stabilize the fully closed active site conformation. It is only in the closed conformation that the active site is sequestered from bulk solvent, thereby forming a protected environment in which reactive carbocation intermediates can be formed without the risk of premature quenching by solvent.

Interestingly, most of the crystal structures reported to date of class I terpenoid cyclases complexed with isoprenoid substrate analogues similarly reveal isoprenoid conformations that are not catalytically competent (21-23). As with aristolochene synthase, this often appears to be a consequence of incomplete or compromised metal binding, e.g., as observed in the complex of 5-epi aristolochene synthase with trifluorofarnesyl diphosphate (22). However, isoprenoid conformations that are not cyclization-competent are occasionally observed in monoterpene cyclase active sites with closed conformations stabilized by complete trinuclear magnesium clusters. For

example, the binding of 3-aza-2,3-dihydrogeranyl diphosphate and 3 Mg^{2+} ions to bornyl diphosphate synthase triggers active site closure, and the position, orientation, and intermolecular contacts of the analogue diphosphate group are nearly identical to those observed in the Mg^{2+}_3–PP_i complex (21). Even so, the conformation of the substrate analogue is inconsistent with that required for cyclization. More recently, cocrystallization of limonene synthase with 2-fluorogeranyl diphosphate yields the structure of the complex with 2-fluoro-(3*S*)-linalyl diphosphate, a catalytic intermediate, bound with an extended conformation that is not cyclization-competent; in contrast, cocrystallization with racemic 2-fluorolinalyl diphosphate yields the helical, cyclization-competent isoprenoid conformation (23). This suggests the possibility that isomerization of geranyl diphosphate to linalyl diphosphate does not require a cyclization-competent substrate conformation – such a conformation could instead be achieved after formation of the linalyl diphosphate intermediate.

Metal Binding. Analyses of enzyme-substrate and enzyme-PP_i complexes reported herein and comparisons with the structure of the enzyme-Mg^{2+}_3-PP_i complex reported previously (11) lead to a proposed model for substrate and metal binding (Figure 6). First, hydrogen bond donors R314 and Y315 would govern the initial molecular recognition of the FPP diphosphate group, perhaps concurrently with binding of the Mg^{2+}_B ion by the NSE/DTE motif. Coordination to Mg^{2+}_B would reorient the substrate diphosphate group, as exemplified by the Mg^{2+}_B-dependent reorientation of the 2F-FPP diphosphate group shown in Figure 4c. Since it is only Mg^{2+}_B that is chelated by three protein residues, it is reasonable to hypothesize that this is the first of the three metal ions to bind (its binding site is largely pre-organized). Some slight conformational changes reflecting the very initial stages of active site closure may be triggered by Mg^{2+}_B and/or substrate binding, e.g., as observed in monomers C and D of the FPP/PP_i and 2F-FPP complexes.

Subsequently, the binding of Mg^{2+}_C by both D90 and the substrate diphosphate group would trigger more substantial conformational changes leading to active site closure, as illustrated by the Mg^{2+}_B-Mg^{2+}_C-PP_i complex in Figure 5c. Finally, the binding of Mg^{2+}_A would complete the formation of the trinuclear metal ion cluster, causing Mg^{2+}_C to shift such that D90 would coordinate to both Mg^{2+}_A and Mg^{2+}_C with *syn-syn* bidentate geometry.

The formation of a complete trinuclear magnesium cluster would complete conformational changes required for full active site closure. In the closed conformation, substrate diphosphate metal ion coordination interactions and hydrogen bond interactions with R175, K226, R314, and Y315 would be fully formed, as observed in the Mg^{2+}_3-PP_i complex (Figure 1c) (11). Upon achieving a cyclization-competent substrate conformation in the closed active site, with the three negative charges of the diphosphate leaving group fully neutralized, substrate ionization (24, 25) and cyclization would be initiated.

At the conclusion of the cyclization cascade, we suggest that metal ion dissociation and the transition to the open active site conformation occurs in reverse sequence: Mg^{2+}_A would dissociate first, but Mg^{2+}_B and Mg^{2+}_C would stabilize the active site conformation in a predominantly closed state, e.g., as observed in the Mg^{2+}_B-Mg^{2+}_C-PP_i complex in Figure 5c. The subsequent dissociation of Mg^{2+}_C would then facilitate the transition to an open active site conformation with the release of product aristolochene, and the release of Mg^{2+}_B-PP_i would conclude the catalytic sequence.

Additional support for the proposed role of metal binding in the conformational transition of the terpenoid synthase active site derives from site-directed mutagenesis studies of another fungal sesquiterpene

cyclase, trichodiene synthase, related to aristolochene synthase by only 15% amino acid sequence identity. The r.m.s. deviation of 349 Cα atoms between unliganded and Mg^{2+}_3-PP_i complexed wild-type trichodiene synthase is 1.4 Å (26). In contrast, D100E trichodiene synthase is defective in Mg^{2+}_C binding and does not achieve a fully closed active site conformation in its complex with Mg^{2+}_A, Mg^{2+}_B, and PP_i; the r.m.s. deviation of 354 Cα atoms between the unliganded and liganded enzymes is only 0.44 Å (27). Therefore, Mg^{2+}_C must play a critical role in triggering the structural transition of the trichodiene synthase active site from the open to the closed conformation, just as Mg^{2+}_C is proposed to play a critical role in triggering the structural transition of the aristolochene synthase active site from the open to the closed conformation.

In accord with the metal binding behavior observed with aristolochene synthase and trichodiene synthase, we conclude that the metal binding sequence summarized in Figure 6 suggests specific functions for the three metal ions in terpenoid cyclase-catalyzed ionization-cyclization reactions: Mg^{2+}_B governs initial enzyme-substrate association; Mg^{2+}_C triggers the conformational transition of the active site from the open state to a predominantly closed state in which metal ion binding motifs on helices D and H are brought closer together; and Mg^{2+}_A completes the trinuclear magnesium cluster and thereby completes the conformational transition to the closed state and initiates the cyclization cascade. Future studies of site-specific mutants in the metal binding site of aristolochene synthase and other terpenoid cyclases will allow us to test this model of metal ion function.

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FOOTNOTES

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The atomic coordinates and structure factors of aristolochene synthase complexed with FPP, 2F-FPP, and DF-FPP have been deposited in the Protein Data Bank with accession codes 3BNX, 3BNY, and 3CKE, respectively.

[2] The abbreviations used are: FPP, farnesyl diphosphate; 2F-FPP, 2-fluorofarnesyl diphosphate; DF-FPP, 12,13–difluorofarnesyl diphosphate; PP_i, inorganic pyrophosphate; BME, β-mercaptoethanol; PEG, polyethylene glycol; GC-MS, gas chromatography-mass spectrometry; CI, chemical ionization.

Figure Legends

Figure 1. Aristolochene synthase reaction. (a) Cyclization of farnesyl diphosphate (FPP) to form aristolochene (OPP = diphosphate, PP_i = pyrophosphate). (b) Superposition of monomer D of unliganded aristolochene synthase in the open conformation (red), the closed conformation in the $Mg^{2+}{}_3$-PP_i complex (green) (11), and the FPP/PP_i complex (blue). The binding of $Mg^{2+}{}_3$-PP_i triggers significant conformational changes in helices G1 and H and loops F–G1 and A–C1. Additionally, the N-terminal portion of the H–α1 loop is incorporated into an extension of helix H; helices C1, D, D1, F, G1, H, and I shift inward, and the H–α1 loop (S231 – G239), disordered in the unliganded enzyme, becomes ordered and caps the active site. In the FPP complex (blue), helices C1, H and loops A–C1 and H–α1 shift slightly toward their expected positions in the closed conformation, reflecting the very initial stages of a transition from the open active site conformation to the closed active site conformation. (c) Aristolochene synthase-$Mg^{2+}{}_3$-PP_i complex (11); PP_i metal coordination and hydrogen bond interactions are shown as red dotted lines.

Figure 2. Enzyme incubation with 2F-FPP. Gas chromatogram of the pentane-extractable product from the incubation of aristolochene synthase with 2F-FPP. The chemical ionization (CI) mass spectrum of the compound eluting at 25.10 min is consistent with a fluorinated sesquiterpene with *m/z* = 222. The compound eluting at 23.37 min exhibits M-1 = 204 and is presumed to be a nonfluorinated degradation product.

Figure 3. Stereoview of FPP binding. (a) Simulated annealing omit map of FPP in monomer A contoured at 3.0σ; enzyme-substrate hydrogen bond interactions are indicated by red dotted lines. (b) Superposition of FPP molecules from monomers A (blue), B (green), and C (magenta). For clarity, only the protein atoms of monomer B are shown. (c) Simulated annealing omit maps of the PP_i anion (blue) and $Mg^{2+}{}_B$ (red) in monomer D contoured at 3σ. Water molecules appear as red spheres and hydrogen bond interactions with PP_i are indicated by red dotted lines. (d) Superposition of the "NSE/DTE" motifs of monomers A (yellow) and D (green) in the FPP/PP_i complex, and monomer D in the $Mg^{2+}{}_3$-PP_i complexed structure (11) (blue), in which the enzyme active site adopts the closed conformation. Note that the $Mg^{2+}{}_B$-PP_i orientation is similar regardless of whether PP_i is complexed with $Mg^{2+}{}_3$ or only $Mg^{2+}{}_B$; moreover, the orientation of the FPP diphosphate group in the absence of Mg^{2+} ions differs significantly from that of metal-coordinated PP_i. Metal coordination interactions are indicated by dashed lines; water molecules are omitted for clarity.

Figure 4. Stereoview of 2F-FPP binding. (a) Simulated annealing omit maps of 2F-FPP (blue) and $Mg^{2+}{}_B$ (red) in monomer D contoured at 3.1σ. Water molecules appear as red spheres and enzyme-substrate hydrogen bond interactions are indicated by red dotted lines. (b) Superposition of the NSE/DTE motifs of monomer D (yellow) in the 2F-FPP complex, and monomer D in the $Mg^{2+}{}_B$-PP_i complex (green) shown in Figure 3c. The interaction of the 2F-FPP diphosphate group with $Mg^{2+}{}_B$ is roughly similar to that observed for PP_i. Metal coordination interactions are indicated by dashed lines; water molecules are omitted for clarity. (c) Superposition of 2F-FPP bound to monomer A (red) and monomer D (blue) illustrates the $Mg^{2+}{}_B$-triggered reorientation of the substrate diphosphate group (for clarity, only the protein atoms of monomer D are shown).

Figure 5. Stereoview of DF-FPP binding. (a) Simulated annealing omit map of DF-FPP bound to monomer B of aristolochene synthase contoured at 3.0σ. Water molecules appear as red spheres and a hydrogen bond between a diphosphate oxygen atom and R314 is indicated by a red dotted line. (b) Superposition of DF-FPP molecules from monomers A (blue), B (green), and C (red), indicating

some degree of disordered binding. For clarity, only the protein atoms of monomer A are shown. (c) Simulated annealing omit maps (contoured at 3σ) of the PP_i anion (blue) and Mg^{2+}_B and Mg^{2+}_C (red) in monomer D. Water molecules appear as red spheres; enzyme-PP_i metal coordination and hydrogen bond interactions are indicated by red dotted lines.

Figure 6. Proposed metal ion binding sequence for catalysis. Model for metal ion binding, conformational transitions between open and closed conformations, and catalysis by aristolochene synthase based on the "snapshots" of metal ion, substrate, and product PP_i binding provided by the crystal structures reported herein.

Table 1: Data Collection and Refinement Statistics

Complex	FPP	2F-FPP	DF-FPP
Resolution (Å)	50.00 – 2.10	61.08 – 1.89	56.30 – 2.4
Unique reflections	80431	104421	57488
Completeness (%) (outer shell)	93.7 (93.5)	88.2 (77.9)	99.6 (99.8)
I/σ(I) overall (outer shell)	11.2 (2.1)	12.3 (3.2)	14.3 (4.4)
R_{merge}(outer shell)[a]	0.099 (0.586)	0.073 (0.502)	0.095 (0.649)
Protein atoms (*N*)[b]	9558	9571	9494
Solvent (*N*)[b]	303	524	245
BME/glycerol atoms (*N*)[b]	20	12	14
Magnesium ions (*N*)[b]	1	1	2
Chloride ions (*N*)[b]	1	0	1
Substrate atoms (*N*)[b]	72	100	78
PP_i atoms (*N*)[b]	9	0	9
R_{cryst} (outer shell)[c]	0.239 (0.314)	0.224 (0.270)	0.241 (0.333)
R_{free} (outer shell)[c]	0.277 (0.350)	0.264 (0.320)	0.290 (0.387)
R.m.s. deviations			
Bonds (Å)	0.007	0.013	0.006
Angles (°)	1.1	1.6	1.1
Dihedral angles (°)	18.8	20.0	18.9
Improper dihedral angles (°)	0.8	1.1	1.7
Ramachandran statistics (%)[d]			
Allowed	91.8	93.8	90.7
Additionally allowed	7.7	5.5	8.3

Generously allowed	0.5	0.7	1.1

[a] $R_{merge} = \Sigma|I_i - \langle I_i \rangle|/\Sigma I_i$, where I_i is the observed intensity for reflection i and $\langle I_i \rangle$ is the average intensity calculated for reflection i from replicate data.

[b] Per asymmetric unit.

[c] $R = \Sigma \, ||F_o| - |F_c||/\Sigma|F_o|$, where R and R_{free} are calculated using the working and test reflection sets, respectively (the test reflection set is excluded from refinement); $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively.

[d] Calculated with PROCHECK (28).

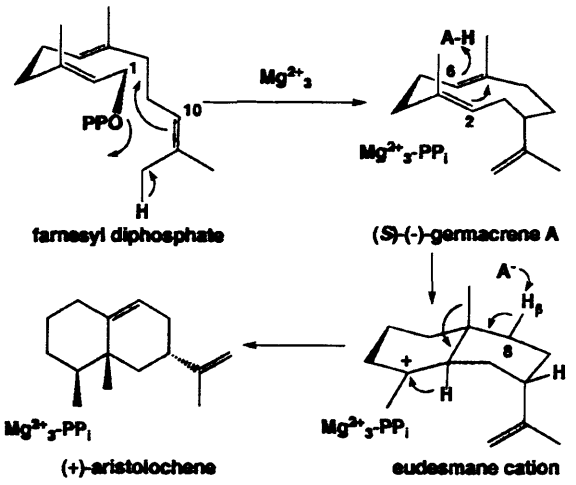

Fig. 1a

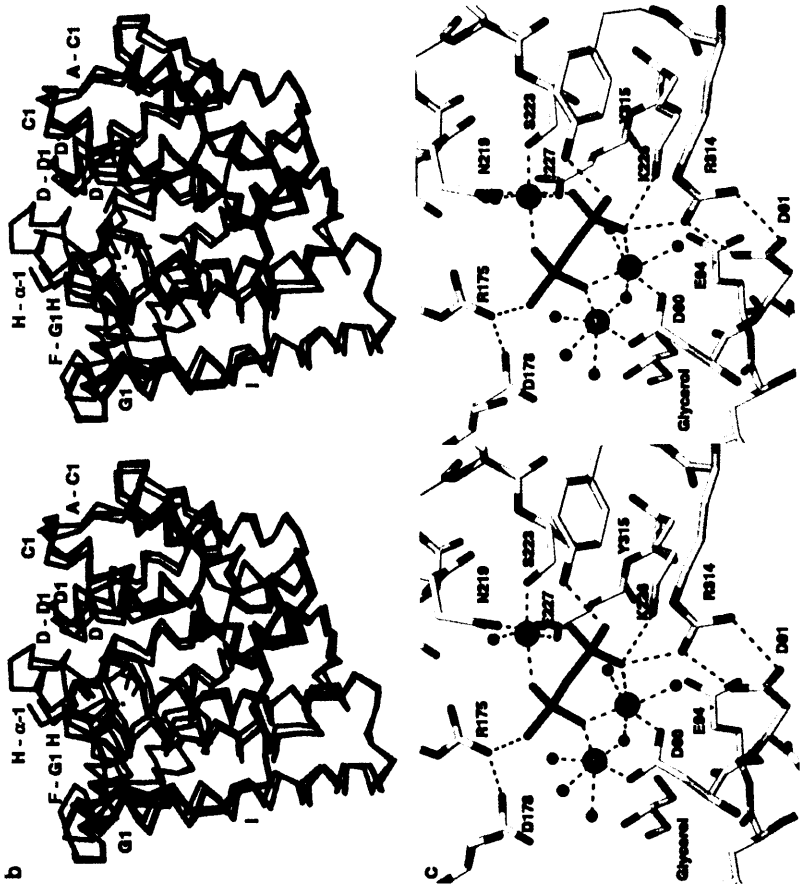

Fig. 1b-c

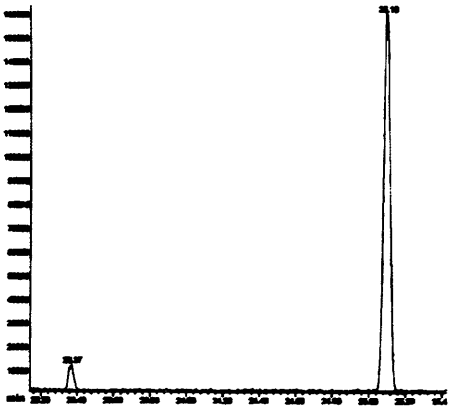

Fig. 2

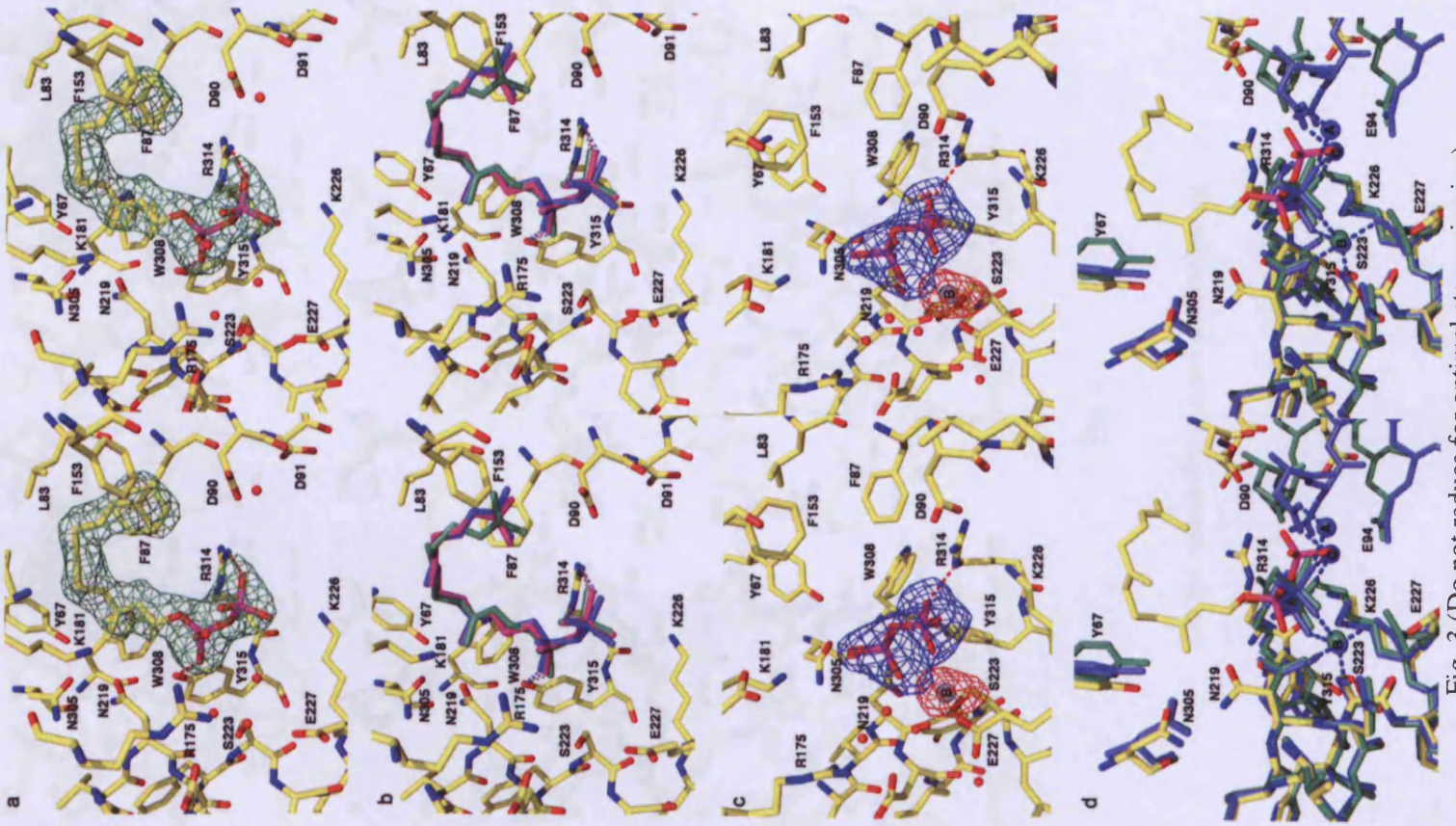

Fig. 3 (Do not reduce for optimal stereoviewing)

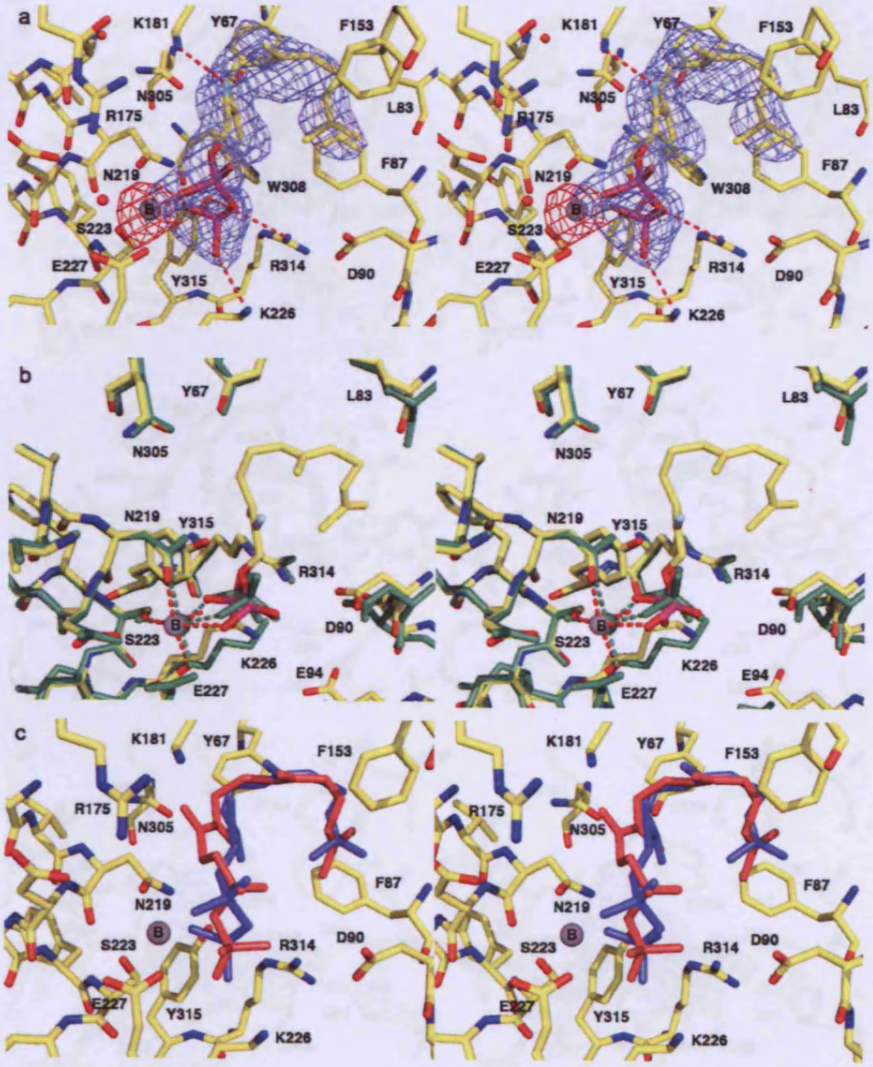

Fig. 4

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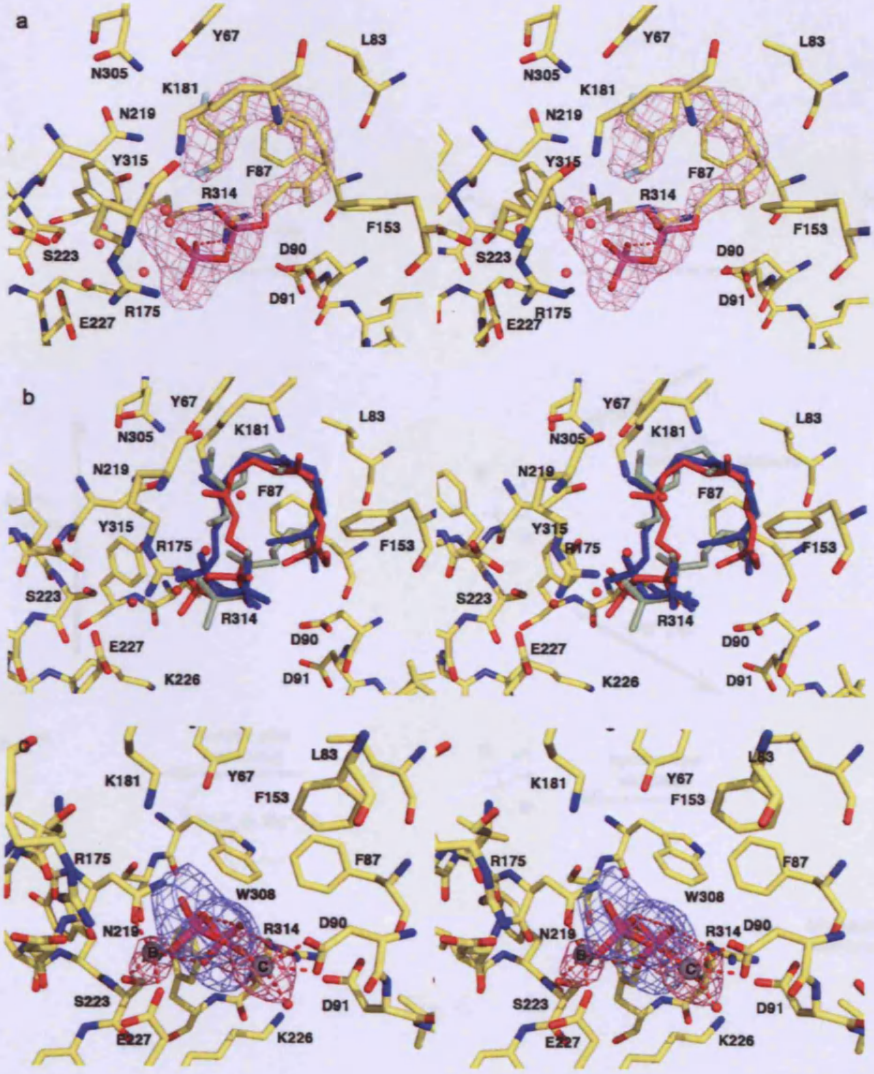

Fig. 5

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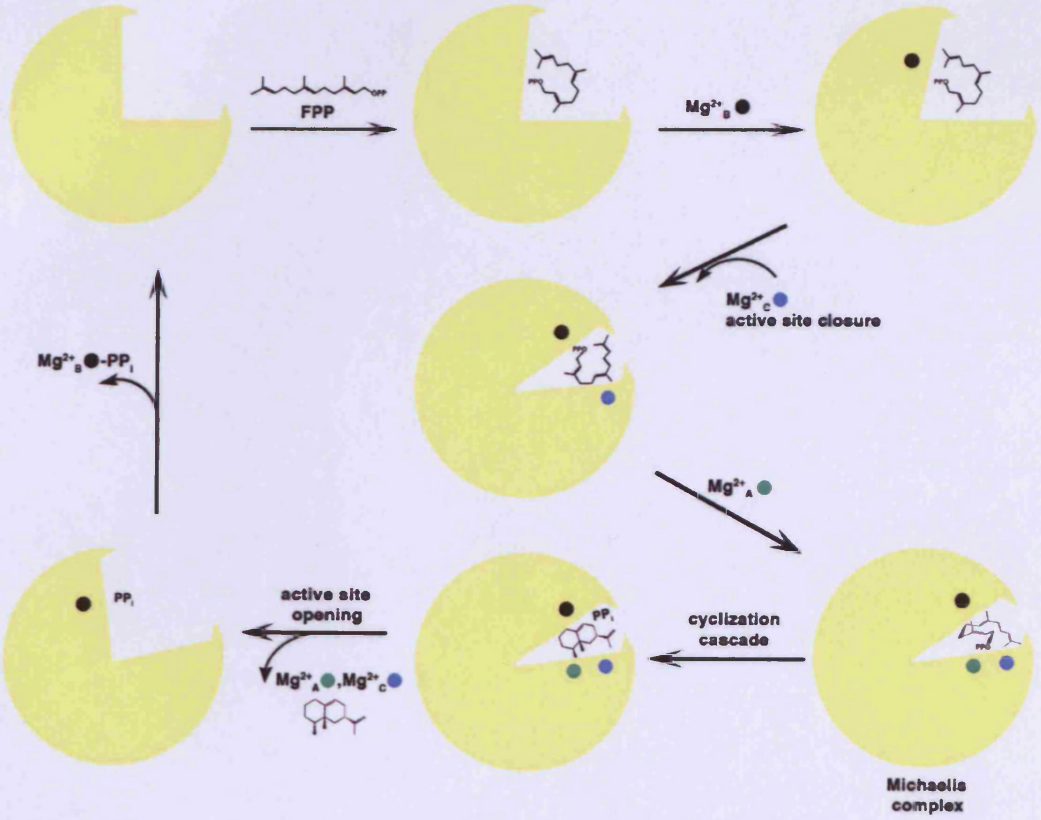

Fig. 6