The biosynthesis of C_5–C_{25} terpenoid compounds

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This review covers recently-published experimental information on the biosynthesis of terpenoids in the range C_5–C_{25}. In addition to sections on the mevalonate and mevalonate-independent (deoxyxylulose phosphate) pathways, the review considers in turn hemiterpenoids, polyprenyl diphosphate synthases, monoterpenoids, sesquiterpenoids, diterpenoids, and sesterterpenoids. The literature from January 1998 to December 2000 is reviewed, with 248 references cited.

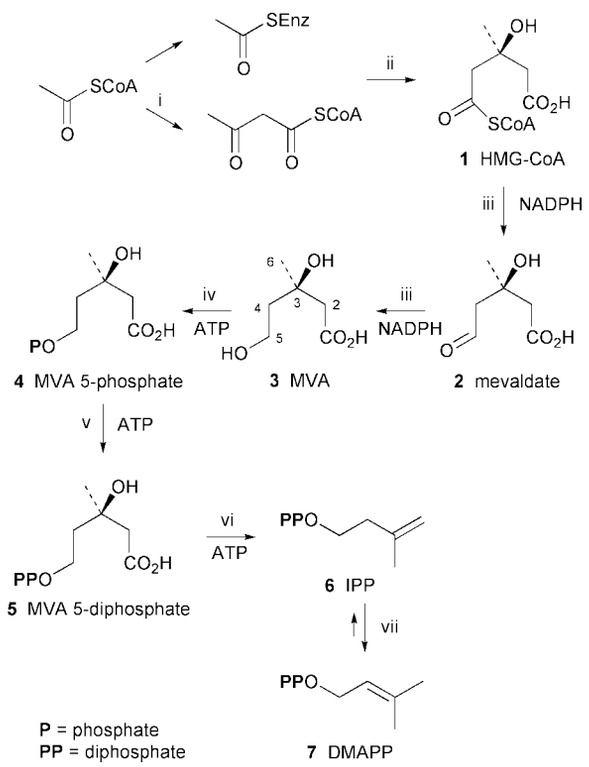
1 Introduction

This report reviews the literature that was published during the three years 1998–2000 on the biosynthesis of terpenoids in the range C_5–C_{25}, and continues the coverage described in earlier volumes of *Natural Product Reports*.[1–3] C_{30} and larger terpenoids are discussed elsewhere under triterpenoids and steroids, and carotenoids. This review describes the biosynthetic pathways, the enzymes and enzyme mechanisms involved, and information about genes encoding for these enzymes. A number of specific aspects that are more appropriate to other journals are not included here. Such topics as the regulation of terpenoid biosynthesis, particularly where the emphasis relates predominantly to steroidal compounds and higher terpenoids, the genetic control of biosynthesis, and biotransformations are not covered. The biosynthesis of meroterpenoids is also generally omitted. These compounds contain a terpenoid unit as part of a more complex structure, and are adequately treated in other reports according to the major substructure, *e.g.* alkaloids, polyketides, and shikimate metabolites.

It is now apparent that the mevalonate pathway, formerly regarded as the universal route to terpenoids and steroids, is much less prominent in secondary metabolism than the more recently discovered mevalonate-independent pathway *via* deoxyxylulose phosphate. The fine details of this latter pathway are now being uncovered, and it is anticipated that the complete sequence will soon be known. Molecular biology is now being used routinely to provide access to biosynthetic enzymes, and this represents a major shift in terpenoid biosynthetic methodology. The molecular biology of plant terpenoid synthases has been reviewed.[4]

2 Mevalonic acid

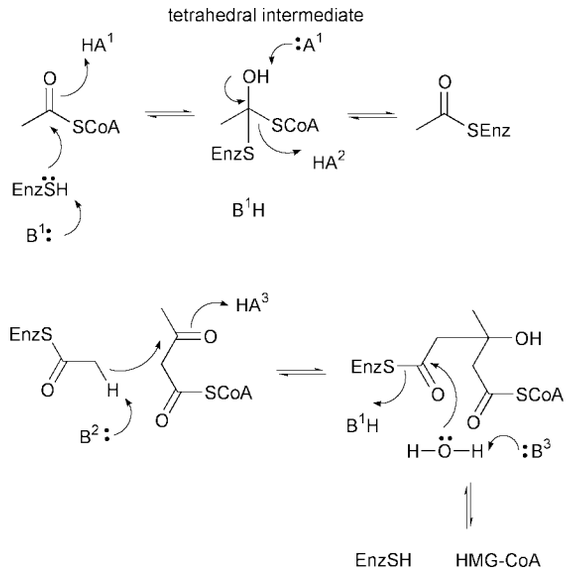
The condensation of acetyl-CoA and acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) **1** is catalysed by the enzyme HMG-CoA synthase (Scheme 1). Two forms of the enzyme are known in mammals, a cytosolic enzyme which



Scheme 1 The mevalonate pathway. *Enzymes*: i, acetoacetyl-CoA thiolase (AACT); ii, HMG-CoA synthase; iii, HMG-CoA reductase (HMGR); iv, mevalonate kinase; v, phosphomevalonate kinase; vi, mevalonate 5-diphosphate decarboxylase; vii, IPP isomerase.

is the starting point for the mevalonic acid (MVA) pathway, and a mitochondrial enzyme which, together with HMG-CoA lyase, is involved in ketone body synthesis. The mitochondrial HMG-CoA synthase is not considered further in this report.

Earlier studies have indicated that a reasonably stable covalent acetyl-*S*-enzyme intermediate forms prior to condensation with acetoacetyl-CoA. This intermediate has now been detected using the avian enzyme. When it is incubated with [1-^{13}C]acetyl-CoA there are large upfield shifts in the ^{13}C NMR spectrum compared to those observed for free acetyl-CoA.[5] This shift (20 ppm for C-1 and 7 ppm for C-2) is probably due to the transient production during acetyl-*S*-enzyme formation of a tetrahedral species with an sp^3-hybridized carbon (Scheme 2). Rapid exchange between such a species (low in steady-state concentration) and a dominant acetyl-*S*-enzyme species (with an sp^2-hydridized carbon) could account for a component of the upfield shift in the observed signal. These studies were



Scheme 2 *Enzyme*: HMG-CoA synthase.

extended using [1,2-$^{13}C_2$]acetyl-CoA, and conducting the reactions in $H_2{}^{18}O$ and ^2H_2O.[6] Analysis of the various shifts observed indicated the involvement of other tetrahedral intermediates, and therefore a minimal mechanism involving only a general base to deprotonate the C-2 methyl group of acetyl-*S*-enzyme and a general acid that protonates the C-3 carbonyl of acetoacetyl-CoA cannot be tenable. The observations point to a more detailed mechanism and possible roles for acid/base catalysts as shown in Scheme 2. By replacing Glu-95 with Ala, catalytic activity of the enzyme was diminished by over five orders of magnitude.[7] This amino acid replacement did not affect active site integrity with respect to initial formation of the acetyl-*S*-enzyme intermediate, or the terminal hydrolysis reaction, but was shown to have defective C–C bond formation. Evidence points to Glu-95 functioning as a general acid in the HMG-CoA synthase reaction. Functional evaluation of 11 invariant amino acids in the enzyme's active site using site-directed mutagenesis has also been reported.[8] Three mutant synthases, D99A, D159A, and D203A, all formed the acetyl-*S*-enzyme intermediate very slowly. The impact of three distinct amino acids on reaction intermediate formation supports the mechanism for acetyl-*S*-enzyme formation that requires formation and collapse of a tetrahedral intermediate, though it is not yet possible to assign precise roles for these amino acids.

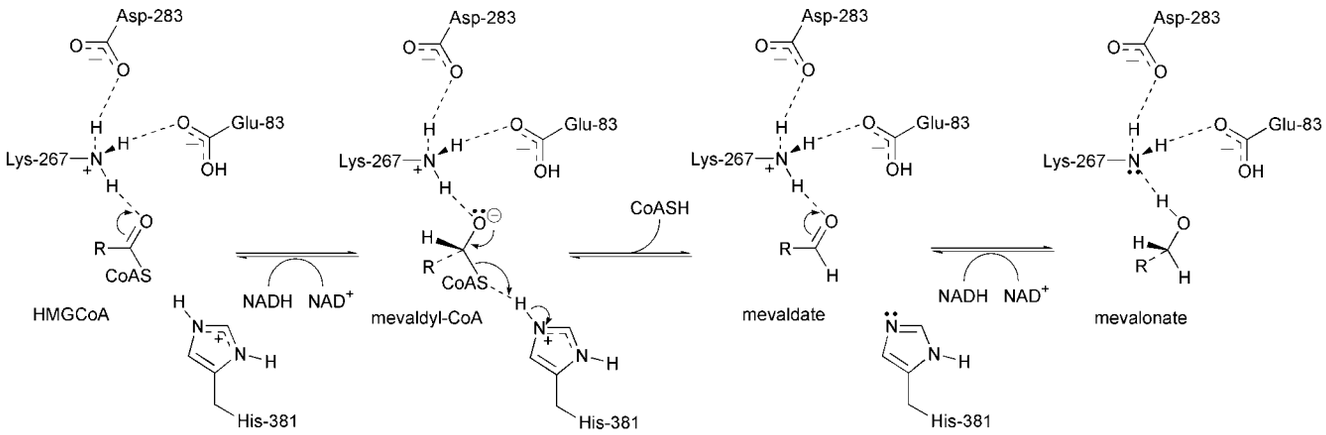
The next enzyme on the mevalonic acid biosynthetic pathway is HMG-CoA reductase (HMGR) which catalyses reductive deacylation of HMG-CoA to mevalonate (MVA) **3** *via* mevaldate **2** and employs two equivalents of NADPH as reductant Scheme 1. This enzyme activity provides an important control mechanism for the flow of metabolites into mevalonate and, especially, into steroid biosynthesis and its study continues to stimulate much research. Eubacterial HMGR from *Streptomyces* sp. strain CL190 has been purified, and the gene encoding for the enzyme cloned.[9] The deduced amino acid sequence revealed several limited motifs which were highly conserved and common to eukaryotic and archaebacterial enzymes. Based on amino acid sequences, two distinct classes of HMGR can be differentiated.[10] Genes that encode class I enzymes are present in all eukaryotes, in many archaea, and in some streptomycetes. Genes encoding class II enzymes are present in some eubacteria. The *mvaA* gene in *Staphylococcus aureus* encodes a class II HMGR.[11] Unlike most other HMGR enzymes, the *S. aureus* enzyme exhibits dual coenzyme specificity for NADP(H) and NAD(H), with NADP(H) preferred. pH profiles suggested His-378 and Lys-263 function in catalysis. The isolation and overexpression of the gene encoding HMGR in *Leishmania major* has been reported.[12] The protein lacks the membrane domain characteristic of eukaryotic enzymes, but exhibits sequence similarity with eukaryotic reductases. Plant genes encoding for HMGR in mulberry (*Morus alba*)[13] and *Tagetes erecta*[14] have also been isolated and characterized. Two cDNAs were found in marigold, one of which encoded a truncated form of the enzyme. Antisense expression of *Arabidopsis thaliana hmg1* gene in tobacco (*Nicotiana tabacum*) was found to decrease general isoprenoid levels.[15]

The crystal structures of two non-productive ternary complexes of HMGR from *Pseudomonas mevalonii* with HMG-CoA/NAD^+ and with MVA/NADH have been determined.[16] In the structure of the apoenzyme reported earlier, the last 50 amino acid residues of the *C*-terminus (the flap domain), including the catalytic residue His-381, were not visible. The structures of the ternary complexes reported here reveal a substrate-induced closing of the flap domain that completes the active site and aligns His-381 with the thioester of HMG-CoA. Lys-267 also appears to be involved in catalysis, its role as a general acid/base being postulated in Scheme 3. Lys-267 facilitates hydride transfer from reduced coenzyme by polarizing the carbonyl group of HMG-CoA, and subsequently of bound mevaldate. In subsequent studies,[17] site-directed mutagenesis was employed to investigate this active site lysine. Replacement of Lys-267 with Ala, His, or Arg resulted in total loss of activity. Then, replacement of Lys-267 with Cys, followed by chemical derivatization allowed the introduction of lysine analogues aminoethylcysteine and carboxyamidomethylcysteine. The latter derivative was inactive, though the former exhibited high catalytic activity. That aminoethylcysteine, but not other basic amino acids, can replace the function of Lys-267 underlines the importance of this residue, and the requirement for a precisely positioned positive charge at the enzyme active site. HMGR from *Pseudomonas mevalonii* is a class II enzyme. If the proposed mechanism involving Lys-267 is general, class I HMGRs should also possess an active site Lys, and indeed, sequence analysis shows three lysines are conserved among all class I enzymes.[18] The three conserved lysines of Syrian hamster HMGR were mutated to Ala; all three mutant enzymes had reduced but detectable activity. Sequence alignments suggested Lys-734 of the hamster enzyme as the most likely cognate of *P. mevalonii* Lys-267, and it is proposed that HMGRs of both classes employ a similar catalytic mechanism involving an active site lysine.

Sequence analysis of flanking regions of the *hmgr* gene in *Streptomyces* sp. strain CL190 revealed five open reading frames, *orfA–E*, which showed similarity to those encoding eukaryotic and archaebacterial enzymes from the mevalonate pathway.[19] An *E. coli* transformant with *hmgr* and *orfABCDE* was shown to grow in the presence of fosmidomycin, a potent inhibitor of the mevalonate-independent pathway (see Section 4), and to produce ubiquinone from labelled acetate with a labelling pattern characteristic of the mevalonate pathway, though the mevalonate pathway is intrinsically absent from *E. coli*. The *hmgr* gene and *orfABCDE* are thus responsible for the mevalonate pathway and constitute a gene cluster in *Streptomyces*. Based on known sequences, the identities of *orfA* to *orfE* were deduced.

3 Hemiterpenoids

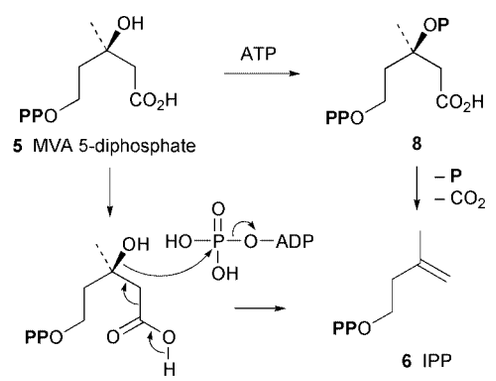
Mevalonate kinase catalyses the first of the ATP-dependent phosphorylations of mevalonate to mevalonate 5-phosphate **4**, then mevalonate 5-diphosphate **5** is produced by the further action of phosphomevalonate kinase. These reactions lead up to formation of isopentenyl diphosphate (IPP) **6** and dimethylallyl diphosphate (DMAPP) **7**, the biogenetic isoprene units (Scheme 1). Purification of mevalonate kinase from the plant *Catharanthus roseus* has been reported.[20] Kinetic studies indicated an ordered sequential mechanism of action, in which mevalonate was the first substrate to bind and ADP was the last



Scheme 3 *Enzyme*: HMG-CoA reductase.

product to leave the enzyme. Activity was dependent on the presence of divalent metal ions, Mg^{2+} and Mn^{2+} being best and equally effective. The activity was strongly inhibited by farnesyl diphosphate. The *Arabidopsis thaliana* gene encoding for mevalonate kinase has been cloned and characterized.[21] Phosphomevalonate kinase activity from *Catharanthus roseus* cells has been partially purified.[22] This enzyme was also dependent on the presence of divalent metal ions, with a preference for Mg^{2+}.

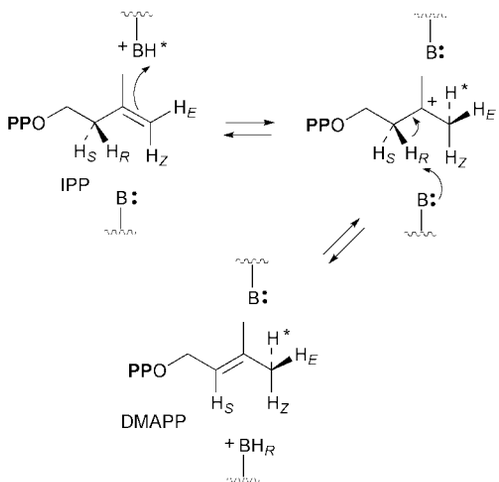
The mechanism of mevalonate 5-diphosphate decarboxylase still awaits full clarification. Whilst a third molecule of ATP is required for the transformation, there had appeared to be little evidence for phosphorylation of the tertiary hydroxy group, and a mechanism in which an ATP molecule facilitated the decarboxylation–elimination had been proposed (Scheme 4).



Scheme 4 *Enzyme*: mevalonate 5-diphosphate decarboxylase.

However, more recent studies (see ref. 1) had given some evidence for involvement of the 3-phosphate intermediate **8**. Sequencing of a full-length cDNA encoding for mevalonate 5-diphosphate decarboxylase in *Arabidopsis thaliana* has been reported.[23] The deduced amino acid sequence shared about 55% identity with yeast, human, and rat enzymes. When expressed in yeast, the *A. thaliana* cDNA complemented strains deficient in the enzyme, though wild-type sterol content was not fully restored.

Isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IPP isomerase) catalyses the 1,3-allylic rearrangement reaction converting IPP into DMAPP *via* a postulated two-base cationic mechanism (Scheme 5). In eukaryotes and yeast, the enzyme removes the *pro-R* proton from C-2 of IPP in the forward reaction, and in the reverse reaction a proton from water is delivered to the *re* face of the C-2-C-3 double bond. Since many bacteria and plant chloroplasts have been shown to employ the mevalonate-independent deoxyxylulose phosphate (DXP) pathway to terpenoids (see Section 4), the stereochemical features of this interconversion have been reinvestigated using enzyme from *E. coli*, an organism which utilizes the DXP pathway.[24] It has been found that enzymes from both *E. coli*

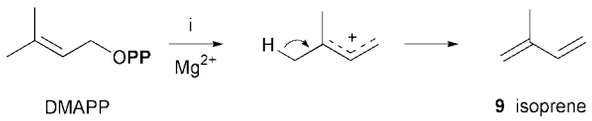


Scheme 5 *Enzyme*: IPP isomerase.

and *Schizosaccharomyces pombe* catalyse the removal of the deuterium from labelled (*R*)-[2-^{2}H]IPP, *i.e.* the same stereochemistry as observed for *Saccharomyces cerevisiae* and rat liver enzymes. When the enzyme was incubated with (*R*)-[2-^{2}H]IPP in the presence of avian FPP synthase, this gave FPP without any deuterium labelling. The eukaryotic FPP synthase is known to remove the *pro-R* hydrogen from IPP during chain elongation. The reverse reaction was investigated by incubating the enzyme with unlabelled DMAPP and ^2H_2O in the presence of GPP and avian FPP synthase, so that the newly formed [2-^{2}H]IPP was immediately converted into FPP. No deuterium labelling was observed in the product. Thus, the reverse reaction also displays the same stereochemical features as eukaryotic isomerases.

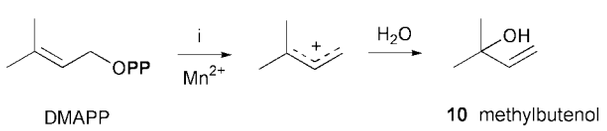
Two cDNAs encoding IPP isomerase in the green unicellular alga *Haematococcus pluvialis* have been identified.[25] Deduced amino acid sequences for the two enzymes were 95% identical; only the smaller of the two proteins was implicated in carotenoid accumulation in the cytoplasm. The presence of isoforms of IPP isomerase in cell cultures of several plant species from the Rubiaceous plants *Cinchona robusta*, *Morinda citrifolia*, and *Rubia tinctorum* has been reported.[26] Additional isoforms appeared inducible, and corresponded to the accumulation of anthraquinones requiring DMAPP for their biosynthesis. The cyanobacterium *Synechococcus* sp. strain PCC6803 is deficient in IPP isomerase activity, consistent with the absence of an obvious homologue for the enzyme in its genome.[27] Incorporation of labelled IPP into terpenoids (primarily C_{20}) in cell extracts occurred only upon priming with DMAPP. Isoprenoid synthesis in *Synechococcus* does not appear to involve interconversion of IPP and DMAPP, and it has been suggested that these materials are synthesized separately (compare similar conclusions in Section 4).

Isoprene **9** is traditionally associated with the terpenoids as the hypothetical building block, but this hemiterpene is also a natural product emitted by a number of plants, often in very large quantities. Isoprene is formed from DMAPP *via* the action of isoprene synthase which catalyses elimination of diphosphate (Scheme 6). The isolation of thylakoid-bound and



Scheme 6 *Enzyme*: i, isoprene synthase.

soluble forms of isoprene synthase from willow (*Salix discolor*) has been described.[28] When solubilized, the thylakoid-bound enzyme exhibited similar catalytic properties to the soluble stromal enzyme, and both contain essential cysteine, histidine and arginine residues, as do other isoprenoid synthases. A methylbutenol synthase activity that catalyses formation of methylbutenol **10** from DMAPP has been detected in needles of grey pine (*Pinus sabiniana*) and partially purified.[29] Like other prenyl diphosphate-utilizing enzymes it was dependent on the presence of a divalent cation, preferably Mn^{2+}, for activity. Based on the analogous biosynthesis of linalool from GPP, a mechanism involving hydration of the DMAPP-derived allylic cation (Scheme 7) is most probable. GPP is not a substrate for



Scheme 7 *Enzyme*: i, methylbutenol synthase.

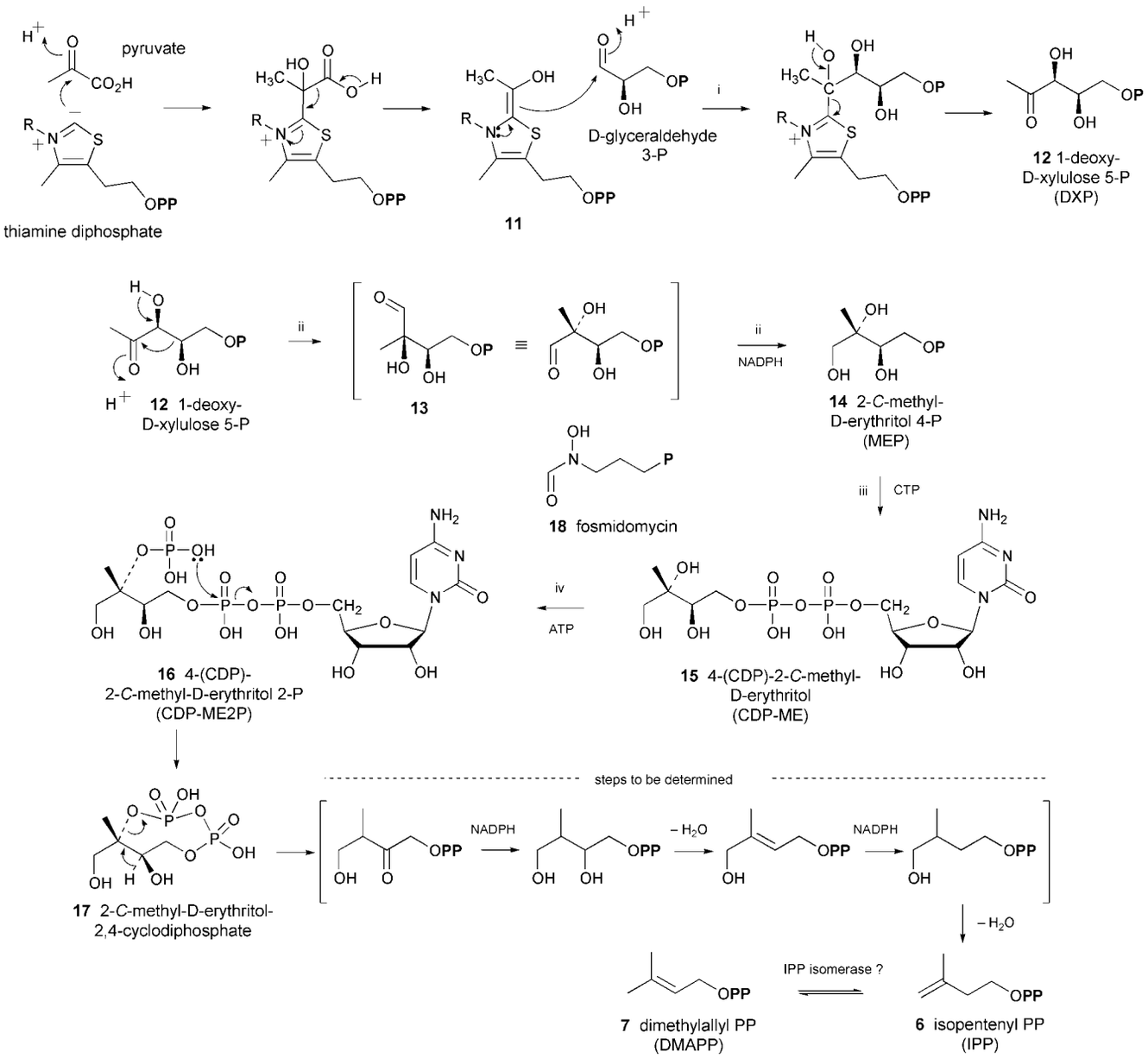
methylbutenol synthase, so the enzyme is distinct from linalool synthase. Like isoprene, methylbutenol is another volatile hemiterpene released from trees, in this case several pine species, and its formation also appears to be regulated by light-dependent reactions in the chloroplast.

4 The mevalonate-independent (deoxyxylulose phosphate) pathway

Considerable evidence has now accumulated that the mevalonate pathway is employed much less frequently in the biosynthesis of terpenoids than is the newly-discovered mevalonate-independent pathway *via* 1-deoxyxylulose 5-phosphate. During the period of review, there have been many further reported examples of its operation, and most of the steps leading from the primary precursors pyruvate and glyceraldehyde 3-phosphate are now delineated, though the late steps concerned in the formation of IPP and DMAPP are not yet clarified. There are now several terminologies commonly in use for this pathway, including mevalonate-independent pathway, non-mevalonate pathway, glyceraldehyde 3-phosphate/pyruvate pathway, deoxyxylulose phosphate (DXP or DOXP) pathway, and methylerythritol phosphate (MEP) pathway. There has been an attempt to obtain agreement that the latter MEP nomenclature should be adopted since MEP is the first committed terpenoid precursor, whilst DXP is also used for the biosynthesis of thiamine and pyridoxol. So far, there is little evidence for any general usage of the MEP terminology. Whereas the mevalonate pathway enzymes are localized in the cytosol, the DXP pathway enzymes appear to be plastid-related. These features broadly account for the observed operation of the two pathways in various classes of terpenoid. Thus, the mevalonate pathway provides cytosolic metabolites, particularly triterpenoids and steroids, plus some sesquiterpenoids. The DXP pathway leads to plastid-related metabolites, monoterpenes and diterpenes, some sesquiterpenes, tetraterpenes (carotenoids), and the prenyl side-chains of chlorophyll and plastoquinones. There are examples of cooperation between the cytosolic and plastidial pathways, especially in the biosynthesis of stress metabolites. The DXP pathway is not known to operate in mammals.

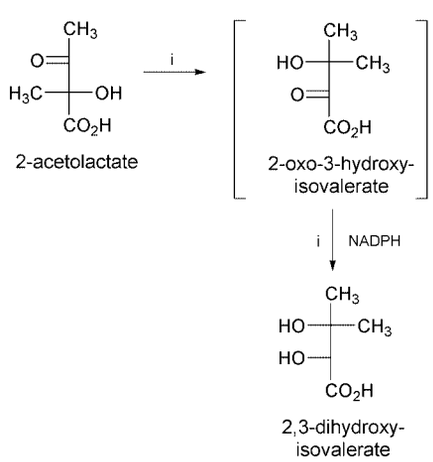
The first reaction of the pathway is a transketolase-like condensation between pyruvate and D-glyceraldehyde 3-phosphate to form 1-deoxy-D-xylulose 5-phosphate (DXP) **12** (Scheme 8). This involves condensation of (hydroxyethyl)thiamine diphosphate **11**, derived from pyruvate, with the aldehyde group of glyceraldehyde 3-phosphate. A gene *dxs* from *E. coli* encoding DXP synthase has been cloned and characterized.[30] The gene was part of an operon that also contains the gene *ispA* which encodes FPP synthase. The enzyme shows features of a typical binding site for thiamine diphosphate, and a histidine residue that has been proposed to participate in proton transfer in transketolase reactions. However, a motif that appears to be involved in substrate binding in transketolases is not conserved in DXP synthase. Cloning of the gene from peppermint (*Mentha* X *piperita*) has also been reported, together with expression of the functional protein in *E. coli*.[31] This enzyme contains a proposed plastid-targeting sequence. Excluding this targeting sequence, the deduced amino acid sequence showed very high identity to DXP synthase enzymes from *E. coli* and *Arabidopsis thaliana*, and also to proteins from the photosynthetic bacterium *Rhodobacter capsulata* and the cyanobacterium *Synechocystis* sp. PCC6803. These appear to form a new class of transketolases distinct from the well-characterized transketolases involved in the pentose phosphate pathway, and their extensive sequence similarity suggests that they are all DXP synthases. Two pepper (*Capsicum annuum*) cDNAs encoding transketolases have been characterized.[32] One of these is primarily involved in plastidial pentose phosphate and glycolytic cycle integration, whilst the second encodes DXP synthase, and is highly expressed during carotenoid biosynthesis in pepper. A *dxs* gene in the unicellular cyanobacterium *Synechococcus leopoliensis* (*Anacystis nidulans*) has been identified and expressed in *E. coli*, resulting in increased synthesis of DMAPP.[33] *Streptomyces* sp. strain CL190 utilizes both mevalonate and mevalonate-independent pathways to terpenoids. The *dxs* gene from this organism has been cloned and overexpressed in *E. coli* to yield recombinant enzyme.[34] This is a soluble enzyme, and most likely a dimer. Except for pH optimum, its enzymic properties were the same as those of recombinant *E. coli* DXP synthase, also overexpressed and purified. A gene *CLA1*, previously isolated from *Arabidopsis thaliana*, has now been shown to encode DXP synthase.[35] In addition to demonstration of the enzymic activity, it has also been shown that this activity complements an albino *cla1-1* mutant. A single base change in the *CLA1* gene is the cause of the *chs5* mutation in *Arabidopsis*, resulting in temperature sensitivity.[36] Application of 1-deoxy-D-xylulose rescues the defect in the *chs5* mutant. An *E. coli* strain engineered to produce the carotenoid lycopene was further transformed with *dxps* genes cloned from *Bacillus subtilis* and *Synechocystis* sp. 6803.[37] This resulted in increased levels of both lycopene and ubiquinone-8 compared to controls.

DXP is transformed into 2-*C*-methyl-D-erythritol 4-phosphate (MEP) **14** in the next step of the pathway. The involvement of this intermediate was demonstrated by preparing mutants of *E. coli* and selecting three that required **14** for growth and survival.[38,39] All the DNA fragments which complemented this synthetic defect were found to contain a *yaeM* gene (in later studies referred to as *dxr*). The deduced amino acid sequence for the encoded protein showed significant homologies to hypothetical proteins with unknown functions from several eubacterial species. The purified recombinant *yaeM* gene product was overexpressed in *E. coli* as a tetramer, and found to catalyse formation of MEP from DXP in the presence of NADPH. NADH was a much poorer reductant, and the enzyme also required a divalent cation, preferably



Scheme 8 The deoxyxylulose phosphate pathway. *Enzymes*: i, 1-deoxyxylulose 5-phosphate synthase (DXP synthase); ii, 1-deoxyxylulose 5-phosphate reductoisomerase (DXP reductoisomerase); iii, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol synthase (CDP-ME synthase); iv, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase (CDP-ME kinase).

Mn^{2+}, but also accepted Co^{2+} or Mg^{2+}. The enzyme is designated 1-deoxy-D-xylulose 5-phosphate reductoisomerase, and catalyses an intramolecular rearrangement and reduction. The rearrangement is pinacol-like, and the anticipated intermediate aldehyde **13** (2-*C*-methylerythrose 4-phosphate) is not released from the enzyme, but is simultaneously reduced by the cofactor NADPH (Scheme 8). The enzyme's amino acid sequence contains a recognizable NADPH-binding domain. There is ample precedent for this rearrangement–reduction in the transformation of 2-acetolactate into 2,3-dihydroxyisovalerate by ketol acid reductoisomerase (Scheme 9) during the biosynthesis of valine, isoleucine, and leucine. The gene encoding a plant homologue of this enzyme has been isolated from peppermint (*Mentha* X *piperita*) and expressed in *E. coli*.[40] Unlike the microbial reductoisomerase, the enzyme encoded by the plant gene is a preprotein bearing an *N*-terminal plastidial transit peptide that directs the enzyme to plastids, where the mevalonate-independent pathway operates in plants. Similarly, a cDNA from *Arabidopsis thaliana* encoding the enzyme has been cloned and expressed.[41] A *dxr* gene from the cyanobacterium *Synechococcus leopoliensis* encoding for the reductoisomerase has been identified and expressed in *E. coli*.[42] In

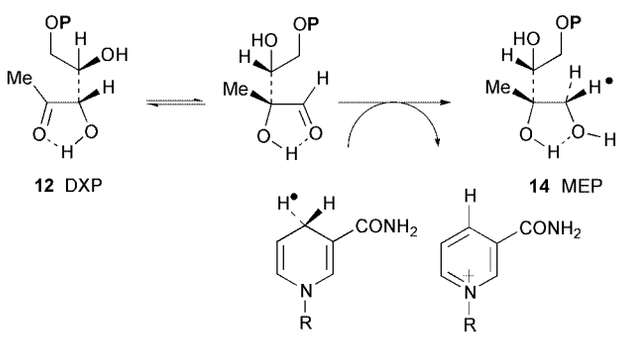


Scheme 9 *Enzyme*: i, ketol acid reductoisomerase.

contrast to the significant increase in DMAPP levels observed when *E. coli* cells overexpressed the DXP synthase gene *dxs*, the overexpression of *dxr* led to no change in DMAPP levels. Overexpression of both genes did not give additional

DMAPP synthesis over that with *dxs*, so it was concluded that DXP synthase but not DXP reductoisomerase catalyses a rate-limiting step in terpenoid biosynthesis.

Identification of some of the catalytic amino acid residues in DXP reductoisomerase from *E. coli* has been achieved *via* overexpression of the *dxr* gene as a histidine-tagged protein.[43] DNA sequencing of *dxr* genes from *dxr*-deficient mutants revealed important base substitution mutations. To characterize the defects, mutant enzymes G14D, E231K, H153Q, H209Q, and H257Q were constructed by site-directed mutagenesis and overexpressed. The results indicated that Glu-231 of the *E. coli* reductoisomerase plays an important role in catalysis, and that His-153, His-209, and His-257, in part, are involved with DXP binding. The stereochemistry of the reduction step has been investigated using a recombinant enzyme from *Synechocystis* sp. PCC6803, which was shown to have 42% identity to the *E. coli* protein.[44] Using [3-^{2}H]DXP as substrate, label was found at the C-1 *pro-S* position of MEP (Scheme 10). The *pro-R*



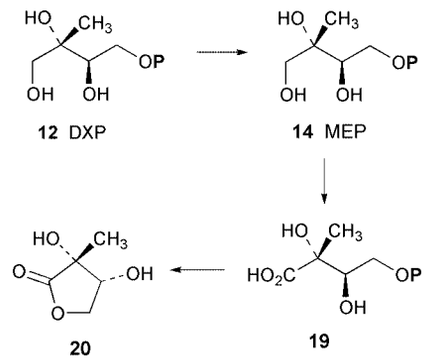
Scheme 10 *Enzyme*: DXP reductoisomerase.

hydrogen at C-1 of MEP therefore derives from NADPH, and hydride delivery is to the *re* face of the proposed aldehyde intermediate. The reductoisomerase has also been shown to be a class B dehydrogenase. Using (4*R*)- and (4*S*)-[4-^{2}H$_1$]NADPH, it was established that only the (4*S*)-labelled coenzyme afforded labelled MEP. The same stereochemical behaviour was found with the *E. coli* reductoisomerase in independent studies.[45]

The phosphonic acid derivative fosmidomycin **18**, an antibiotic isolated in 1980 from *Streptomyces lavendulae*, has proved to be a specific inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase.[46] This compound was chosen for study *via* a database search for antibiotics which were active towards *E. coli* and *Bacillus subtilis*, both of which synthesize terpenoids using the deoxyxylulose phosphate pathway, but was inactive towards *Staphylococcus aureus* which possesses the mevalonate pathway. Fosmidomycin was found to strongly inhibit purified recombinant *E. coli* reductoisomerase through a mixed competitive and noncompetitive mechanism, and its inhibitory effect on *E. coli* could be reversed by the addition of 2-*C*-methylerythritol. Fosmidomycin has close structural similarity to the hypothetical enzyme-bound intermediate 2-*C*-methylerythrose 4-phosphate **13**, which presumably accounts for its inhibitory properties. In independent studies,[47] fosmidomycin was also identified as an inhibitor of the DXP pathway *via* its known herbicidal as well as antibacterial activity. It was demonstrated to inhibit cell multiplication and pigment accumulation in the red alga *Cyanidium caldarium*, formation of carotenoids in green ripening tomato fruits, formation of carotenoids and chlorophylls in etiolated barley leaves, accumulation of prenyl pigments in duckweed (*Lemna gibba*), and emission of isoprene from leaves of *Populus nigra*, *Platanus* X *acerifolia*, and *Chelidonium majus*. Since the antibiotic had no inhibitory activity on recombinant *E. coli* DXP synthase, the reductoisomerase was proposed to be its target.

The biosynthesis of 2-*C*-methylerythritol in leaves of *Liriodendron tulipifera* has been investigated using a variety of labelled 1-deoxy-D-xylulose substrates.[48] In particular, the transformation of [2,3,4,5-^{13}C$_4$]deoxyxylulose into [1,2,3,4-^{13}C$_4$]-2-*C*-methylerythritol proved the occurrence of an intramolecular rearrangement exactly as seen in the deoxyxylulose pathway. Additionally, 1-deoxy-D-xylulose proved to be a precursor of β-carotene in *Liriodendron*. It is proposed that deoxyxylulose is converted into the 5-phosphate by a plant kinase, and then into 2-*C*-methylerythritol 4-phosphate by the deoxyxylulose phosphate pathway, leading to terpenoids. In certain plants, such as *Liriodendron*, dephosphorylation results in formation of 2-*C*-methylerythritol. When [3-^{2}H]deoxyxylulose was used as substrate, the 2-*C*-methylerythritol which was formed was labelled specifically in the H*Si* position of C-1.[49] This is entirely consistent with the stereochemistry of reduction seen above with the *E. coli* reductoisomerase, and also compares with the mechanistically related steps which occur during the biosynthesis of valine and isoleucine.

Most feeding experiments reported have employed deoxyxylulose or methylerythritol as substrates, since these are more readily available than the phosphate derivatives regarded as intermediates in the DXP pathway. However, the phosphates have also been demonstrated as precursors of terpenoids in feeding experiments in higher plant cells.[50] Labelled DXP was produced *via* the cloned synthase enzyme, whilst labelled MEP was obtained synthetically. Both substrates were efficiently converted into β-carotene and geranylgeraniol in chromoplasts of *Capsicum annuum*, though this was absolutely dependent on the presence of ATP. Similar results were obtained with chromoplasts of *Narcissus pseudonarcissus*, and chloroplasts from several other plant species. In the *Capsicum* chromoplast system, fosmidomycin completely inhibited the incorporation of DXP into β-carotene, though incorporations of MEP and IPP were not affected. The feeding of labelled deoxyxylulose to *Ipomoea purpurea* plants leads to formation of a metabolite, identified as 2-*C*-methyl-D-erythrono-1,4-lactone **20**.[51] Similar transformation occurs in a variety of plant systems. This compound had previously been isolated as a stress metabolite in certain plants. It is postulated that DXP is first converted into MEP, which is then oxidized to a carboxylic acid **19** (Scheme 11). Dephos-



Scheme 11

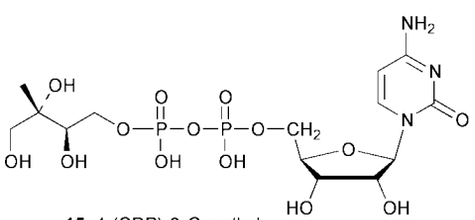
phorylation then yields the free acid which can spontaneously form the lactone **20**.

After 2-*C*-methylerythritol 4-phosphate, the deoxyxylulose phosphate pathway proceeds *via* 4-diphosphocytidyl-2-*C*-methyl-D-erythritol (CDP-ME) **15** (Scheme 8). This compound was identified by preparing mutants of *E. coli* blocked between MEP and IPP.[52] However, since such mutations would be lethal, an *E. coli* transformant possessing mevalonate pathway enzymes beyond mevalonate was constructed as the parent strain. Mutants able to grow only upon addition of mevalonate were identified. These mutants facilitated cloning of several genes that complemented the defects, and led to preparation of one gene product that converted 2-*C*-methylerythritol 4-phosphate into CDP-ME in the presence of CTP. A second gene product converted CDP-ME into the next intermediate in the pathway, CDP-ME2P **16** (see below). In independent

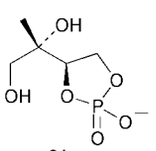
studies, a gene from *Arabidopsis thaliana* bearing similarity to the *ipsD* gene of *E. coli* was cloned, and a fragment encoding the protein minus a potential plastid-targeting sequence was expressed in a recombinant *E. coli* strain.[53] This protein was shown to catalyse formation of CDP-ME from MEP. From deduced amino acid sequences, the catalytic domain of the plant enzyme is about 30% identical with the *E. coli* enzyme.

Phosphorylation of the 2-hydroxy group of CDP-ME gives 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate (CDP-ME2P) **16** (Scheme 8). This was obtained by incubation of the gene product encoded by *ychB* from *E. coli* with CDP-ME in the presence of ATP.[54] Independently, it was surmised from a study of the distribution in various organisms of the *dxs*, *dxr*, and *ygbP* genes that the *ychB* gene in the *E. coli* genome may also encode an enzyme involved in the DXP pathway.[55] The recombinant protein obtained by expression of this gene was purified and shown to phosphorylate CDP-ME in an ATP-dependent manner. Further, a ^{14}C-labelled sample of the product was converted efficiently into carotenoids by isolated chromoplasts of *Capsicum annuum*. The putative catalytic domain of a predicted tomato protein with similarity to this *E. coli* kinase has been expressed in *E. coli*.[56] The purified protein similarly catalysed phosphorylation of CDP-ME to the 2-phosphate. Divalent metal ions, preferably Mg^{2+}, were required for activity. Neither the tomato enzyme nor the *E. coli* orthologue catalysed the phosphorylation of isopentenyl monophosphate. This contrasts with the reported activities of a partially purified *ychB* gene product and an orthologue from *Mentha* X *piperita*, both of which phosphorylated isopentenyl monophosphate to IPP, and which had thus been concluded to be the final step in IPP biosynthesis.[57] By comparison with the phosphorylation of CDP-ME, this phosphorylation of isopentenyl monophosphate to IPP was extremely slow, and regarded as metabolically irrelevant.[56]

The last currently delineated step in the DXP pathway is the transformation of 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate **16** into 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate **17** (Scheme 8). This is catalysed by the enzyme encoded by the *ygbB* gene, found to be closely linked to *ygbP* which encodes CDP-ME synthase.[58] The *E. coli* gene was expressed in a recombinant *E. coli* strain to give a soluble enzyme which converted **16** into the cyclodiphosphate **17** and CMP. The enzyme required Mn^{2+} or Mg^{2+} but no other cofactors. ^{14}C-Labelled cyclodiphosphate **17** was efficiently incorporated into carotenoids by isolated chromoplasts from *Capsicum annuum*. The enzyme was also found to form a cyclophosphate **21** from CDP-ME (Scheme 12), but the product was not incorporated



15 4-(CDP)-2-*C*-methyl-D-erythritol

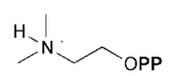


21

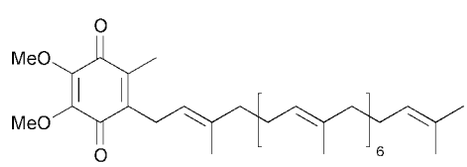
Scheme 12

into carotenoids, and is assumed not to be a metabolic intermediate. The cyclic diphosphate **17** had been isolated earlier from cultures of bacteria exposed to oxidative stress, but had been interpreted as a dead-end product derived from the DXP pathway. Its formation from **16** is formulated as nucleophilic attack of the 2-phosphate to form the phosphoanhydride, displacing CMP as leaving group (Scheme 8).

The remaining steps in the pathway to IPP have yet to be confirmed, but are suggested to be an intramolecular elimination followed by reductions and dehydrations (Scheme 8).[59] In the mevalonate pathway, IPP is converted into DMAPP by the action of IPP isomerase, but in the deoxyxylulose phosphate pathway there is growing evidence that this isomerism may not occur. Thus, secretory cells specialized for monoterpene biosynthesis from peppermint (*Mentha* X *piperita*) incorporated labelled pyruvic acid into geranyl diphosphate. However, in the presence of 2-(dimethylamino)ethyl diphosphate **22**, a transition state analogue inhibitor of IPP isomerase, there was a decrease in GPP production and an accumulation of IPP, with no detectable DMAPP.[60] This was indicative of IPP, not DMAPP, being the terminal product from the pathway. Feeding experiments in *E. coli* using deoxyxylulose as a precursor have shown that label from [3-^{2}H]deoxyxylulose is found in ubiquinone **23** in the (*E*)-methyl group in the terminal unit, and in all other positions derived from the terminal methylene group of IPP.[61] However, label from [4-^{2}H]deoxyxylulose is retained exclusively in the double bond corresponding to the DMAPP starter unit, but not in any of the remaining double bonds generated from IPP in the elongation process. This is in striking contrast to the situation observed for the mevalonate pathway in eukaryotic organisms where the same H-atom of IPP (H*Re*) is lost both in the isomerization to DMAPP and in the elimination step of the elongation process. An IPP isomerase has been demonstrated in *E. coli*, and it shares the same stereochemical features with eukaryotic enzymes (see above).[24] Thus, one concludes that either the stereochemistry of elongation is different from that of the isomerization, or, alternatively, IPP and DMAPP may be formed independently from a common intermediate.



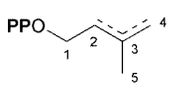
22 2-(dimethylamino)ethyl diphosphate (DMAEPP)



23 ubiquinone-8

Genetic evidence for the branching of pathways to IPP and DMAPP in *E. coli* has been presented.[62] This required creation of an *E. coli* strain impaired in the synthesis of MEP *via* disruption of the *dxr* gene and therefore unable to synthesize IPP and DMAPP. However, in addition it had the engineered ability to synthesize IPP from exogenously supplied mevalonate *via* insertion of genes encoding for mevalonate pathway enzymes converting mevalonate into IPP. Finally, a derivative strain harbouring a disruption to *idi*, the gene encoding IPP isomerase, was constructed. The absence of IPP isomerase was known to have no apparent effects on growth and survival, confirming *idi* was a non-essential gene. Since the engineered *E. coli* strain with the double disruption at *dxr* and *idi* genes grew in the presence of methylerythritol, it was concluded that, if there is only one route for the biosynthesis of either IPP or DMAPP in *E. coli*, isomerization is not accomplished by IPP isomerase encoded by *idi*. The presence of a second IPP isomerase gene was excluded, and the results are best explained in terms of a branching point after MEP resulting in independent synthesis

of IPP and DMAPP. Further evidence is provided by labelling studies in an *E. coli* strain defective in DXP synthase and which synthesizes its isoprenoids exclusively from exogenous methylerythritol.[63] Hydrogen atoms from [1,1-$^2H_2$]methylerythritol were incorporated without loss into the prenyl chain of ubiquinone-8 **23** on carbons derived from C-4 of IPP and on the (*E*)-methyl group of DMAPP. Label from C-5 of [3,5,5,5-$^2H_4$]methylerythritol was incorporated into ubiquinone-8 and menaquinone-8 **24**, residing on the methyl groups derived from the C-5 methyl of IPP and the (*Z*)-methyl group of DMAPP (see **25**). Thus, there was no change in oxidation state of these carbon atoms during the reaction sequence between MEP and IPP. Further, no deuterium scrambling was observed between C-4 and C-5 of IPP/DMAPP, suggesting complete stereoselectivity of IPP isomerase, if involved. However, the C-3 deuterium atom from this substrate was preserved only in the DMAPP starter unit, and was completely missing from the IPP-derived extender units. Since the IPP isomerase gene in *E. coli* is non-essential,[64] it was concluded that different routes to IPP and DMAPP are operative. A study of lutein and phytol (from chlorophylls) biosynthesis in cell cultures of *Catharanthus roseus* has also demonstrated inconsistencies.[65] Deuterium label from position 3 of deoxyxylulose was incorporated into both IPP- and DMAPP-derived isoprene units, retaining about 75% of the label compared to an internal standard ^{13}C label, and this is located preferentially in the (*E*)-hydrogen atom of IPP. This preferential labelling rules out DMAPP as the compulsory precursor of IPP. The 4-^2H label from deoxyxylulose was completely washed out, most probably as a consequence of the isomerization and elongation process. This is in marked contrast to the situation in *E. coli*, where this label is retained in the DMAPP starter unit. This could reflect differences in stereospecificity of IPP isomerase in the two systems, but could also indicate independent formation of IPP and DMAPP.



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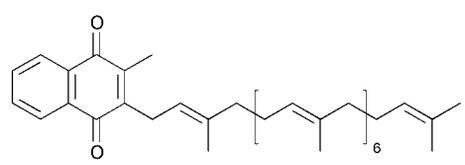
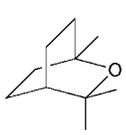


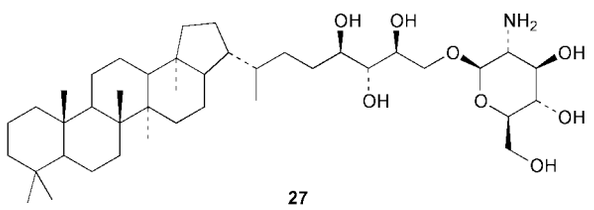
24 menaquinone-8

When [2-^{13}C,4-$^2H_1$]deoxyxylulose was fed to twiglets of *Eucalyptus globulus*, the monoterpene cineole **26** isolated gave further data that IPP and DMAPP are formed in independent rather than sequential steps.[66] Thus the formation of five isotopomers could be detected, and their structures and relative abundance showed that ^{13}C label was incorporated to the same extent into both C_5 units. However, whilst the ^2H label was retained to an extent of 57% in the starter DMAPP unit, it was completely or almost completely lost in the unit derived from IPP. Since it was already known that the corresponding hydrogen in GPP is fully retained during cineole biosynthesis, it follows that the two olefinic hydrogens of GPP must have different metabolic origins.



26 cineole

Ubiquinone-8 **23** biosynthesis in *E. coli* from [1,1,1,$^2H_3$]-deoxyxylulose shows intact incorporation of the methyl group of deoxyxylulose, labelling methyl groups originating from the methyl group of IPP and the (*Z*)-methyl of DMAPP.[67] Triterpenoids **27** and **28** of the hopane series in the bacterium *Zymomonas mobilis* are biosynthesized *via* the DXP pathway, and, after feeding [1-^2H]glucose, deuterium labelling was found on all carbons derived from C-2 and C-4 of IPP.[68] Due to peculiar metabolic pathways in this bacterium, [1-^2H]glucose is converted into NADP^2H, so these incorporations represent the result of reductive steps during the biosynthesis. Deuterium at C-4 of the IPP units arises because of the NADPH-dependent reduction in the DXP to MEP conversion catalysed by the reductoisomerase. However, the presence of deuterium at C-2 indicates an additional reduction step, consistent with the proposed sequence in Scheme 8. Phytol **29** biosynthesis in the cyanobacterium *Synechocystis* sp. UTEX 2470 from a number of deuterium-labelled glucose substrates has been investigated.[69] Similarly, labelling at C-4 in IPP units from [6,6-$^2H_2$]glucose as precursor was explained by the participation of labelled NADPH. In addition, since in no experiment was deuterium retained at C-2 in IPP units, the involvement of an intermediate such as the 2-ketone (Scheme 8) was postulated. During the biosynthesis of the monoterpene linalyl acetate **30** in *Mentha citrata*, labelling studies using [3-$^2H_3$]alanine (as a source of pyruvate) and [6,6-$^2H_2$]glucose have established that the conversion effectively retains five hydrogens of DXP, three from the C-1 methyl group and two from C-5.[70]



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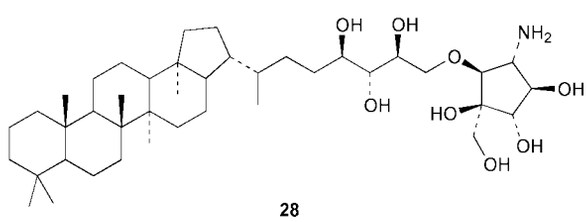

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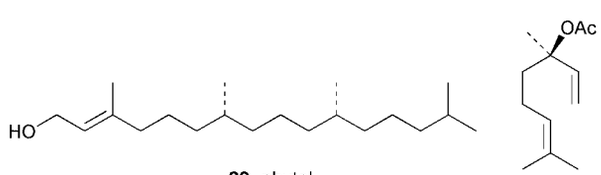


29 phytol

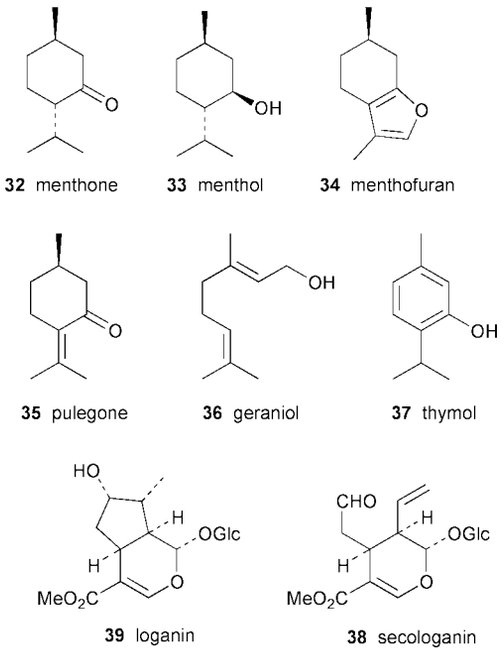
30 linalyl acetate

To identify genes that might encode enzymes that catalyse later steps in the DXP pathway, the occurrence of known DXP pathway genes in bacteria, yeast, and *Arabidopsis* was ascertained, and genes that exhibited the same pattern of occurrence were identified.[71] In addition, bacterial genomic libraries and plant cDNA libraries were screened for genes that increased the accumulation of lycopene in *E. coli* engineered to produce this compound. Common to both approaches was the identification of homologues of the *E. coli lytB* gene as prospective DXP pathway genes. It has been proposed that the *lytB* gene encodes an enzyme for a step at or subsequent to the point at which the pathway branches to form IPP or DMAPP. A mutant of the cyanobacterium *Synechocystis* strain PCC 6803 with an insertion in the promoter region of *lytB* grew slowly and produced easily bleached colonies, whilst insertions in the coding region were lethal. Supplementation with the alcohol analogues of IPP and DMAPP alleviated the growth impairment. The *Synechocystis lytB* gene and a *lytB* cDNA from the plant *Adonis aestivalis* were each found to enhance accumulation of

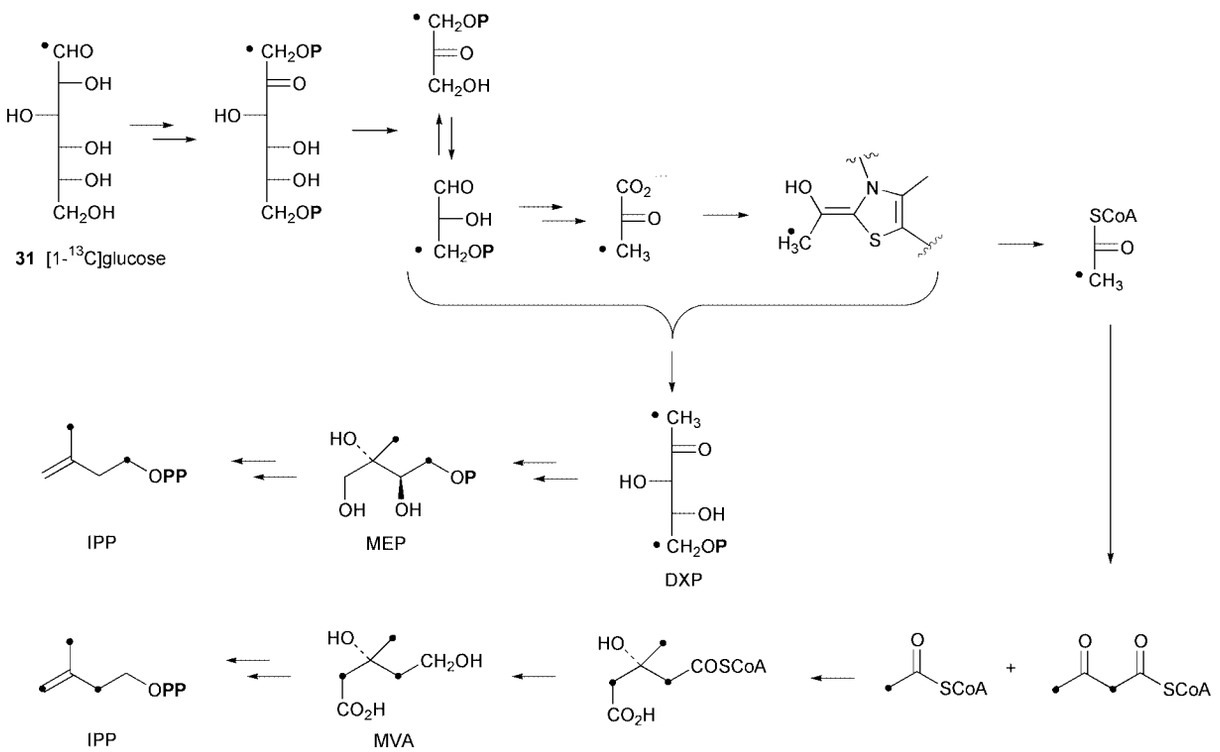
carotenoids in engineered *E. coli*. Combined with a *dxs* cDNA, the individual effects of *dxs* and *lytB* were multiplied. In contrast, a combination of lytB and *ipi*, a cDNA encoding IPP isomerase, was no more effective in enhancing carotenoid production than *ipi* alone. Thus, the ratio of IPP and DMAPP produced *via* the DXP pathway is influenced by the enzyme LytB, though its nature has yet to be established.

Whether a terpenoid is synthesized in nature *via* the deoxyxylulose phosphate pathway, or *via* the alternative mevalonate pathway, is readily established by the labelling patterns found in feeding experiments using glucose precursors, *e.g.* [1-^{13}C]-glucose (**31**, Scheme 13). Catabolism of glucose through glycolysis and subsequent incorporation of the resulting metabolites gives characteristic labelling patterns according to the pathway followed. Through this, or related methodologies, the widespread utilization of the deoxyxylulose phosphate pathway has been established. The hemiterpene isoprene **9** is formed in the bacterium *Bacillus subtilis* by the DXP pathway, as it is in plants.[72] In the monoterpene field, labelled deoxyxylulose is incorporated into menthone **32**, menthol **33**, menthofuran **34**, and eucalyptol (cineole) **26** in *Mentha* X *piperita*, into pulegone **35** in *Mentha pulegium*, into geraniol **36** in *Pelargonium graveolens*, and into thymol **37** in *Thymus vulgaris*.[73] However, incorporations were rather lower than the incorporations into chlorophylls and carotenoids. The iridoid secologanin **38** in *Catharanthus roseus* is DXP-derived,[74] as is loganin **39** in *Rauwolfia serpentina*.[75] In the latter studies, a retrobiosynthetic approach was also employed, in which the labelling patterns observed in protein-derived amino acids were used to reconstruct the labelling patterns of phosphoenolpyruvate, pyruvate and acetyl-CoA, and these patterns were subsequently used to predict labelling in DMAPP and IPP derived *via* the alternative pathways. The observed labelling patterns in loganin were in excellent agreement with the DXP pathway prediction. The previously reported low incorporations of mevalonate into loganin can be attributed to metabolite exchange (crosstalk) between the two terpenoid pathways, the extent of which can be established from a quantitative analysis of general carbon metabolism.

A number of sesquiterpenoids have been shown to be of mevalonate rather than DXP origin, which has been thought

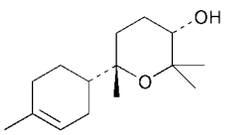


to relate to biosynthetic location in the cytoplasm. However, recent studies on the biosynthesis of sesquiterpenes bisabolol oxide A **40** and chamazulene **42** in flowers of chamomile (*Matricaria recutita*) has established they have mixed origins.[76] Two of the isoprene units were predominantly formed from the DXP pathway, but the third, comprising the last extender unit, was of mixed origin, and mevalonate also contributed to its formation. It was hypothesized that a GPP C_{10} unit was created in the plastid from DXP, and was then transferred to another subcellular compartment that has access to IPP formed *via* both pathways. In further studies,[77] these findings were reinforced by feedings of labelled deoxyxylulose. This precursor was efficiently incorporated into all three isoprene units of bisabolol oxide A, chamazulene, and also bisabolol oxide B **41**, but there was a significantly lower incorporation into the biogenetically terminal unit. It was estimated that the DXP pathway contributed approximately 90% of label to the first two

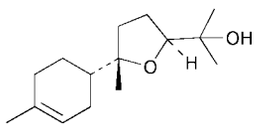


Scheme 13

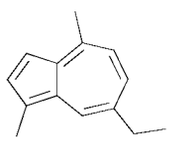
units, but only 50% to the third. Abscisic acid **43** is formed by cyclization of FPP in fungi, but by degradation of a C_{40} carotenoid in plants (see Section 7). Feeding experiments with [1-^{13}C]-glucose have shown that in the tulip tree (*Liriodendron tulipifera*) abscisic acid is derived from DXP, whilst in the fungi *Botrytis cinerea* and *Cercospora pini-densiflorae* it has its origins in mevalonate.[78] Furthermore, abscisic acid from *L. tulipifera* had labelling analogous to that in β-carotene **44** from the same feeding, consistent with a carotenoid origin. Lipiferolide **45** from *L. tulipifera* was also DXP-derived.



40 bisabolol oxide A



41 bisabolol oxide B

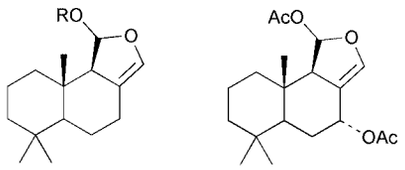


42 chamazulene

In marked contrast, the drimane esters **46**–**48** found in the marine molluscs *Dendrodoris limbata* and *D. grandiflora* have been shown to be derived from glucose precursors *via* the mevalonate pathway.[79] Similarly, the gymnomitranes β-barbatene **49**, gymnomitr-3(15)-en-4α-ol **50**, and gymnomitran-4-one **51** were all products of the mevalonate pathway in the liverworts *Reboulia hemispaerica* and *Bazzania trilobata*.[80]

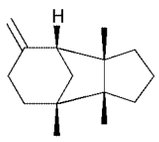
In fungus-infected barley (*Hordeum vulgare*) plants the cyclohexenone derivative blumenin **52** has been shown to originate from DXP, and is probably derived from a sesquiterpenoid precursor.[81] A group of so-called homoterpenes (C_{11}) formed in certain plants as a response to insect damage are known to be degraded sesquiterpenes (see Section 7). In a study of the relative roles of [$^2H_2$]deoxyxylulose and [$^2H_5$]mevalonate in the formation of volatile terpenoids including homoterpenes, the DXP pathway was found to be the major pathway.[82] Thus, in suitably treated lima beans (*Phaseolus lunatus*), deoxyxylulose labelled the monoterpenes ocimene **53** and linalool **54** to a particularly high extent, whereas both deoxyxylulose and mevalonate could serve as precursors of the homoterpene 4,8-dimethylnona-1,3,7-triene **55**. A higher degree of labelling was achieved in **55** by using a statin inhibitor of HMGR in the mevalonate pathway, thus indicating that biosynthesis could follow both routes. The diterpene-derived analogue of **55**, 4,8,12-trimethyltrideca-1,3,7,11-tetraene **56**, was also labelled by deoxyxylulose. Similar data were obtained with other metabolites in some other plant species, and it was apparent that the DXP pathway is a major pathway to volatile plant terpenoids. Although deoxyxylulose is preferentially channeled into monoterpene and diterpene biosynthesis in the plastids, there is also a large contribution to sesquiterpene biosynthesis in the cytosol, due to the plant's ability to manipulate resources between cytosol and chloroplast.

In the diterpenoid field, steviol **57** is synthesized in *Stevia rebaudiana via* the DXP pathway,[83] as are bicyclic and tetracyclic diterpenes such as scoparic acid **58** and scopadulcic acid **59** in leaves of *Scoparia dulcis*.[84] In the latter studies, labelling

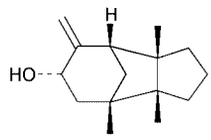


46 R = fatty acid
47 R = Ac

48



49 β-barbatene



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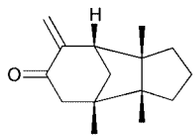

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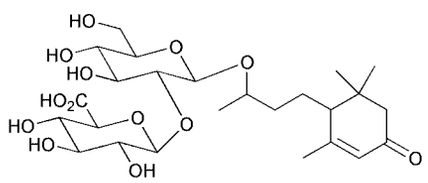
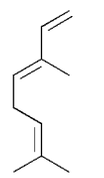
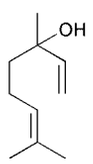


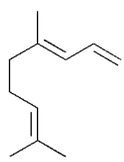
52 blumenin



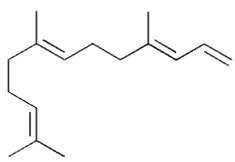
53 ocimene



54 linalool

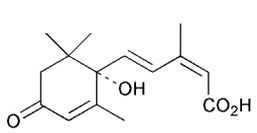


55

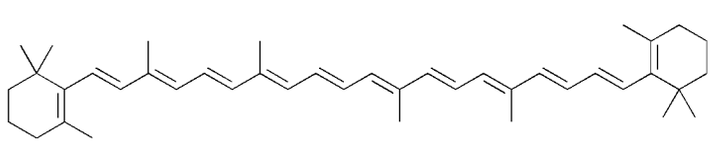


56

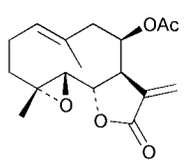
patterns showed phytol **29** was also produced *via* the DXP pathway, whilst the sterol β-sitosterol **60** originated from the mevalonate pathway. Phytol biosynthesis has also been studied in the cyanobacterium *Synechocystis* sp. UTEX 2470, where the DXP pathway was also shown to be operative.[85] However, in cultured cells of the liverwort *Heteroscyphus planus*, feeding experiments with [2-^{13}C]glycerol and [6,6-$^2H_2$]glucose along with earlier results demonstrated simultaneous operation of both mevalonate and DXP pathways.[86] There was also equal incorporation of either methyl or carboxy carbons of acetate into all carbons of the phytyl side-chain of chlorophyll (*via* double-labelled acetate) with complete loss of methyl hydrogens. This indicated that CO_2 evolved from the carboxy carbon through the tricarboxylic acid cycle may be reutilized *via* the reductive pentose phosphate cycle followed by the glycolytic pathway. Biosynthesis of the neoverrucosane diterpene, 8α-acetoxy-13α-hydroxy-5-oxo-13-*epi*-neoverrucosane **61**, in the liverwort *Fossombronia alaskana* is predominantly (>95%) *via* the DXP pathway.[87] This contrasts markedly with studies in the eubacterium *Chloroflexus aurantiacus* where the structurally related verrucosane verrucosan-2β-ol **62** was shown to be of mevalonate origin.[88] The diterpenoid moiety of brasilicardin A **63** from the pathogenic actinomycete *Nocardia brasiliensis* is also formed *via* the DXP pathway.[89] In the two diatoms *Phaeodactylum tricornutum* and *Nitzschia ovalis*, isoprenoid biosynthesis has been investigated using a variety of precursors.[90] Acetate was the preferred carbon source for cytosolic sterol biosynthesis using the mevalonate pathway, but phytol biosynthesis in the chloroplast involved the DXP pathway and CO_2



43 abscisic acid (ABA)

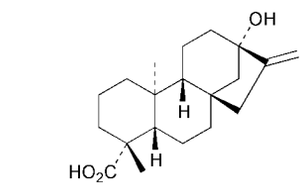


44 β-carotene

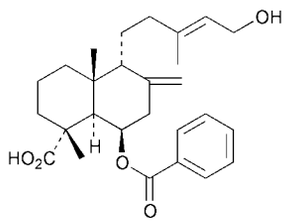


45 lipiferolide

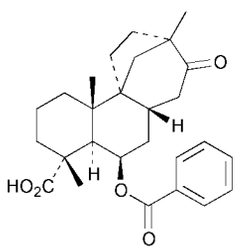
proved the best carbon source. Thus, the diatoms demonstrated the same type of compartmentation for isoprenoid biosynthesis as seen in plants.



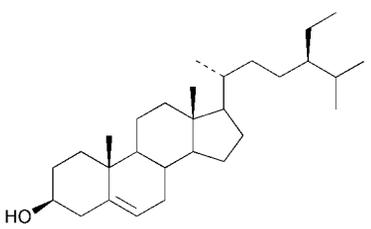
57 steviol



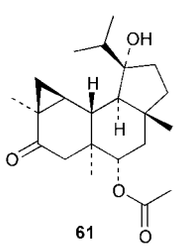
58 scoparic acid A



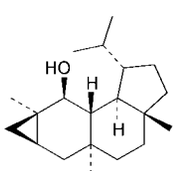
59 scopadulcic acid B



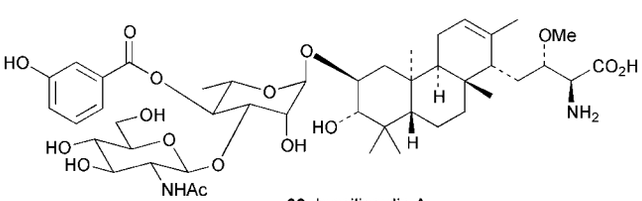
60 β-sitosterol



61



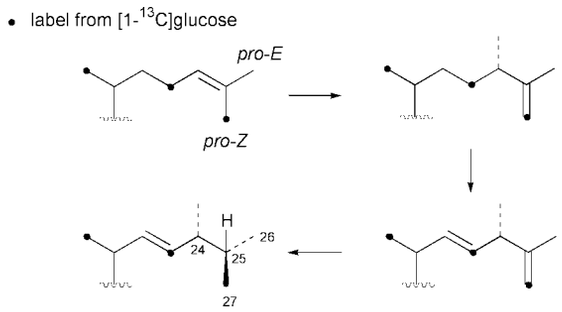
62 verrucosan-2β-ol



63 brasilicardin A

Incorporation of [1-^{13}C]glucose has been used to differentiate between involvement of the DXP and mevalonate pathways in terpenoid biosynthesis in a number of unicellular algae.[91] In the green algae *Chlorella fusca* and *Chlamydomonas reinhardtii*, all isoprenoids examined (the sterols chondrillasterol **64**, 22,23-dihydrochondrillasterol **65**, ergost-7-enol **66**; phytol **29**; carotenoids lutein **67** and β-carotene **44**) were synthesized from DXP, as they were in another previously investigated green alga *Scenedesmus obliquus*. This organism also used DXP to synthesize ubiquinone **23**. In the red alga *Cyanidium caldarium* and in the Chrysophyte *Ochromonas danica* there appeared a clear dichotomy, as seen in the higher plants. Thus, sterols porifasterol **68**, 7-dehydroporifasterol **69** and ergosterol **70**, were formed from mevalonate, but chloroplast isoprenoids (phytol, β-carotene) originated from DXP. The Euglenophyte *Euglena gracilis* synthesized both ergosterol and phytol *via* the mevalonate pathway. Both phytol and β-carotene originated from DXP in the cyanobacterium *Synechocystis* PCC 6714. The sterol ergosterol **70** is also a product of the DXP pathway in the yeast-like alga *Prototheca wickerhamii*.[92] It was also established that the *pro-Z* methyl group of cycloartenol **71** is derived from C-5 of IPP and that protonation at C-25 of the $\Delta^{25(27)}$-sterol intermediate takes place from the *si*-face of C-25 to form the isopropyl *pro-R* methyl group (Scheme 14).

Ubiquinone biosynthesis also varies according to organism. [2,3-$^{13}C_2$]- and [2,4-$^{13}C_2$]-Deoxyxylulose substrates were incorporated into ubiquinone-8 **23** in *E. coli*, giving a labelling pattern consistent with the rearrangement of DXP into MEP.[93] On the other hand, studies in tobacco (*Nicotiana tabacum*) cells[94] have shown that sterols (campesterol **72**, 22,23-dihydrobrassicasterol **73**, β-sitosterol **60** and stigmasterol **74**) which are synthesized in the cytoplasm, and the prenyl chain of ubiquinone-10 **75** (located in the mitochondria) were derived from the same mevalonate-derived pool of IPP. However, the prenyl chain of plastoquinone-9 **76** was DXP-derived, in keeping with its chloroplastic location. The results fit the known compartmentation of these long chain polyprenols, and indicate the complete enzymic independence of the pathways. Menaquinone-9(H_4) **77** is produced by the DXP pathway in *Actinoplanes* sp. A40644, though a terpenoid derivative BE-40644 **78** was shown to be derived mainly from mevalonate.[95] It was also deduced that the DXP pathway operates at the early stages of fermentation, and that its contribution is replaced by the mevalonate pathway when secondary metabolite production begins.



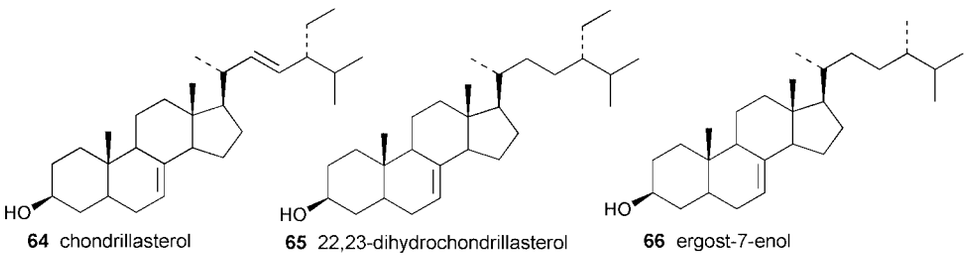
Scheme 14

Whilst detailed information about meroterpenoid biosynthesis is deliberately omitted from this report, the relative importance of the DXP and mevalonate pathways in supplying the terpenoid portion of meroterpenoids is of interest. The DXP pathway has been found to be operative in the biosynthesis of the flavonoid glabrol **79** in *Glycycrrhiza glabra*,[96] the furocoumarins psoralen **80**, xanthotoxin **81**, bergapten **82** and isopimpinellin **83** in *Apium graveolens*,[97] the coumarin antibiotic novobiocin **84** in *Streptomyces niveus*[98] and *Streptomyces spheroides*,[99] and the bitter acid humulone **85** in *Humulus lupeus*.[100] Lucidin 3-primveroside **86**, an anthraquinone formed by combination of an isoprene unit with *o*-succinylbenzoate, derives its isoprene unit from DXP in *Rubia tinctorum*.[101] The monoterpenoid moiety of the indole alkaloid teleocidin B-4 **87** from *Streptomyces blastmyceticum* is also DXP-derived.[102] Isoprenoid units of shikonin **88** in *Lithospermum erythrorhizon*,[103] boviquinone-4 **89** in *Suillus bovinus*,[104] and paraherquamide A **90** in *Penicillium fellutanum*[105] have been shown to originate from mevalonate.

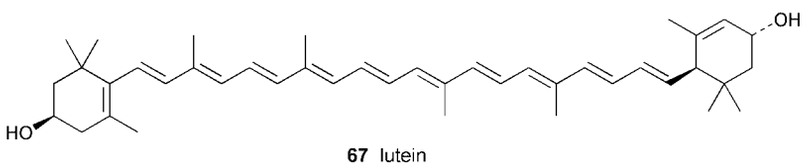
A phylogenetic study of the distribution of genes of the two pathways suggests the mevalonate pathway is characteristic of archaebacteria, that the DXP pathway is characteristic of eubacteria, and that eukaryotes have inherited their genes for IPP biosynthesis from prokaryotes.[106] The occurrence of genes specific to the DXP pathway is restricted to plastid-bearing eukaryotes, indicating that these genes were acquired from the cyanobacterial ancestor of plastids. Lateral gene transfer between eubacteria subsequent to the origin of plastids appears to have played a major role in the evolution of this pathway. Similar conclusions were obtained from an independent survey, suggesting lateral gene transfer explains many features of IPP biosynthesis in bacteria, archaea and eukaryotes.[107] Several reviews covering the deoxyxylulose phosphate pathway are available,[108, 109] including a recent Report.[110]

5 Polyprenyl diphosphate synthases

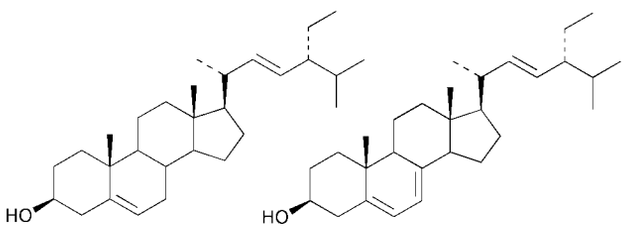
Polyprenyl diphosphate synthases (prenyltransferases) are responsible for the alkylation steps involving DMAPP and one



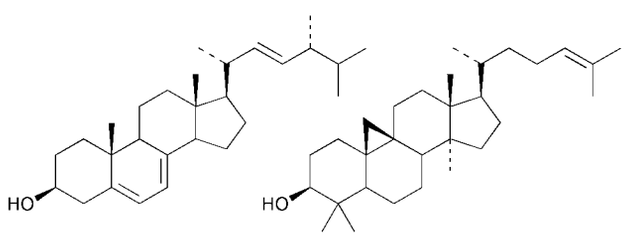
64 chondrillasterol **65** 22,23-dihydrochondrillasterol **66** ergost-7-enol



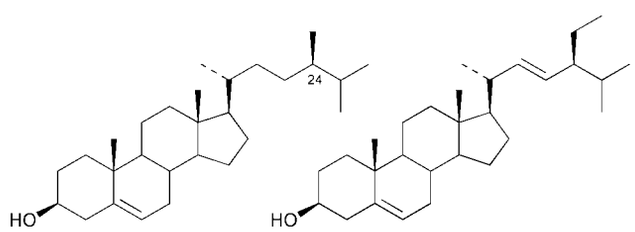
67 lutein



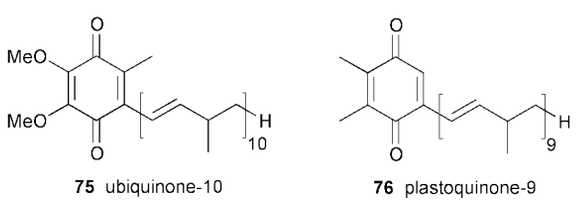
68 porifasterol **69** 7-dehydroporifasterol



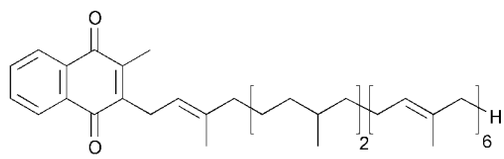
70 ergosterol **71** cycloartenol



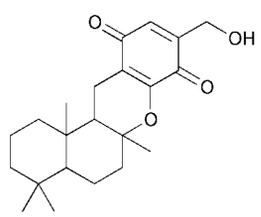
72 campesterol (24*R*)
73 22,23-dihydrobrassicasterol (24*S*)I **74** stigmasterol



75 ubiquinone-10 **76** plastoquinone-9



77 menaquinone-9(H_4)

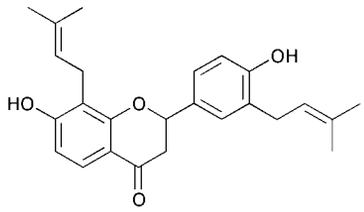


78 BE-40644

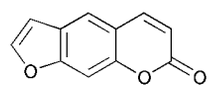
or more IPP residues, reactions which provide the polyprenyl diphosphate precursors for the various terpenoid families. Available evidence points to the initial ionization of the chain starter unit to provide an allylic cation prior to addition of IPP extender units. Many polyprenyl diphosphate synthase enzymes are relatively non-specific and catalyse the condensation of DMAPP with IPP units, building up polyprenyl diphosphate chains of different lengths. Farnesyl diphosphate synthase catalyses the successive condensations of IPP with both DMAPP and geranyl diphosphate (GPP) **91** to give farnesyl diphosphate (FPP) **92**. Geranylgeranyl diphosphate (GGPP) synthase catalyses the further condensation step between FPP and IPP giving GGPP **93** (Scheme 15); in fungi and animals this enzyme appears specific to FPP, whereas in plants and bacteria DMAPP, GPP and FPP are all accepted.

Geranyl diphosphate synthase from oil glands of spearmint (*Mentha spicata*) has been purified to yield two proteins, which contained amino acid sequences that matched cDNA clones from screening of a peppermint (*Mentha* X *piperita*) cDNA library.[111] Expression of each cDNA individually yielded no detectable prenyltransferase activity, but co-expression produced functional GPP synthase. This enzyme is a heterodimer, with both subunits required to produce catalytically active native and recombinant enzyme.

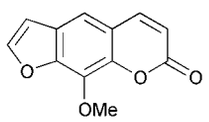
A cDNA encoding for farnesyl diphosphate synthase has been characterized in tomato (*Lycopersicon esculentum*) fruit.[112] Tomato FPP synthase genes appear to be encoded by a small multigenic family. Characterization of FPP synthase from an insect has been reported.[113] The cDNA isolated from the moth *Agrotis ipsilon* encodes a polypeptide which shares regions of homology with FPP synthase from other organisms, but has special features, such as an extra *N*-terminal extension of about 70 amino acids. A full-length cDNA from the plant *Gossypium arboreum* encoding FPP synthase has been transferred into *Artemisia annua* using an *Agrobacterium tumefaciens* transformation system.[114] The foreign gene was shown to be expressed in certain lines, and a consequence of this was a 2–3 times improvement in the synthesis of the sesquiterpene artemisinin (see Section 7). FPP synthase catalyses chain elongation of DMAPP *via* GPP through two addition steps, but product selectivity of avian enzyme has been altered to favour synthesis of GPP.[115] Site-directed mutagenesis has been used to modify residues that form the binding pocket for the hydrocarbon residue of the allylic substrate. From a number of reasonable changes tried, two substitutions, namely A116W and N144′W,



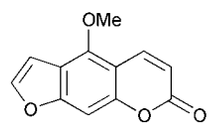
79 glabrol



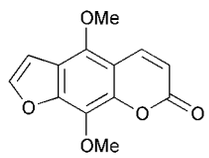
80 psoralen



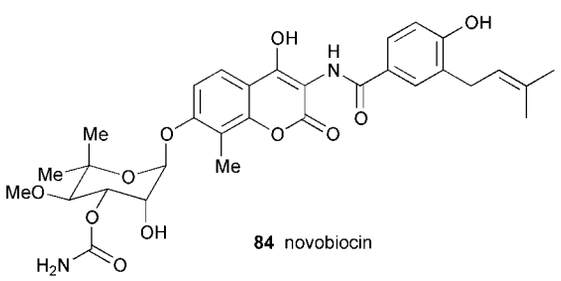
81 xanthotoxin



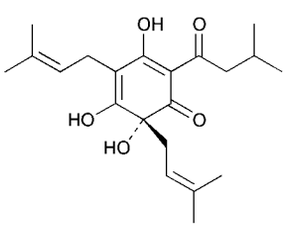
82 bergapten



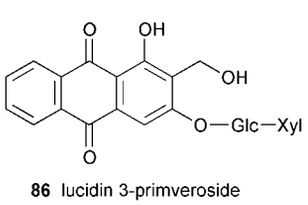
83 isopimpinellin



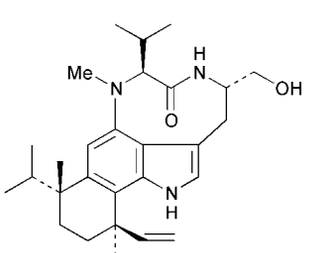
84 novobiocin



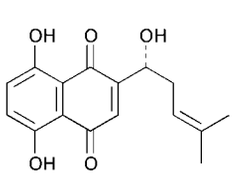
85 humulone



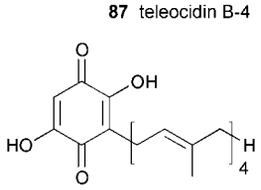
86 lucidin 3-primveroside



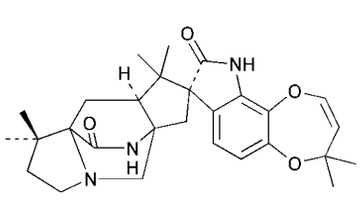
87 teleocidin B-4



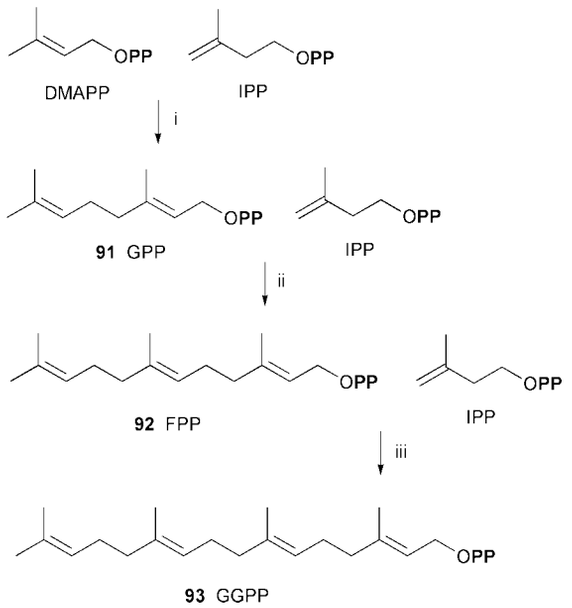
88 shikonin



89 boviquinone-4



90 paraherquamide A



93 GGPP

Scheme 15 *Enzymes*: i, geranyl diphosphate synthase; ii, farnesyl diphosphate synthase; iii, geranylgeranyl diphosphate synthase.

strongly discriminated against binding of GPP when compared with the wild-type enzyme. Although all three enzymes converted GPP into FPP, when incubated with DMAPP, the two mutant enzymes produced substantially lower levels of FPP. Similarly, mutant FPP synthase proteins of *Bacillus stearophilus* have been constructed with single amino acid substitutions before the first aspartate-rich motif.[116] The mutated enzyme in which serine at the fourth position before the motif was replaced by phenylalanine exclusively produced GPP from DMAPP, and had very little affinity towards GPP. There was less selectivity with the corresponding tyrosine mutant, whilst the tryptophan mutant proved inactive.

Geranylgeranyl diphosphate synthase has been isolated from cell cultures of *Taxus baccata* and purifed.[117] This enzyme was a homodimer. The most effective substrate was FPP, and although DMAPP was accepted, it was a much poorer substrate. GGPP synthase activity is rapidly induced when *T. baccata* cells are treated with methyl jasmonate, leading to a sharp increase in taxane diterpenoid production.[118] A cDNA encoding GGPP synthase has been cloned from a methyl jasmonate-induced *Taxus canadensis* cell library.[119] Recombinant enzyme was expressed in yeast. The deduced amino acid sequence of this gymnosperm enzyme resembled those of GGPP synthases from angiosperms, except for a 90–100 residue sequence at the *N*-terminus, which corresponds to a plastidial transit peptide. The enzyme was most effective with GPP as substrate, but also accepted DMAPP (33% activity) and GPP (66% activity). The full-length preprotein and a truncated version in which the transit peptide had been removed were successfully transformed to complement a GGPP-defective yeast mutant. However, overexpression in wild-type yeast proved toxic, probably due to depletion of endogenous FPP. GGPP synthase genes from sunflower (*Helianthus annuus*)[120] and humans[121] have also been cloned and characterized. Overexpression of an archeal GGPP synthase from *Sulfolobus acidocaldarius* has been achieved by creating fusion proteins that retain thermostability better than the native enzyme, and have higher activity.[122] The fusion proteins existed in dimer, tetramer, octamer or dodecamer forms, and their product specificities varied somewhat according to the oligomerization. Similar techniques were used to obtain GGPP synthase from the extremely thermophilic bacterium *Thermus thermophilus*.[123] A dimeric fusion protein retained thermostability and had higher specific activity than the native enzyme. The deduced amino acid sequence for this enzyme suggested it was

not typical of bacterial GGPP synthases, but had similarities with archaeal and eukaryotic short chain prenyltransferases. In *Arabidopsis thaliana*, there exists a small gene family for GGPP synthases encoding five isozymes and a related protein. Each gene appears to be expressed in different tissues during plant development, GGPP being synthesized by the organelles themselves instead of being transported.[124]

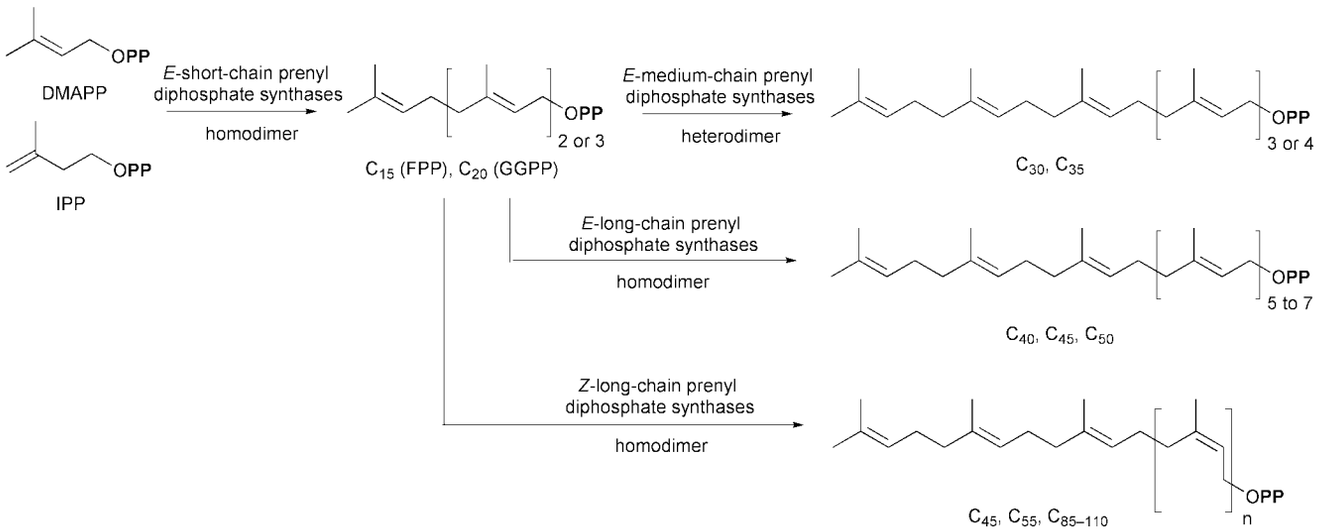
cDNAs encoding GGPP synthase have been isolated from two diterpene producing plants, *Scoparia dulcis* and *Croton sublyratus*.[125] Both cloned genes showed high amino acid sequence homology (60–70%) to other plant enzymes and contained highly conserved aspartate-rich motifs, and were expressed in *E. coli* to yield active enzymes. Two mutant *S. dulcis* enzymes were then constructed in which small amino acids at the fourth and fifth positions before the first aspartate-rich motif were replaced with aromatic amino acids, or in which amino acids from the aspartate-rich motif were deleted. Both mutants behaved as FPP synthase, and almost exclusively produced FPP from DMAPP and IPP, demonstrating that amino acids in the region of the aspartate-rich motif play essential roles in product length determination. Using *Sulfolobus acidocaldarius* GGPPsynthase and *Bacillus stearothermophilus* FPP synthase, mutations at the 5th, 8th, and 11th positions before the first aspartate-rich motif were investigated to establish their effect on chain length.[126] The side-chains of these amino acids are situated on the same side of an α-helix, and it had already been demonstrated that a single mutated GGPP synthase enzyme F77S produced mainly a C_{25} product (see ref. 1). Double mutated GGPP synthase (L74G and F77G) mainly produced a C_{35} product accompanied by significant amounts of C_{30} and C_{40}. A triple mutated enzyme (I71G, L74G, and F77G) produced C_{40} with some C_{35} and C_{45}. Similar effects were observed in the FPP synthase system. It was deduced that there exists a passage in which the growing polyprenyl chain resides as it elongates, and that bulky amino acids in the original passage block further elongation and control chain length.

Hexaprenyl diphosphate synthase catalyses the condensation of FPP with three molecules of IPP to afford hexaprenyl diphosphate, the precursor of the side-chain of menaquinone-6. In *Micrococcus luteus*, this enzyme consist of two dissociable components, designated A and B, with no prenyltransferase activity until they are combined. A similar situation exists in *Bacillus subtilis* for heptaprenyl diphosphate synthase, which provides the precursor of the side-chain of menaquinone-7, and these two-component systems are thus quite distinct from the other homodimeric prenyltransferases catalysing the synthesis of shorter or longer chain prenyl diphosphates. Genes encoding both components A and B of hexaprenyl diphosphate synthase from *Micrococcus luteus* have been cloned and characterized,[127] and also those for components I and II of heptaprenyl diphosphate synthase from *Bacillus subtilis*.[128] For the latter enzyme, the individual components were each overexpressed in *E. coli*, and studies indicated that the two essential subunits in the presence of FPP and Mg^{2+} form a stable ternary complex which seems to represent the catalytically active state of the synthase. No such complex is formed in the presence of IPP and Mg^{2+}. It was also demonstrated that component I possessed specific affinity for FPP, this affinity significantly increasing in the presence of component II. It is believed that component I first binds FPP, then associates with component II in the presence of Mg^{2+} to form the ternary complex which then catalyses the four consecutive chain extensions. Then the complex dissociates with concomitant release of the product. Alignment of amino acid sequences for component I and the corresponding subunits of *Bacillus stearothermophilus* heptaprenyl diphosphate synthase and *Micrococcus luteus* hexaprenyl diphosphate synthase showed three regions of high similarity.[129] Site-directed mutagenesis experiments suggested some of the conserved residues in region B of component I are involved in the binding of allylic substrate as well as determining the chain length of reaction product. In *Bacillus stearothermophilus* heptaprenyl diphosphate synthase, an aspartate-rich motif is conserved in component II, and it appears that amino acids around this region may regulate chain length as found in other systems.[130] To test this theory, a mutated enzyme containing the substitution I76G in component II was shown to catalyse condensations beyond C_{35}, mainly C_{40}. Two mutated enzymes A79Y and S80F, which have a single replacement to the aromatic residue at the fourth or fifth position before the first aspartate-rich motif, mainly yielded a C_{20} product. The results strongly suggest that there is a common mechanism to control product chain length in both short- and medium-chain prenyl diphosphate synthases. In the wild-type heptaprenyl diphosphate synthase the prenyl chain can grow on the surface of the small residues at positions 79 and 80, and elongation is precisely blocked at C_{35} by isoleucine 76. Several amino acid residues in the larger subunits of *Micrococcus luteus* hexaprenyl diphosphate synthase and *Bacillus subtilis* heptaprenyl diphosphate synthase were substituted by site-specific mutagenesis.[131] In both enzymes, replacement of the Ala at the fifth position upstream of the first aspartate-rich motif with bulky amino acids resulted in shortening the chain length of the products. A double combination of mutant subunits of the heptaprenyl diphosphate synthase (I-D97A, II-A79F) yielded exclusively GGPP. Other combinations produced a C_{40} prenyl diphosphate, or chain lengths up to C_{50}. These results suggest that these medium-chain prenyl diphosphate synthases control chain length by a novel mechanism, in which both subunits cooperatively participate (Scheme 16).

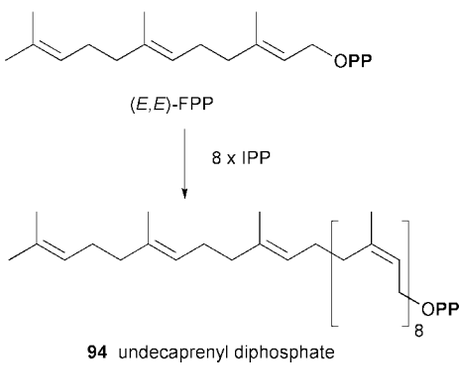
Decaprenyl diphosphate is required for the side-chain of ubiquinone-10. The gene encoding for decaprenyl diphosphate synthase in *Gluconobacter suboxydans* has been cloned and expressed in *E. coli*.[132] Enzyme activity was noted with GPP, FPP, and GGPP as allylic substrates. Single amino acid substitutions introduced upstream of aspartate-rich regions led mainly to mutant enzymes with reduced catalytic activity or a shortening of chain length in the product. However, a A70G mutant produced undecaprenyl diphosphate, whereas a A70Y mutant was completely inactive, indicating that Ala-70 is important for enzyme activity and the determination of product chain length.

In bacteria, undecaprenyl diphosphate synthase catalyses the (*Z*)-prenyl chain elongation onto all-(*E*)-FPP as a primer to yield undecaprenyl diphosphate **94** with (*E*,*Z*)- mixed stereochemistry (Scheme 17). Cloning of the gene for undecaprenyl diphosphate synthase from *Micrococcus luteus* and overproduction of the enzyme in *E. coli* has been reported.[133] The deduced primary structure of the (*Z*)-prenyl chain elongating enzyme was discovered to be totally different from those of (*E*)-prenyl chain elongating enzymes which are known to contain characteristic conserved regions, especially the aspartate-rich motifs. Undecaprenyl diphosphate synthase has been isolated from *E. coli* and studied with respect to kinetics and product distribution.[134] In the presence of excess FPP, intermediates in the range C_{25}–C_{50} accumulated, whereas under conditions where both enzyme and FPP were in excess of IPP, C_{20}–C_{30} products were favoured.

Studies with extracts from *Mycobacterium smegmatis* and *M. tuberculosis* have shown that *M. smegmatis* synthesizes mainly decaprenyl diphosphate and heptaprenyl diphosphate, whilst *M. tuberculosis* produces only decaprenyl diphosphate.[135] Data from both organisms suggest that GPP is the allylic substrate for two distinct prenyl diphosphate synthases, one located in the cell membrane that synthesizes (*E*,*Z*)-FPP, and the other present in the cytosol that synthesizes (*E*,*E*,*E*)-GGPP. In *M. smegmatis*, (*E*,*Z*)-FPP and (*E*,*E*,*E*)-GGPP are both utilized by membrane-associated prenyl diphosphate synthase activities to generate decaprenyl diphosphate and heptaprenyl diphosphate respectively. However, in *M. tuberculosis*, (*E*,*E*,*E*)-GGPP



Scheme 16



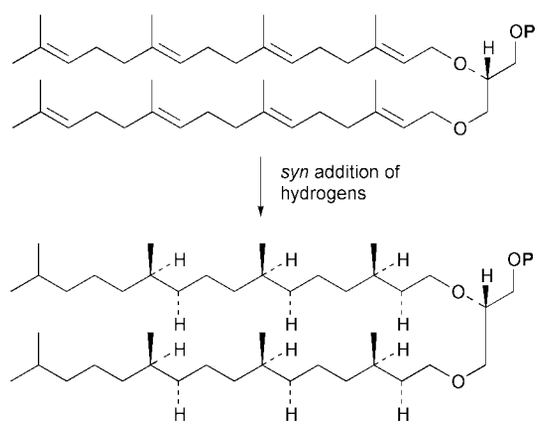
Scheme 17

is not utilized for synthesis of heptaprenyl diphosphate. Gene cloning from *M. tuberculosis* has provided two unusual (*Z*)-isoprenyl diphosphate synthase activities.[136] One of these adds an isoprene unit to GPP producing (*E*,*Z*)-FPP, whilst the second enzyme adds seven more isoprene units to this substrate to generate decaprenyl diphosphate.

The enzyme dehydrodolichol diphosphate synthase is a (*Z*)-prenyltransferase that catalyses synthesis of the dehydrodolichol diphosphate which is used as a precursor of dolichyl phosphate. The cloning and characterization of a cDNA from *Arabidopsis thaliana* that encodes for this enzyme has been reported.[137] The identity of the cloned enzyme was confirmed by functional complementation of a yeast mutant defective in the enzyme.

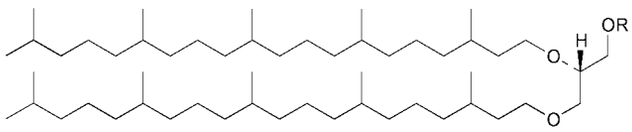
The phytyl side-chain of chlorophylls, tocopherols, and phylloquinone is a reduced form of the geranylgeranyl group. A cDNA encoding a pre-geranylgeranyl reductase from *Arabidopsis thaliana* has been cloned and expressed in *E. coli*.[138] The deduced primary structure displays a characteristic dinucleotide binding domain. Geranylgeranyl reductase sequentially catalyses the reduction of geranylgeranyl-chlorophyll *a* into phytyl-chlorophyll *a*, as well as the reduction of free GGPP into phytyl diphosphate. Due to its multifunctionality and hydrophobicity, this enzyme may participate in chlorophyll, tocopherol and phylloquinone pathways. A tobacco (*Nicotiana tabacum*) cDNA sequence encoding geranylgeranyl reductase has also been reported.[139] Using transgenic plants expressing antisense RNA, it was concluded that the tobacco enzyme provides phytol for both tocopherol and chlorophyll synthesis.

A novel prenyltransferase gene encoding farnesylgeranyl diphosphate (FGPP) synthase has been isolated from the hyperthermophilic archaeon *Aeropyrum pernix*.[140] This was expressed in *E. coli* as a glutathione *S*-transferase fusion protein and produced FGPP from either FPP or GGPP substrates. FGPP is required for synthesis of C_{25}–C_{25} diether lipids **95** in this organism. A characteristic feature of archaea is the production of membrane lipids, the basic core of which is usually 2,3-di-*O*-phytanyl-*sn*-glycerol **96**. Aspects of the biosynthesis of this material have been studied in *Haloarcula japonica*.[141] [$^2H_9$]Mevalonolactone was highly incorporated into the phytanyl chains of the core lipid, with total enrichment about 70%. Saturation of the geranylgeranyl group to the phytanyl group was shown to take place through *syn* addition of hydrogen (Scheme 18).

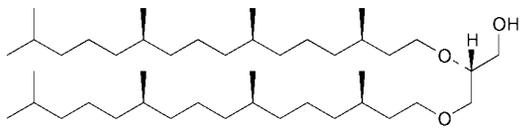


Scheme 18

Biosynthesis of these lipids is believed to proceed *via* digeranylgeranylglyceryl phosphate derived from *sn*-glycerol 1-phosphate and GGPP. Similar feeding experiments with the thermophilic methanogens *Methanococcus janaschii* and *Methanobacterium thermoautotrophicum* produced some unexpected labelling patterns in the metabolites diphytanylglycerol **96**, 36-membered lipid **97**, and 72-membered lipid **98**.[142] It was concluded that an unusual double bond migration had occurred, and that this isomerization must have taken place after construction of the digeranylgeranylglyceryl group (Scheme 19). Further, the labelling patterns in the 36- and 72-membered lipids showed the retention of two deuterium atoms at the C-16 position, excluding higher oxidized states such as aldehyde or carboxylate as an intermediate for C–C bond formation. These results further support the involvement of an isomerized intermediate having a terminal methylene group, and a plausible mechanism is proposed *via* protonation and hydride transfer from NAD(P)H (Scheme 20).



95 C_{25}–C_{25} diether lipid



96 2,3-di-*O*-phytanyl-*sn*-glycerol

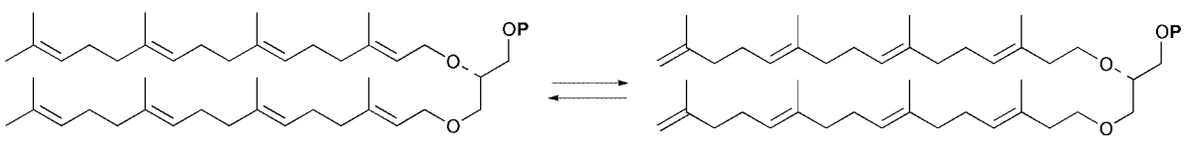
A review on enzymatic aspects of isoprenoid chain elongation has been published.[143]

6 Monoterpenoids

The conversion of geranyl diphosphate (GPP) **91** into simple cyclic monoterpenes involves initial isomerization to (+)-(3*S*)-linalyl diphosphate (LPP) **100** or to (−)-(3*R*)-LPP **102**. GPP is typically bound to the enzyme as a complex with a divalent metal ion. Ionization to the allylic linalyl cation **99** allows formation of LPP, and the opportunity for cyclization *via* the stereochemically favourable linalyl cation **101** (Scheme 21). A series of cation–diphosphate pairs participates in the sequence, and both the isomerization and cyclization reactions are catalysed by a single enzyme.

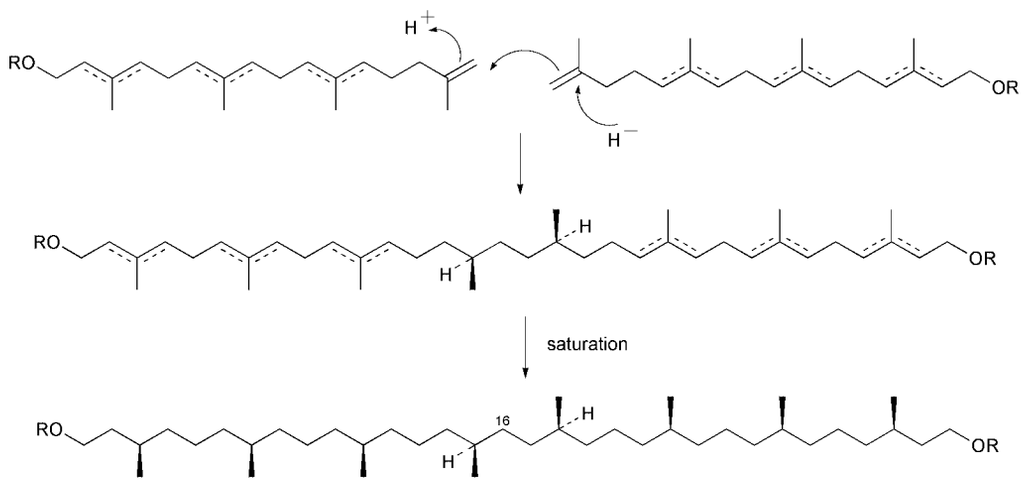
Two clones with high sequence similarity to plant monoterpene cyclases were obtained from an *Artemisia annua* cDNA library and expressed in *E. coli*.[144] The deduced peptide sequences were 88% identical, and had 42% identity with limonene synthase from *Mentha spicata*. The two recombinant enzymes transformed GPP into the acyclic monoterpene (3*R*)-linalool **103** as the sole product in the presence of divalent cations. There was no activity with a range of other possible substrates. Although linalool cannot be detected in the essential oil of *A. annua*, an increase in transcripts for the two genes is detectable after wounding the leaves and stems. The *Arabidopsis* genome project has produced sequences with similarity to members of the terpene synthase family of genes. Expression of a putative terpene synthase gene allowed production of a protein which was indeed a monoterpene synthase enzyme.[145] This enzyme converted GPP into the acyclic monoterpenes β-myrcene **104** and (*E*)-β-ocimene **105**, plus small amounts of cyclic monoterpenes (+)- and (−)-limonene **106**, 2-carene **107**, and tricyclene **108** (Scheme 22). *Arabidopsis* has not previously been reported as a producer of terpenoid secondary metabolites.

The enzyme limonene synthase from spearmint (*Mentha spicata*) catalyses the isomerization–cyclization of GPP to

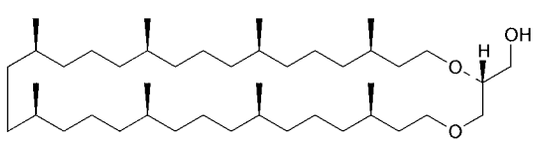


digeranylgeranylglyceryl phosphate

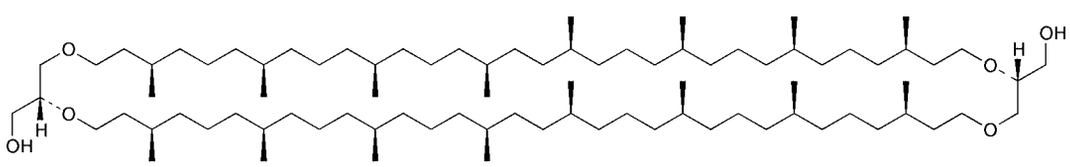
Scheme 19



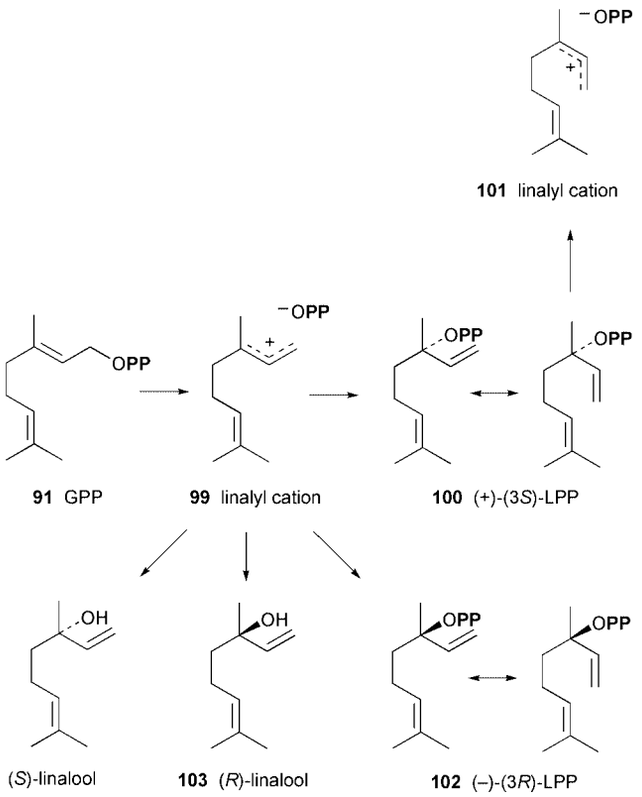
Scheme 20



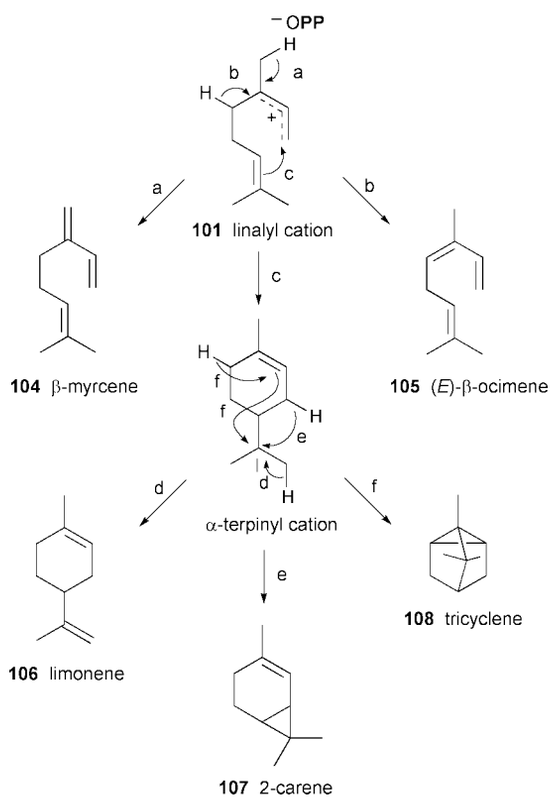
97



98

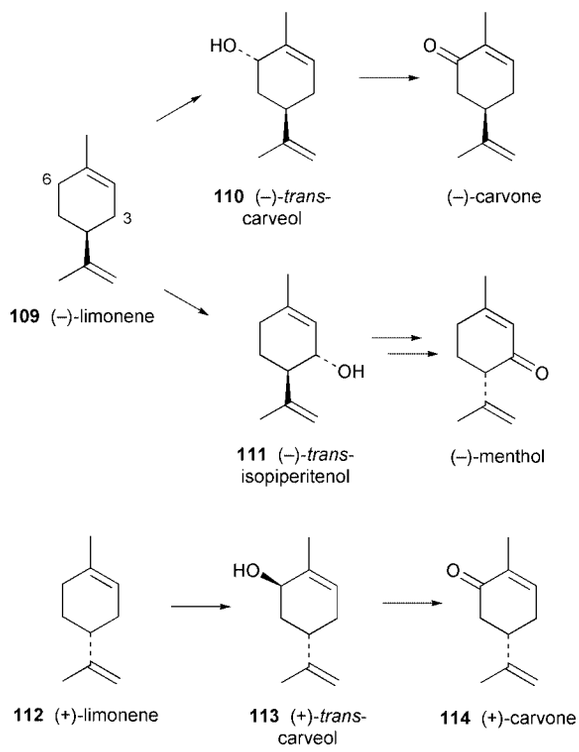


Scheme 21



Scheme 22

(−)-(4*S*)-limonene **109**, and exists as a preprotein bearing a long plastidial targeting sequence. This causes difficulties for expressing the full-length protein in *E. coli*, and, to obtain a recombinant enzyme, the targeting sequence has been removed.[146] A soluble pseudomature form of the enzyme that is catalytically more efficient than the native enzyme was thus obtained. Truncation up to and including Arg-58, or substitution of Arg-59, yielded enzymes that are incapable of converting GPP, but are able to cyclize exogenously supplied (3*S*)-linalyl PP **100**. This indicates a role for the tandem arginines in the diphosphate migration step accompanying formation of the intermediate LPP. Cyclic monoterpenes in *Mentha* species derived from limonene are characterized by additional oxygenation, which may be predominantly at C-3 (*e.g.* peppermint) or at C-6 (*e.g.* spearmint), according to species. Regiospecific cytochrome P-450-dependent hydroxylases catalyse these hydroxylations. The microsomal limonene-6-hydroxylase has been purified from the oil glands of spearmint and amino acid sequences used to design probes for a cDNA library.[147] A full-length cDNA was isolated and expressed in a baculovirus–*Spodoptera* system to provide a functional 6-hydroxylase enzyme converting (−)-(4*S*)-limonene **109** into (−)-*trans*-carveol **110** (Scheme 23). Two closely related full-length cDNA

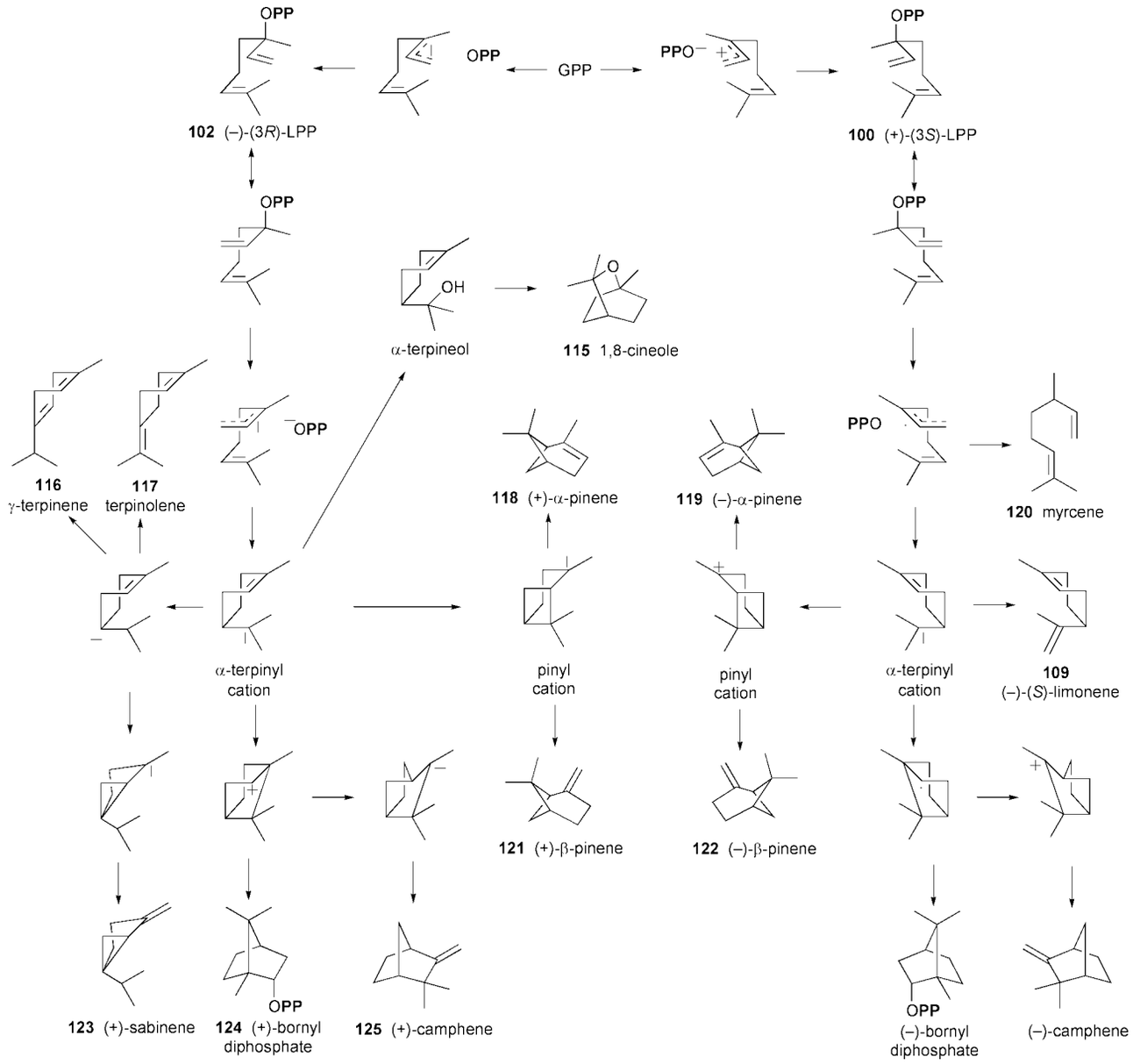


Scheme 23

species from a peppermint (*Mentha* X *piperita*) oil gland cDNA library were similarly expressed and shown to be limonene 3-hydroxylase, which converted (−)-(4*S*)-limonene into (−)-*trans*-isopiperitenol **111**. Both enzymes bear a typical amino-terminal membrane anchor, consistent with their microsomal location. Their primary sequences were 70% identical and 85% similar. In further studies,[148] a combination of domain swapping and reciprocal site-directed mutagenesis between the two enzymes demonstrated that exchange of a single residue (F363I) in the spearmint limonene-6-hydroxylase changed the enzyme so that it now possessed the full regiospecificity and catalytic efficiency of the peppermint limonene-3-hydroxylase.

Microsomal preparations from caraway (*Carum carvi*) catalyse the 6-hydroxylation of (+)-limonene **112** to (+)-*trans*-carveol **113**, the key intermediate in the biosynthesis of carvone **114** (Scheme 23).[149] The enzyme met all the established criteria for cytochrome P-450-dependent mixed function oxidases.

Common sage (*Salvia officinalis*) produces a broad range of cyclic monoterpenes with a variety of carbon skeletons (Scheme 24). cDNAs encoding three multiproduct monoterpene synthases have been isolated and functionally expressed in *E. coli*.[150] The major products obtained from GPP substrate were (+)-bornyl diphosphate **124** (75%), 1,8-cineole **115** (79%), and (+)-sabinene **123** (63%), respectively. However, significant

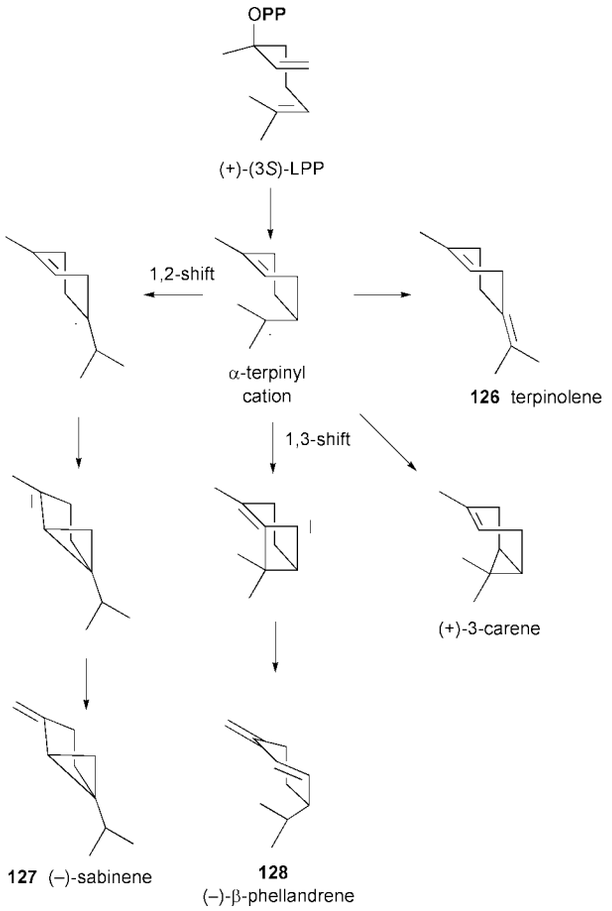


Scheme 24

minor products characterized from the bornyl diphosphate synthase were (+)-α-pinene **118**, (+)-camphene **125**, and (±)-limonene **109/112**. The 1,8-cineole synthase also produced (+)- and (−)-α-pinene **118/119**, (+)- and (−)-β-pinene **121/122**, myrcene **120**, and (+)-sabinene **123**; the (+)-sabinene synthase also produced γ-terpinene **116** and terpinolene **117** (Scheme 24). All three proteins appear to be translated as preproteins bearing an amino-terminal plastid-targeting sequence. The recombinant bornyl diphosphate synthase was a homodimer, whereas the other two enzymes were monomers. The distribution and stereochemistry of the products produced by the recombinant (+)-bornyl diphosphate synthase suggests this enzyme might represent both (+)-bornyl diphosphate synthase and (+)-pinene synthase which had previously been assumed to be two distinct enzymes though they had never been satisfactorily resolved.

(−)-Sabinene **127** is the major monoterpene produced by a European strain of the liverwort *Conocephalum conicum*. A partially purified sabinene synthase has been obtained from cultured plants and shown to possess the same general properties as monoterpene synthases from gymnosperms and angiosperms.[151] A North American strain of the liverwort produces (+)-bornyl acetate as its major monoterpene, and the formation of bornyl diphosphate was similarly demonstrated as a cyclization product from GPP. As in cell-free extracts from some plant species, the first-formed bornyl diphosphate is rapidly hydrolysed to borneol by phosphatases in the extract.

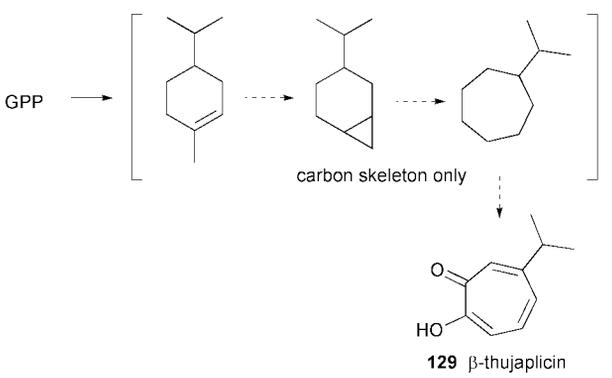
Analysis of the oleoresin from several tissues of loblolly pine (*Pinus taeda*) showed the derived turpentine to consist mainly of (+)-α-pinene **118** and (−)-β-pinene **122**. Cell-free extracts from xylem tissue yielded three monoterpene synthases which together accounted for the monoterpene isomer and enantiomer content of the turpentine.[152] The major products of these three enzymes, incubated with GPP, were (+)-α-pinene **118**, (−)-α-pinene **119**, and (−)-β-pinene **122**, respectively. In most properties, these enzymes resembled other monoterpene synthases. Four full-length cDNA species have been isolated from a wounded Grand fir (*Abies grandis*) cDNA library and shown to encode four different monoterpene synthase enzymes.[153] These were expressed in *E. coli* and characterized as (−)-camphene synthase, (−)-β-phellandrene **128** synthase, terpinolene **126** synthase, and an enzyme that produces both (−)-limonene **109** and (−)-α-pinene **119**. The origins of those metabolites not already covered in Scheme 24 can be formulated as in Scheme 25. These enzymes were all translated as preproteins bearing an amino-terminal plastid targeting sequence of 50–60 amino acid residues. cDNA truncation allowed deletion of the transit peptide and functional expression of pseudomature forms of



Scheme 25

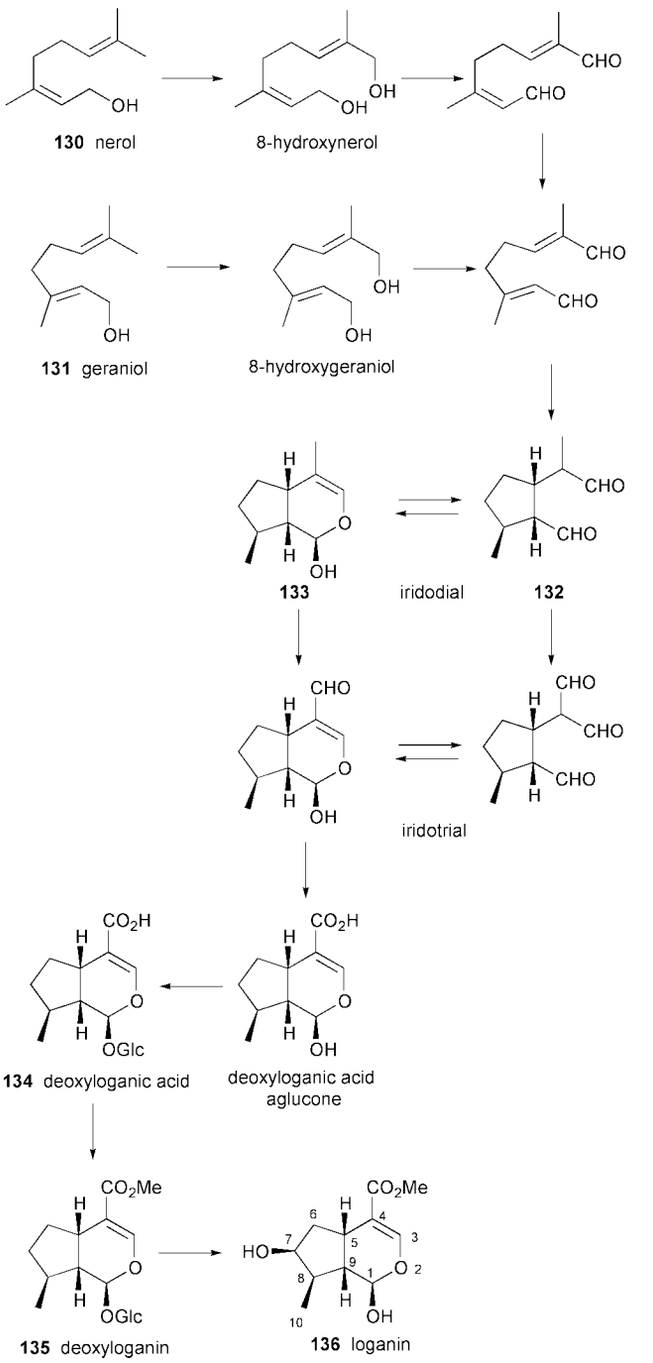
the enzymes with no change in product outcome. Sequence comparison revealed these enzymes resembled more closely sesquiterpene and diterpene synthases from conifers than they did mechanistically-related monoterpene synthases from angiosperms.

The tropolone β-thujaplicin **129** from *Cupressus lusitanica* has been shown to be monoterpenoid in origin.[154] ^{14}C-Labelled geraniol was incorporated into β-thujaplicin in a *C. lusitanica* cell culture, and experiments with glucose and mevalonate showed the mevalonate-independent pathway to be operative. Based on the labelling patterns obtained from ^{13}C-glucose feedings, a skeletal rearrangement of an initial limonane-type monoterpene is indicated, in which a methyl group is incorporated into the seven-membered ring (Scheme 26).



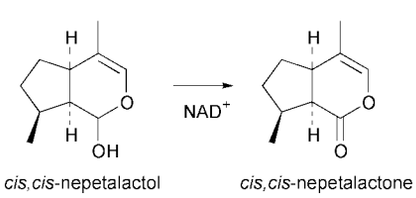
Scheme 26

Iridoids are derived from geraniol **131**, or the (*Z*)-isomer nerol **130**, *via* early hydroxylation of the 8-methyl (Scheme 27). The dialdehyde iridodial **132** and/or its derived lactol **133** are regarded as pivotal intermediates in the pathway to other structures, *e.g.* loganin **136**. Whether nepetalactones are derived



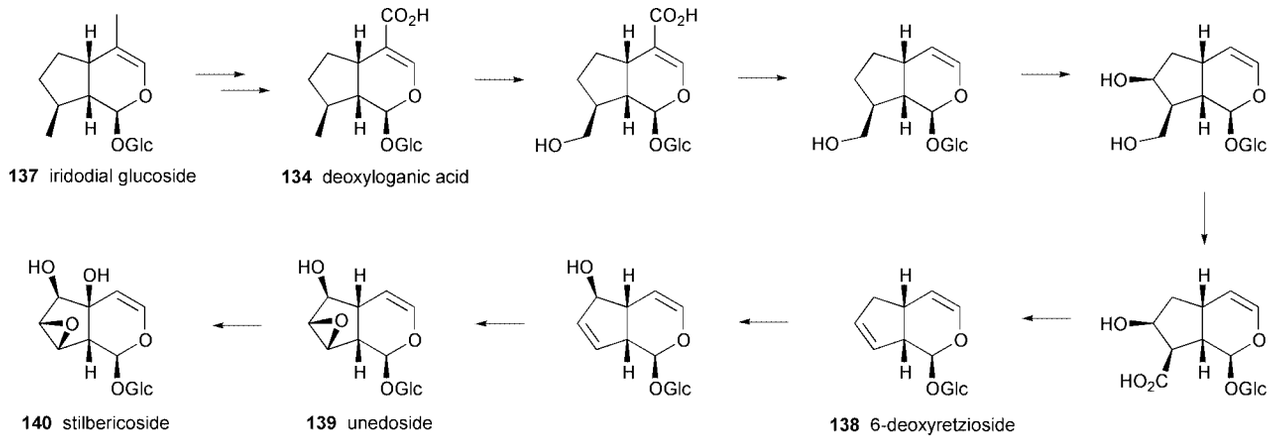
Scheme 27

directly from iridodial by a Cannizzaro-type reaction or *via* the corresponding lactol has been investigated in cell-free extracts from catmint (*Nepeta racemosa*).[155] Support for the lactol intermediate was obtained by partial purification of an enzyme from young leaves that catalysed the NAD^+-dependent oxidation of *cis*,*cis*-nepetalactol to *cis*,*cis*-nepetalactone (Scheme 28).

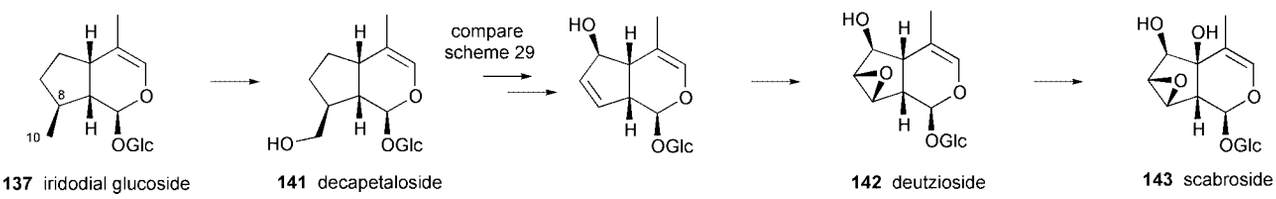


Scheme 28

In some iridoids, C-10 is lost *via* hydroxylation then decarboxylation. The biosynthesis of such structures has been investigated further in recent studies.[156] Using *Thunbergia alata* plants,

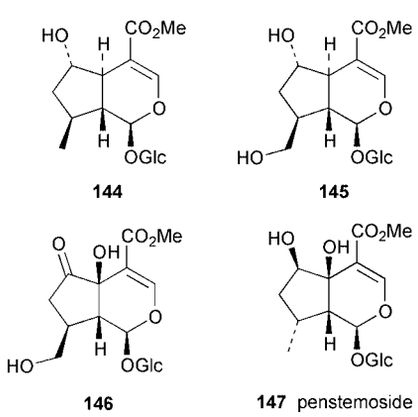


Scheme 29



Scheme 30

6-deoxyretzioside **138** was shown to be incorporated into stilbericoside **140** rather more efficiently than was deoxyloganic acid **134**, supporting its proposed intermediacy (see ref. 2) in the pathway to **140** (Scheme 29). In *Deutzia schneideriana*, iridodial glucoside **137** but not its 8-epimer was incorporated into scabroside **143** and deutzioside **142**, whilst in *D. scabra*, decapetaloside **141** but not the isomeric 7-hydroxyiridodial glucoside was a precursor of these two compounds. These experiments help to confirm the pathway shown in Scheme 30, where loss of C-10 is believed to be analogous to that in the biosynthesis of stilbericoside. Deoxyloganic acid **134** was also found to be a precursor of unedoside **139** in *Nuxia floribunda*,[157] and this compound seems a likely intermediate in the pathway to stilbericoside. Deoxyloganic acid, but not 8-epideoxyloganic acid, was also incorporated into the *trans*-fused iridoid glycosides (5αH)-6-epidihydrocornin **144** and 10-hydroxy-(5αH)-6-epidihydrocornin **145** in *Penstemon secundiflorus*.[158] This observation indicates that formation of the *trans*-fusion is therefore a late event, and does not occur during cyclization of the open chain monoterpene in iridoid formation. Label from deoxyloganic acid was also found in 10-hydroxyhastatoside **146** (which bears an 8β-methyl group), while 8-epideoxyloganic acid labelled penstemoside **147** (which has an 8α-methyl group), showing

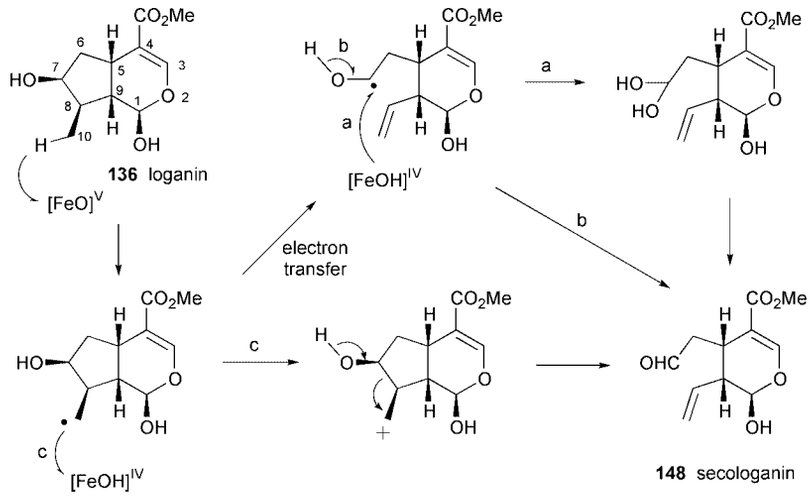


biosynthetic pathways from both epimers of deoxyloganic acid in the same plant.

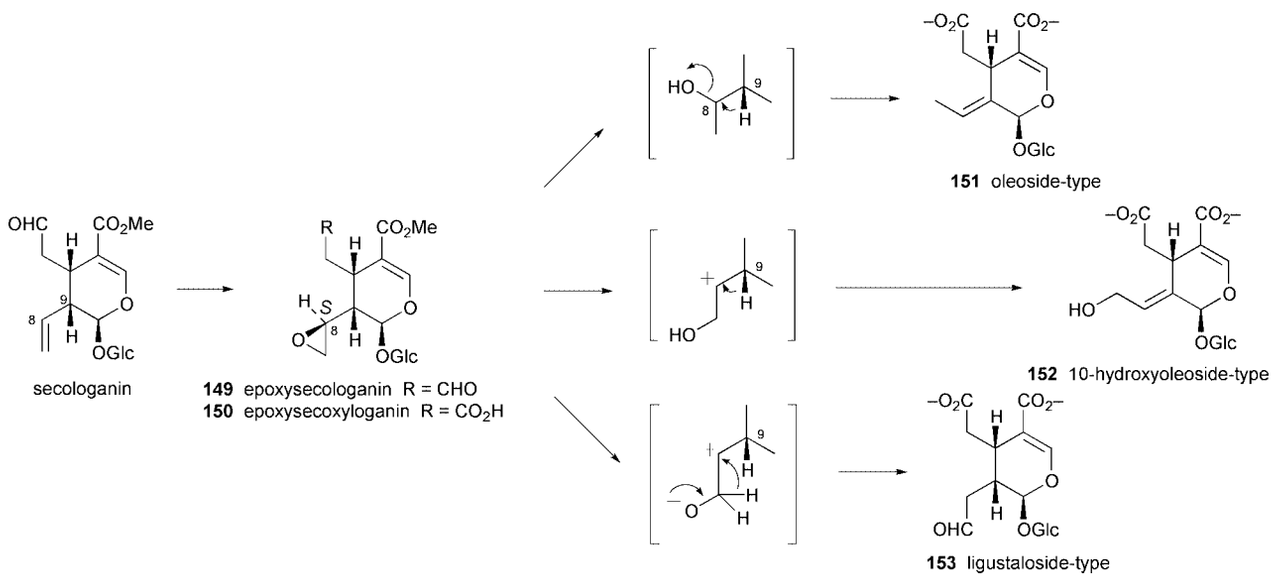
The conversion of loganin **136** into secologanin **148** by an oxidative cleavage of the cyclopentane ring is an important biosynthetic step in that secologanin then becomes a precursor for many alkaloids, especially the terpenoid indole alkaloids. Whilst *Lonicera japonica* is known to contain loganin and a number of secologanin derivatives, cell suspension cultures do not accumulate iridoid or secoiridoid glucosides.[159] However, the cells did have the ability to convert loganin into secologanin. They also converted 7-deoxyloganin **135**, but not geraniol, into both loganin and secologanin. This suggested a lack of enzymes converting geraniol into iridoids in the cell cultures.

The enzyme secologanin synthase has been detected in microsomal preparations from cell suspension cultures of *Lonicera japonica*.[160] The enzymic reaction required NADPH and O_2 cofactors, was blocked by CO and several other cytochrome P-450 inhibitors, and so is concluded to belong to the cytochrome P-450 monooxygenases. The enzyme was specific for loganin, and other substrates tested, including loganic acid, 10-hydroxyloganin, 7-epiloganin, 7-dehydrologanin, 7-dehydrologanic acid, and 8-epiloganin, were not accepted. The mechanism proposed for the loganin–secologanin conversion (Scheme 31) parallels that in the formation of furocoumarins, and involves abstraction of a hydrogen radical from C-10 followed by cleavage of the C–C bond. The resultant C-7 carbon radical is converted into an aldehyde through hydroxylation or desaturation. Alternatively, successive removal of a hydrogen radical and an electron could yield a carbocation, and C–C cleavage could then be formulated as an ionic mechanism.

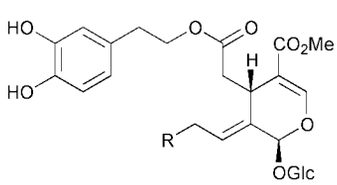
The biosynthesis of a range of secoiridoids has been investigated using plants of the Oleaceae.[161] These fall into three types: oleoside **151**, 10-hydroxyoleoside **152**, and ligustalolide **153**. Initially, labelled secologanin was shown to be a precursor of ligustaloside B **157** in *Ligustrum japonicum*, then C-8 stereoisomers of 8,10-epoxysecologanin **149** and 8,10-epoxysecoxyloganin **150** were administered separately to *Olea europaea*, *Osmanthus fragrans*, and *Ligustrum japonicum*. (8*S*)-8,10-epoxysecologanin was a significantly better precursor than the (8*R*)-isomer for oleuropin **154** in *Olea europaea*, for 10-acetoxyoleuropin **155** in *Osmanthus fragrans*, and for ligustaloside A **156** and ligustaloside B **157** in *L. japonicum*. Both stereoisomers of 8,10-epoxysecoxyloganin were poorly incorporated. The results were interpreted in terms of (8*S*)-8,10-epoxysecologanin being a precursor of all three types of secoiridoid, and the mechanisms shown in Scheme 32 are proposed.



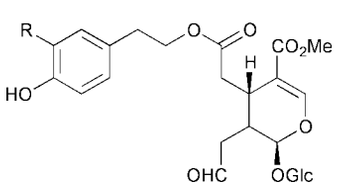
Scheme 31 *Enzyme*: secologanin synthase.



Scheme 32



154 oleuropin R = H
155 10-acetoxyoleuropin R = OAc

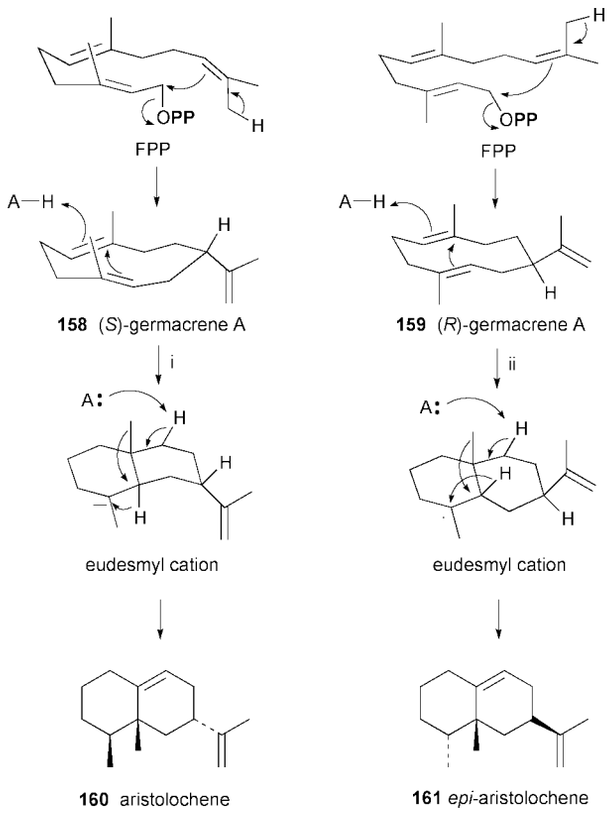


156 ligustaloside A R = OH
157 ligustaloside B R = H

7 Sesquiterpenoids

Farnesyl diphosphate (FPP), the product of FPP synthase, is the precursor to a wide variety of sesquiterpenoids. Under certain circumstances, farnesol is also capable of initiating terpenoid biosynthesis, *via* phosphorylation to FPP. Two kinase activities converting farnesol into farnesyl monophosphate and subsequently FPP have been demonstrated in microsomal fractions from cultures of tobacco *Nicotiana tabacum*.[162] CTP acted as the phosphate donor. Geranylgeraniol was similarly converted into mono and diphosphates. Labelled farnesol was readily incorporated into sterols by tobacco cultures, though upon treatment with elicitor, label was directed away from sterol synthesis into formation of sesquiterpene phytoalexins such as capsidiol.

Aristolochene synthase accomplishes the cyclization of FPP to germacrene A and its subsequent rearrangement *via* the eudesmyl cation to (+)-aristolochene **160** (Scheme 33). The *Aspergillus terreus* enzyme has been purified, and the gene coding for this enzyme identified, sequenced and expressed in *E. coli*.[163] The *A. terreus* protein had 70% identity in its deduced amino acid sequence with the enzyme previously isolated from the blue cheese mould *Penicillium roqueforti*. The crystal structure of recombinant aristolochene synthase from *P. roqueforti* has been reported.[164] The structure reveals active site features that participate in the cyclization. The enzyme active site is a template to enforce the correct substrate conformation throughout the cyclization cascade, and an aspartate-rich segment DDVIE is the coordination site for the Mg^{2+} ions necessary to trigger initial carbocation formation. In the formation of the germacrene A intermediate **158**, there is no apparent general base to assist the deprotonation step, which may thus be assisted by the diphosphate leaving group. The phenolic hydroxy group of Tyr-92 appears to be the general acid/base involved in the cyclization of germacrene A to the eudesmyl cation, and proton removal after the subsequent rearrangements. Active site aromatic residues appear to stabilize carbocation intermediates through the sequence.



Scheme 33 *Enzymes*: i, aristolochene synthase, ii, 5-*epi*-aristolochene synthase.

Whilst the *Penicillium roqueforti* enzyme synthesizes aristolochene by way of (*S*)-germacrene A **158**, the *Nicotiana tabacum* enzyme 5-*epi*-aristolochene synthase produces the diastereoisomeric product by way of (*R*)-germacrene A **159** (Scheme 33). This is a consequence of the stereospecificity of the cyclization cascade conferred by the enzyme active site. Although the germacrene A intermediate is not released during normal catalysis in either enzyme, its intermediacy in the *Penicillium roqueforti* system has been deduced by indirect methods (see ref. 1). The intermediate role of germacrene A in the *Nicotiana tabacum* system has now also been demonstrated.[165] Based on the three-dimensional structure of tobacco 5-*epi*-aristolochene synthase, proton donation by Tyr-520 was considered responsible for activation of germacrene A to initiate formation of the eudesmyl cation (see ref. 1). Using site-directed mutagenesis, a Y520F point mutation was introduced into the enzyme, which effectively blocked this protonation step. This protein metabolized FPP much less efficiently than the original enzyme, but yielded a single product indistinguishable from authentic germacrene A, establishing both the intermediate role of this sesquiterpene and the function of Tyr-520.

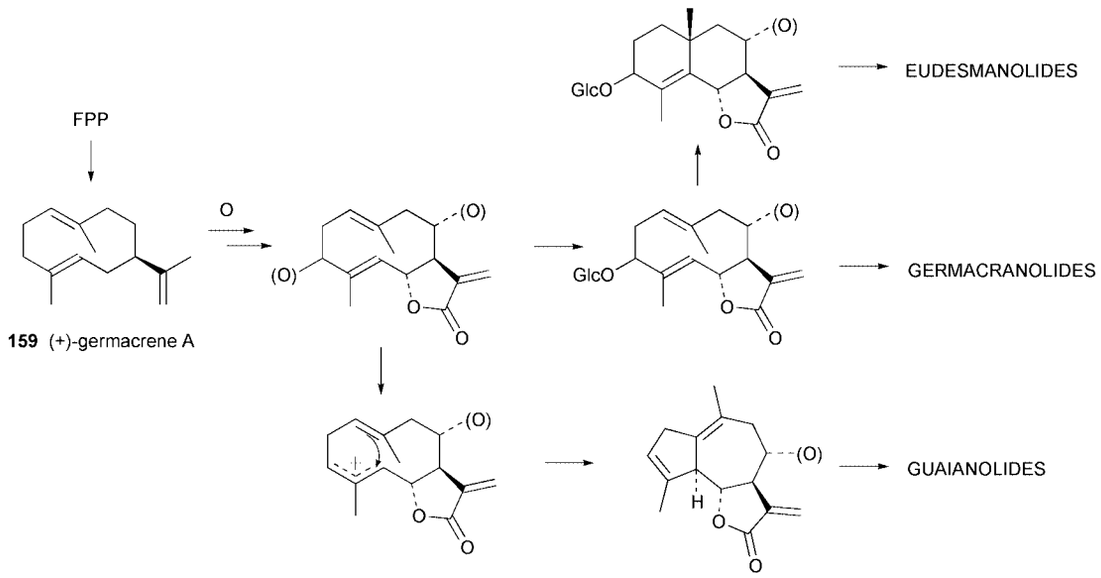
Roots of chicory (*Cichorium intybus*) contain high levels of bitter sesquiterpene lactones comprising guaianolides, eudesmanolides and germacranolides, which are thought to arise from a common precursor, probably germacrene A. (+)-(*R*)-Germacrene A synthase has been isolated and purified from chicory roots.[166] The release of germacrene A from the enzyme contrasts with the aristolochene/*epi*-aristolochene systems above, where this intermediate is enzyme-bound and immediately cyclized further. It is suggested that further cyclizations in chicory to give the various sesquiterpene lactones may be initiated by oxidation reactions, especially epoxidations (Scheme 34).

The predominant sesquiterpene in the leaf oil of tomato (*Lycopersicon esculentum* cv Cherry) is germacrene C **165**, with smaller amounts of germacrenes A **162**, B **163** and D **164**. Soluble enzyme preparations from leaves catalysed divalent metal ion-dependent cyclization of FPP to these germacrenes.[167] cDNA isolation, characterization and bacterial expression in *E. coli* led to proteins that converted FPP into the four germacrenes (C: 64%; A 18%; B 11%; D 7%). None of the expressed proteins was active with GGPP, though one truncated protein converted GPP into the monoterpene limonene. The germacrene C synthase activity corresponded to a deduced polypeptide whose sequence compared most closely (50% identity) with that of δ-cadinene synthase from cotton. The biosynthetic relationships accounting for formation of the four germacrenes are postulated as shown in Scheme 35. Germacrene C could be formed by two alternative mechanisms from a germacryl cation, invoking either 1,3- or 1,2-hydride shifts.

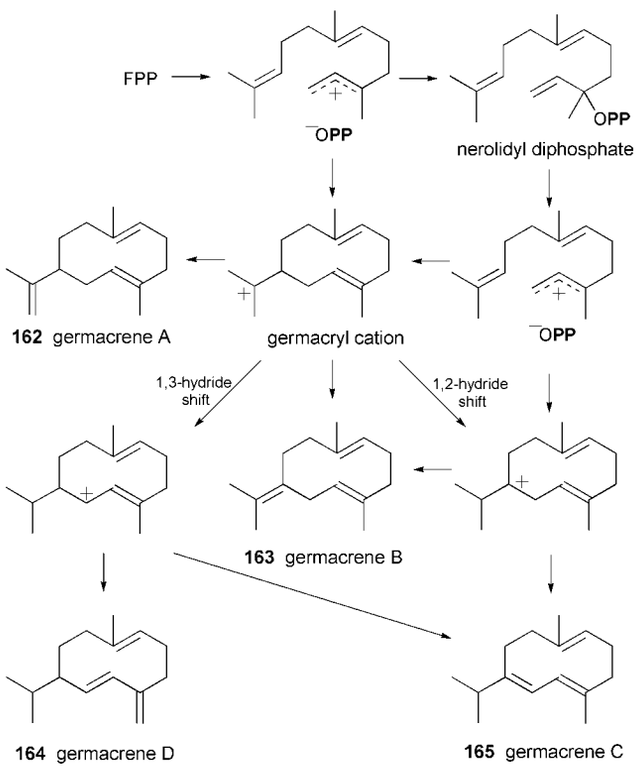
Germacrene D in the essential oil of *Solidago canadensis* is a mixture of the two enantiomers, and their formation has been studied to ascertain whether this involves one or two enzymes.[168] Enzyme extracts from young leaves converted FPP into both isomers, but purification allowed separation of two active fractions which were shown to be enantioselective synthases, producing either (+)-germacrene D **166** or (−)-germacrene D **167**. The involvement of separate enzymes in the formation of enantiomers has been demonstrated previously in monoterpene biosynthesis.

Two cDNA species isolated from a cDNA library of grand fir (*Abies grandis*) have been expressed in *E. coli* and shown to encode proteins with the capability of synthesizing a remarkable number of sesquiterpene products.[169] The enzymes have been named δ-selinene synthase and γ-humulene synthase based on the principal products formed. Each enzyme produced three major products: these were δ-selinene **168** (25%), germacrene B **163** (17%) and guaia-6,9-diene **169** (10%) from δ-selinene synthase, and γ-humulene **170** (29%), sibirene **171** (15%) and longifolene **172** (12%) from γ-humulene synthase. However, the former enzyme yielded a total of 34 sesquiterpene products, and the latter 52, which together accounted for many of the sesquiterpenes identified in the oleoresin from grand fir. The deduced amino acid sequences of the two proteins were 83% similar and 65% identical, with high similarity to known monoterpene and diterpene synthases from grand fir. The multi-step, multi-product reactions catalysed by these enzymes are amongst the most complex of any terpenoid cyclase so far described.

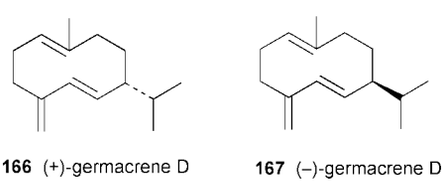
Infection of cotton (*Gossypium* species) with the fungus *Verticillium dahliae* induces formation of aromatic phytoalexins such as gossypol (see below) that are ultimately derived from cadinane sesquiterpenes. The formation of an early intermediate (+)-δ-cadinene **174** in enzyme preparations from *G. barbadens*e has been investigated by feeding ^{2}H-labelled FPP and nerolidyl PP precursors.[170] Labelling patterns generated were consistent with the sequence shown in Scheme 36, in which the nerolidyl cation cyclizes to a germacradienyl cation **173** followed by a 1,3-hydride shift, a second cyclization, then proton loss. Thus, (1-*RS*)-[1-^{2}H]-FPP was converted into [5-^{2}H]- and [11-^{2}H]-δ-cadinene, demonstrating the 1,3-hydride shift, and a $^{2}H_{5}$-labelled sample of nerolidyl PP was converted with retention of all labels. δ-Cadinene synthase activity is not present until several hours after infection with the fungus, and precedes production of the gossypol-related phytoalexins. In previous investigations, three cDNAs for the (+)-δ-cadinene cyclase (CAD1) had been characterized in *G. arboreum*, and, on the basis of sequence similarities, these were grouped into two subfamilies *cad*1-C (2 members) and *cad*1-A (1 member). A new member of the CAD1 family has been isolated from a *G. arboreum* cDNA library, and has also been assigned to the *cad*1-C family.[171] Desoxyhemigossypol **175** is a key intermediate in the later stages of the gossypol **178** pathway which proceeds *via* hemigossypol **177** (Scheme 37). A methyltransferase enzyme which specifically methylates the 6-position of desoxyhemigossypol giving **176** has been isolated from *G. barbadense* tissue

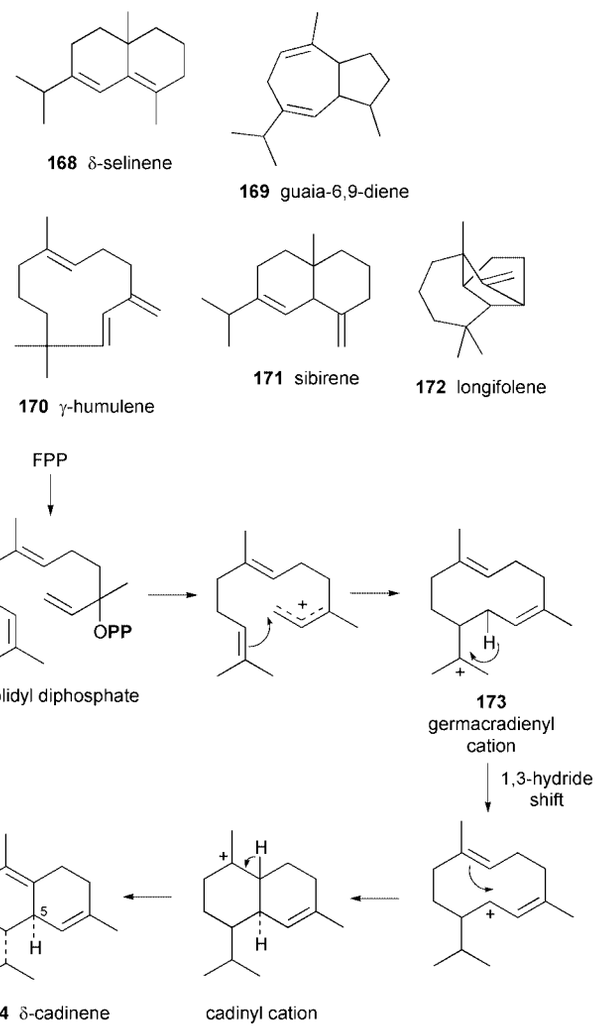


Scheme 34



Scheme 35

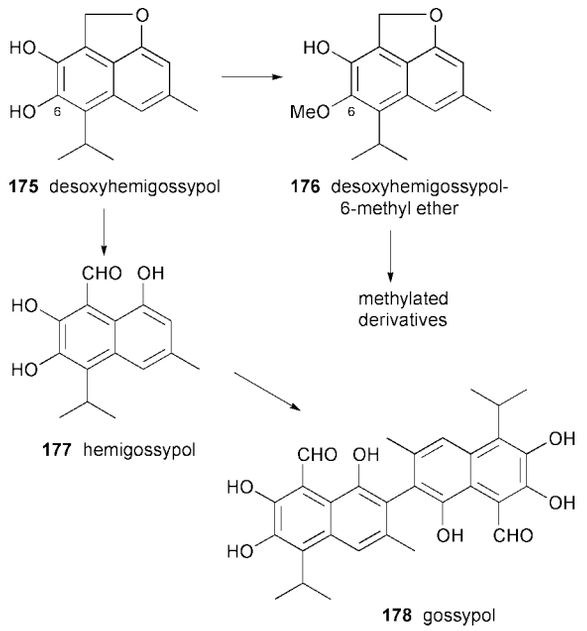




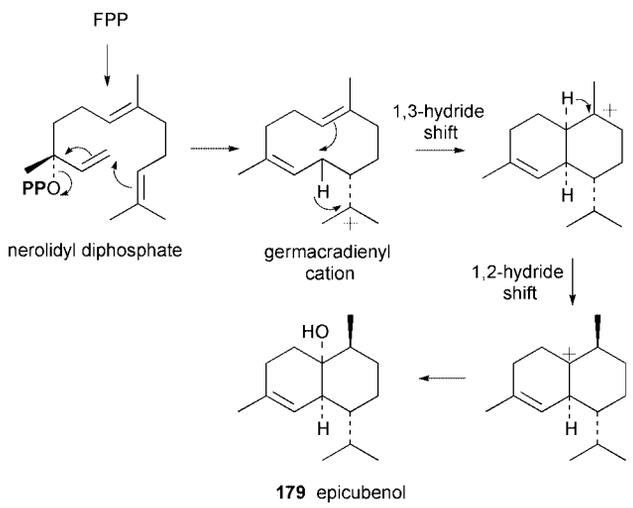
Scheme 36 *Enzyme*: δ-cadinene synthase.

infected with *Verticillium dahliae*.[172] Although gossypol and some of the other cotton phytoalexins do not contain *O*-methyl groups, a series of methylated derivatives are formed, and characterized by being less toxic to fungi. The enzyme is a dimer, does not require Mg^{2+}, and appears specific for desoxyhemigossypol, not methylating any of a wide range of diphenol or dinaphthol substrates tested.

Epicubenol synthase from *Streptomyces* sp. LL-B7 catalyses the cyclization of FPP to epicubenol **179** (Scheme 38). The origin of the introduced hydroxy group has been investigated by incubating the partially purified enzyme with FPP in the presence of ^{18}O-labelled water, demonstrating exclusive derivation from water.[173] Although direct formation of the alcohol by quenching of the cation with water is the most reasonable mechanistic possibility, in the monoterpene field, borneol is known to arise by hydrolysis of the diphosphate, which is formed by recapture of the diphosphate leaving group of the GPP substrate.



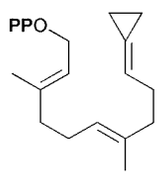
Scheme 37



Scheme 38 *Enzyme*: epicubenol synthase.

(*E*)-α-Bisabolene synthase is one of two inducible sesquiterpene synthases of grand fir (*Abies grandis*), and a cDNA encoding this enzyme has been isolated from a wound-induced grand fir stem library.[174] This was functionally expressed in *E. coli* and shown to produce (*E*)-α-bisabolene **180** as the sole product from FPP in the presence of a divalent cation Mg^{2+} or Mn^{2+}. The expressed synthase resembles other terpenoid synthases in sequence, except for a large 216-amino acid amino terminal insertion. A similar large insertion has also been found in the diterpene synthases abietadiene synthase and taxadiene synthase, but has not been noted previously in grand fir monoterpene and sesquiterpene synthases. When incubated with GPP, monoterpene synthase activity was also noted, the enzyme producing (4*R*)-limonene **112** (Scheme 39). However, GGPP was not utilized (compare *epi*-cedrol synthase, below). The presence of an Arg^{25}–Arg^{26} tandem motif, an element previously found only in monoterpene synthases that require an isomerization step in the reaction sequence, is consistent with the cyclization proceeding through the tertiary allylic intermediate nerolidyl PP (Scheme 39). Labelled (*E*)-α-bisabolene **180** was also shown to be a precursor of other metabolites in cell suspension cultures of grand fir. The predominant product identified was todotamuic acid **181** (Scheme 40).

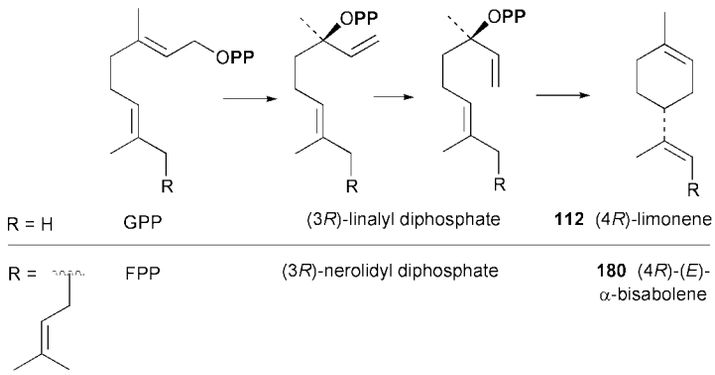
The biosynthesis of trichothecene mycotoxins proceeds from FPP *via* the intermediate hydrocarbon trichodiene **182**. Trichodiene formation is catalysed by the enzyme trichodiene synthase, and involves preliminary isomerization to (3*R*)-nerolidyl diphosphate, then cyclization and subsequent rearrangements (Scheme 41). In experiments to target the proposed active site base responsible for the final deprotonation reaction, the 10-cyclopropylidene analogue **183** of farnesyl diphosphate was incubated with recombinant *Fusarium sporotrichioides* trichodiene synthase.[175] The cyclopropylidene analogue proved to be a mechanism-based inhibitor of the enzyme, and also acted as a substrate in that three unidentified sesquiterpene hydrocarbon products were detected.



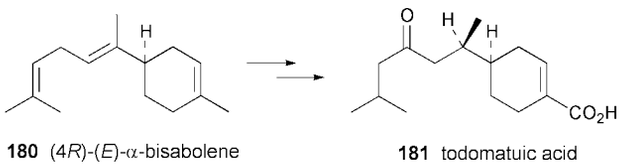
183

During formation of the trichothecene skeleton, trichodiene **182** is oxygenated at several positions and subjected to further cyclization. Isotrichodiol **186**, isotrichotriol **187**, and isotrichodermin **188** feature among the proven intermediates in the pathway building up the substitution pattern of 3-acetyldeoxynivalenol (3-AcDON) **189** in *Fusarium culmorum* (Scheme 42). The first two oxygenation steps have now been shown to be hydroxylation at position 2 leading to 2α-hydroxytrichodiene **184**, followed by epoxidation to give 12,13-epoxy-9,10-trichoene-2α-ol **185**.[176] ^{13}C-Labelled **184** and **185** were specifically incorporated into 3-AcDON and also into sambucinol (SOL) **193** in *F. culmorum* cultures. Neither 2β-hydroxytrichodiene nor 12,13-epoxytrichodiene were precursors. In some experiments, incorporations into preSOL **192**, a known precursor of SOL, and into a dead-end metabolite apotrichodiol **191** were also recorded. Both **184** and **185** could also be detected at low levels in the cultures. SOL, preSOL and apotrichodiol are believed to arise from isotrichodiol **186** *via* the unsubstituted trichothecene EPT **190**, whereas the oxygenated trichothecenes branch away *via* isotrichotriol **187** (Scheme 42).

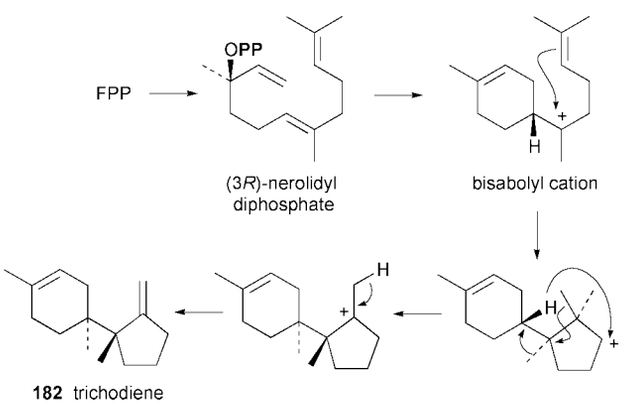
An interesting observation is that the 3-*O*-acetyl of isotrichodermin **188** is actually lost and then replaced during incorporation into 3-AcDON **189** (see ref. 1). 3-Acetylation appears to significantly reduce toxicity in trichothecenes, and thus this esterification may play some role in protecting the host organism from its toxic metabolites. A gene *Tri101* responsible for the 3-*O*-acetylation reaction has been cloned from a *Fusarium graminearum* cDNA library and expressed in the yeast *Schizosaccharomyces pombe*.[177] Yeast transformants resistant to trichothecenes all contained a cDNA for *Tri101*. The protein TRI101 expressed in *E. coli* was demonstrated to catalyse specifically the 3-*O*-acetylation of several representative trichothecenes. The resistance gene in *F. graminearum* is not part of the trichothecene biosynthetic gene cluster. In further studies,[178] the gene was shown to be located between the two 'house-keeping' genes for UTP-ammonia ligase and phosphate permease. Its isolation from the trichothecene gene cluster is attributed to independent evolution. Disruption of a homologue of*TRI101* in *F. sporotrichioides* produced mutants unable to synthesize T-2 toxin **194** which accumulated isotrichodermol **195** and small amounts of trichothecenes not normally encountered in cultures of the parent strain.[179] Again, this is consistent with the enzyme TRI101 converting isotrichodermol into isotrichodermin. Disruption of another *F. sporotrichioides* gene, *TRI11*, also results in altered trichothecene metabolism, and in this case accumulation of isotrichodermin **188**.[180] The nucleotide sequence of *TRI11* predicts a polypeptide of the cytochrome P-450 family, and it is suggested that it may encode a C-15 hydroxylase involved in trichothecene



Scheme 39 *Enzyme*: (*E*)-α-bisabolene synthase.



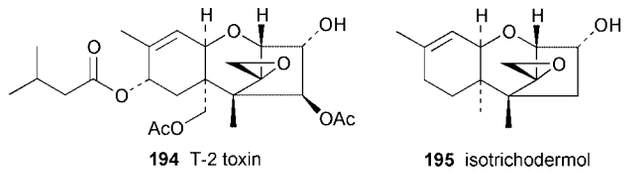
Scheme 40



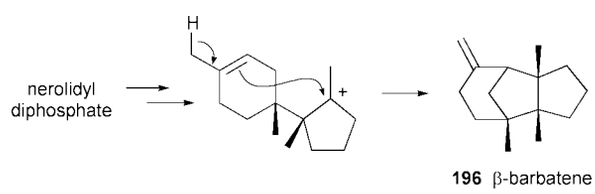
Scheme 41 *Enzyme*: trichodiene synthase.

biosynthesis. A novel gene *Tri102* in the gene cluster of *F. graminearum* is involved in toxin transport,[181] and a similar function is ascribed to the gene *TRI12* in *F. sporotrichioides*.[182]

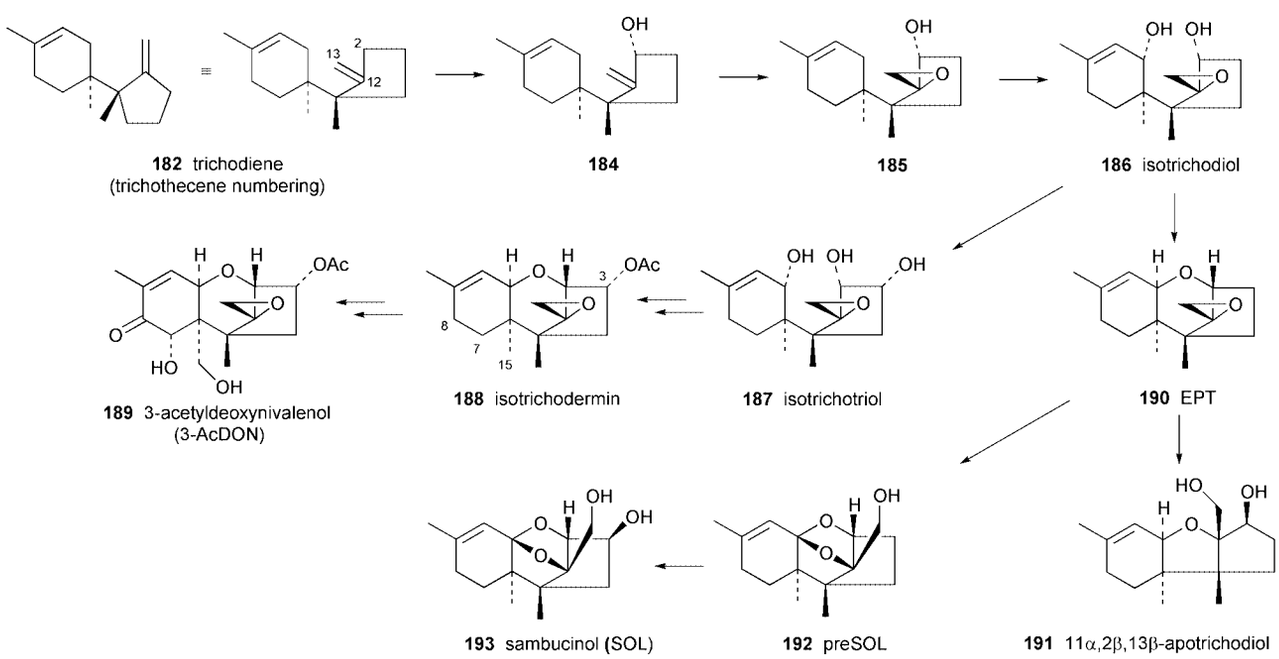
Cultures of the liverwort *Heteroscyphus planus* also produce



the irregular sesquiterpene β-barbatene **196** which has been proposed to be related biogenetically to the trichothecenes. Indeed, recent evidence now confirms it appears to share the early pathway almost as far as trichodiene, though instead of losing a proton to form trichodiene, a further cyclization occurs.[183] Thus, labelling experiments with deuteriated mevalonates, ^{13}C- and ^{2}H-labelled acetates, labelled glycerol and glucose initially showed the exclusive operation of the mevalonate pathway. Labelling patterns then confirmed the 1,4-hydride shift, and double 1,2-methyl shifts, as in the formation of trichodiene (Scheme 41). β-Barbatene **196** formation is then formulated as a consequence of an additional ring cyclization, followed by loss of a proton (Scheme 43).

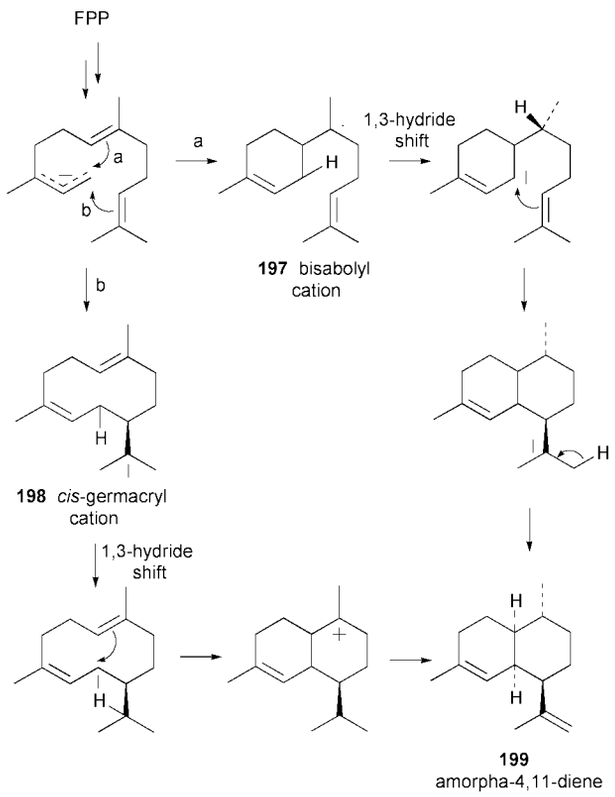


Scheme 43



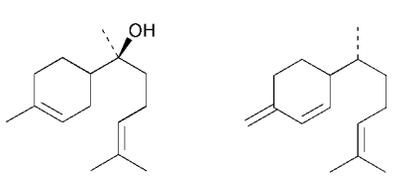
Scheme 42

Artemisinin (qinghaosu) (see below) is a sesquiterpene derivative containing a peroxide linkage that has been shown to be crucial for the antimalarial activity displayed by this compound. The cadinane derivative artemisinic acid is known to be a precursor of the highly modified structure of artemisinin, but a number of very different hypothetical sequences have been proposed over the years. Recent studies are now allowing parts of the pathway to be defined with more certainty. The initial step appears to be formation of amorpha-4,11-diene **199** (Scheme 44).[184] Small amounts of **199** could be extracted from



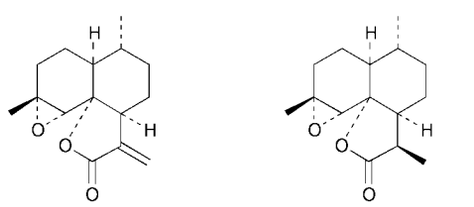
Scheme 44 *Enzyme*: amorpha-4,11-diene synthase.

the leaves. A number of sesquiterpene synthase activities from leaves of *Artemisia annua* could be demonstrated, one of which catalysed formation of **199** from FPP. The enzyme possessed properties typical of sesquiterpene synthases. A cDNA clone encoding this enzyme was subsequently isolated.[185] This allowed an amino acid sequence for the enzyme to be deduced, showing some 32–51% identity with other sesquiterpene cyclases. When expressed in *E. coli*, the recombinant enzyme catalysed formation of a range of olefinic and oxygenated sesquiterpene products, of which amorpha-4,11-diene (91.2%) predominated. Formation of amorpha-4,11-diene is formulated as involving initial cyclization of FPP to a bisabolyl cation **197** or to a *cis*-germacryl cation **198** (Scheme 44). The formation of α-bisabolol **200** and β-sesquiphellandrene **201** as minor products, and a lack of germacrene products, seems to favour the intermediacy of the bisabolyl cation, however. Essentially identical conclusions were obtained in independent studies on the same system.[186]



200 α-bisabolol **201** β-sesquiphellandrene

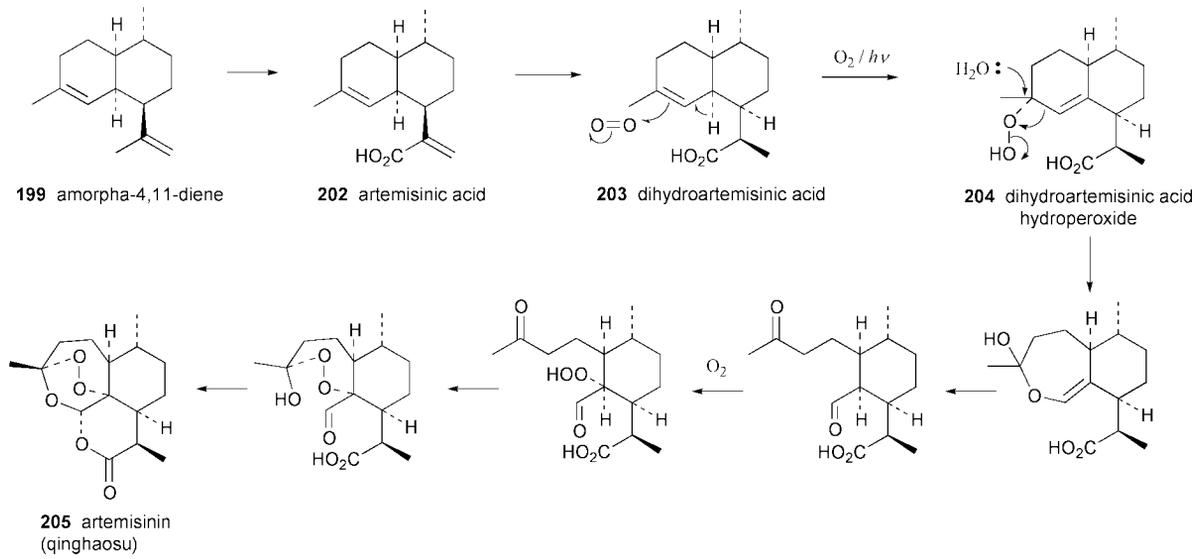
Artemisinic acid **202** would arise by a terminal oxidation of amorpha-4,11-diene **199** (Scheme 45). Isolation of dihydroartemisinic acid **203** from leaves of *Artemisia annua* and an interesting oxidative conversion of this compound into artemisinin **205** led to the suggestion that a similar chemical, non-enzymic sequence may occur in nature.[187] When dihydroartemisinic acid was subjected to photooxidation with singlet oxygen, it gave initially the hydroperoxide **204**. This reaction is analogous to photooxidation of polyunsaturated fatty acids to hydroperoxides. Ring cleavage of the hydroperoxide induced by air oxidation (triplet oxygen) and subsequent ring formations generates the artemisinin system (Scheme 45). Very strong support for this hypothesis is provided by the subsequent isolation of the hydroperoxide **204** from *Artemisia annua*.[188] The presence of dihydroartemisinic acid **203** and its hydroperoxide **204** in the plant, and the mild oxidizing conditions under which **203** can be converted into artemisinin **205**, provide evidence that a non-enzymic photochemical conversion is responsible for the late stages of artemisinin biosynthesis. Indeed, the presence of high levels of dihydroartemisinic acid in *A. annua* is suggested to give the plant protection against oxidizing species, such as singlet oxygen, reacting with these species, and that artemisinin is the by-product of this scavenging.[189] On the other hand, arteannuin B **206** has also been implicated in earlier feeding experiments as an intermediate between artemisinic acid and artemisinin. Recent studies have shown that the reduced form, dihydroarteannuin B **207**, can be converted by an enzyme system from *A. annua* into artemisinin, with the addition of cofactors ATP, NADPH, Mg^{2+} and Mn^{2+} optimizing yields.[190] Further clarification of this pathway is awaited with interest.



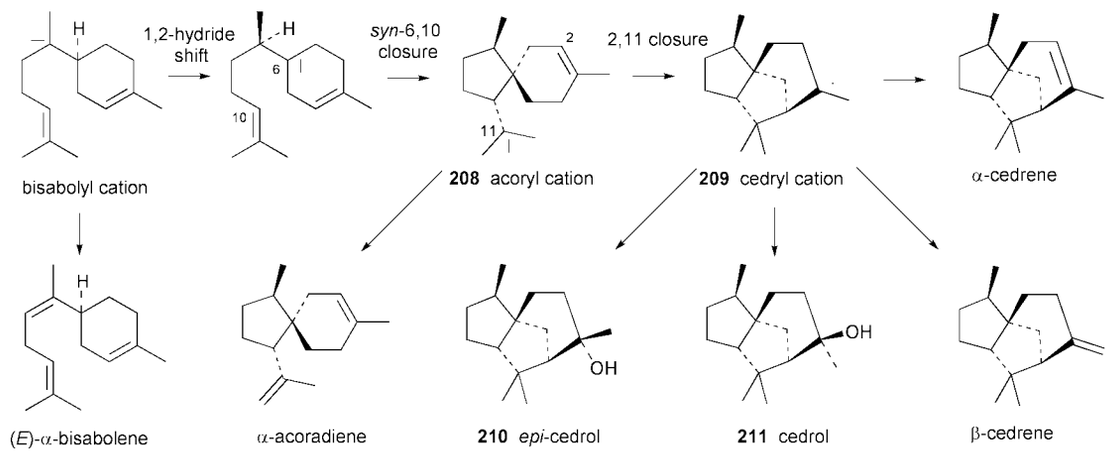
206 arteannuin B **207** dihydroarteannuin B

A cDNA clone encoding *epi*-cedrol synthase has been isolated from *Artemisia annua*.[191] The deduced amino acid sequence of the enzyme was 32–43% identical with sequences of other known sesquiterpene cyclases. When expressed in *E. coli*, the recombinant enzyme catalysed formation of both olefinic (3%) and oxygenated (97%) sesquiterpenes from FPP, the major component being *epi*-cedrol **210**. The enzyme was not active with GGPP as substrate, but did convert GPP into monoterpenes (limonene, terpinolene, γ-terpinene, myrcene, α-terpinene), though at a lower rate (compare α-bisabolene synthase, above). The proposed sequences to *epi*-cedrol (and some other metabolites) are shown in Scheme 46. This involves 1,2-hydride shift from the bisabolyl cation, followed by *syn*-6,10 ring closure generating the acorane skeleton. The cedryl cation **209** originates *via* 2,11-closure from the acoryl cation **208**. When quenched by water, *epi*-cedrol **210** and cedrol **211** are produced in a ratio of 96:4, perhaps due to preferential facial attack by coordinated water at the active site, or because of steric hindrance from the methyl groups. The high proportion of oxygenated products (quenching by water) relative to olefinic products (loss of proton) is rather unusual among known sesquiterpene cyclases, and, most interestingly, the cedrols are not significant metabolites in *A. annua*. Cloning of *epi*-cedrol synthase from *A. annua* has also been accomplished in independent studies, with similar results.[192]

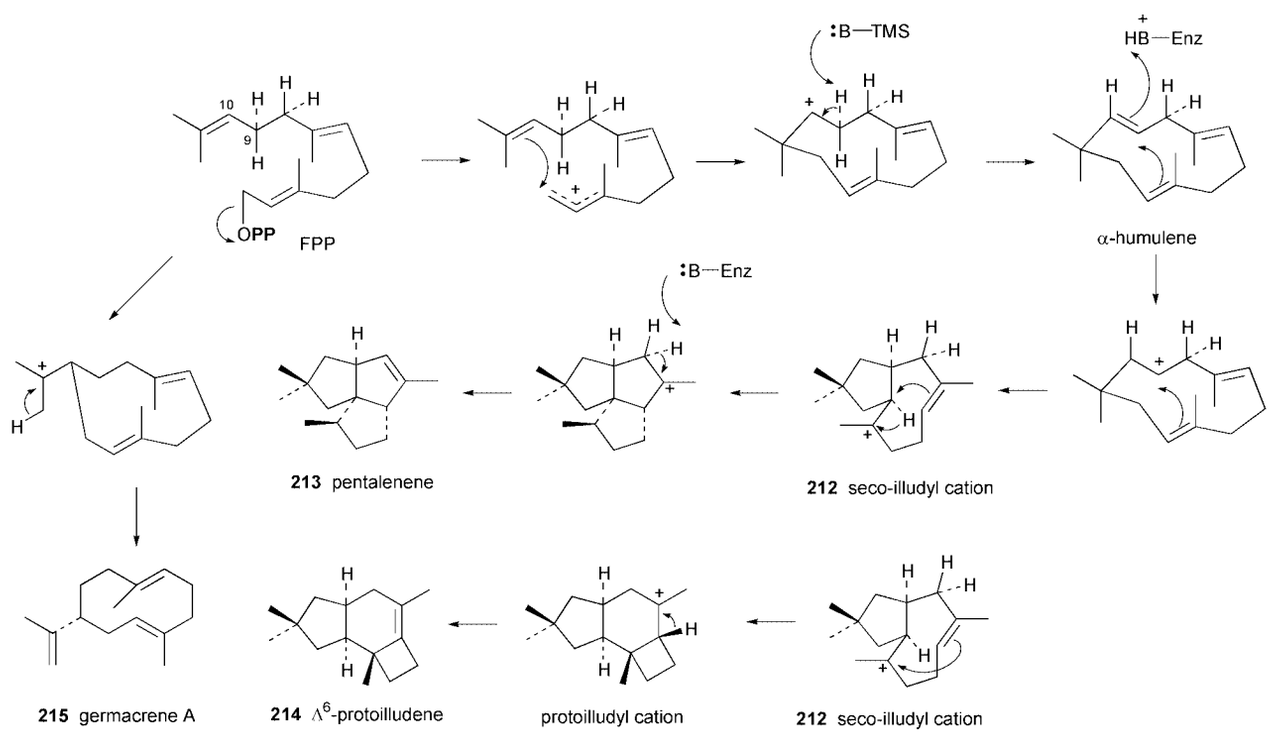
Pentalenene synthase catalyses the conversion of FPP into pentalenene **213** in *Streptomyces* UC5319, and extensive studies have supported the cyclization mechanism shown in Scheme 47. It had been postulated that a single active-site base might be responsible for the successive deprotonation–reprotonation–deprotonation steps, and X-ray studies had indicated that a His-309 residue appeared ideally suited for abstraction of the



Scheme 45



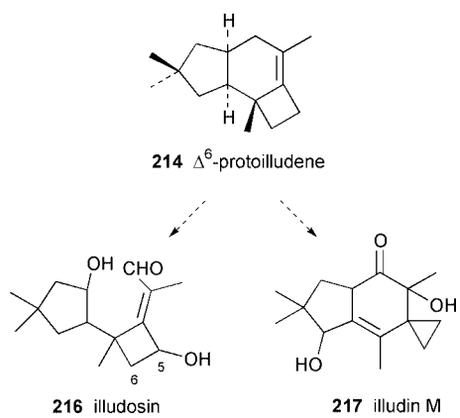
Scheme 46 *Enzyme*: *epi*-cedrol synthase.



Scheme 47 *Enzyme*: pentalenene synthase.

relevant protons. To investigate the proposed role of H309, a series of mutant enzymes was constructed, in which this histidine was replaced by alanine, serine, cysteine, or phenylalanine.[193] Unexpectedly, all four mutants retained substantial cyclase activity, without any really substantial changes in kinetic parameters. The major product in each case was pentalenene **213**, accompanied by up to 20% of two additional sesquiterpenes, Δ^6-protoilludene **214** and germacrene A **215**. Δ^6-Protoilludene **214** formation is readily explained by cyclization of the natural seco-illudyl cation intermediate **212** (Scheme 47), whilst the production of germacrene A requires an alternative Markovnikov addition to the 10,11-double bond of FPP. The results establish that H309 is not required for cyclase activity, but normally it could play a role in the final deprotonation step since no other basic amino acid residue is apparently positioned correctly. In the mutant enzymes, some other group must perform this function. Notably, the deprotonations leading to **214** and **215** involve protons chemically and geometrically distinct from that lost in the generation of pentalenene.

Sites of incorporation of ^{13}C-labelled acetates and [1-^{13}C, 2-$^{2}H_3$]acetate into the fomannosane sesquiterpenes illudosin **216** and illudin M **217**, produced by the fungus *Omphalotus nidiformis*, lead to the conclusion that FPP is cyclized *via* the humulyl and protoilludyl cations as shown in Scheme 47.[194] This is essentially as expected from earlier studies in the fomannosane series, apart from the formation from [1-^{13}C,2-$^{2}H_3$]-acetate of some labelled illudosin species with two deuteriums attached to C-6 and also ^{13}C at C-5. A mechanism has been proposed in which some exchange occurs *via* non-stereospecific reprotonation during the carbocation cyclization–rearrangement, giving the appearance of an intramolecular rearrangement. Δ^6-protoilludene **214** is almost certainly an intermediate (Scheme 48).



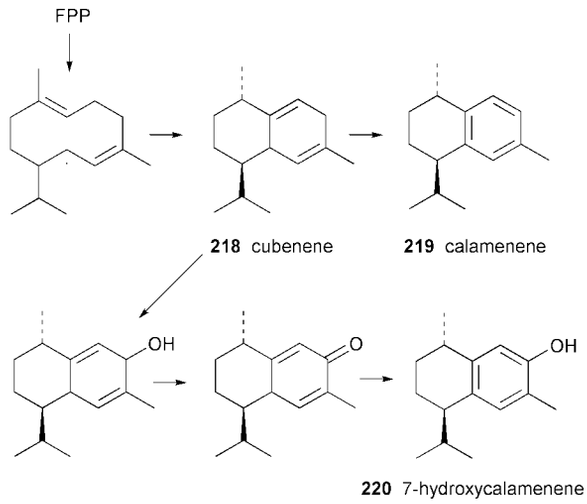
214 Δ^6-protoilludene

216 illudosin **217** illudin M

Scheme 48

The biotransformation of cubenene **218**, but not calamenene **219**, to 7-hydroxycalamenene **220** in cultures of the liverwort *Heteroscyphus planus* suggests this metabolite is formed by hydroxylation of the methylene carbon between the unconjugated double bonds, rather than by an aromatic hydroxylation (Scheme 49).[195]

NMR analyses of striatol **222** produced by suspension cultures of the liverwort *Ptycanthus striatus* in the presence of [2-^{13}C]- and [4,4-$^{2}H_2$]-labelled mevalonates have established that cyclization occurs between the distal and central double bond in FPP, followed by a concerted sequence of 1,2-migrations of hydrogen and a methyl group.[196] These, and the stereochemical consequences, are shown in Scheme 50. The early part of this pathway leading to cation **221** has also been encountered in the biosynthesis of pinguisane sesquiterpenoids, also metabolites of certain liverworts, so that cation **221** may be a common intermediate to striatanes and pinguisanes (see ref. 1). The same organism also synthesizes the uncommon tricyclic sesquiterpenes kelsoene **224** and prespatane **226** which appear structurally



218 cubenene **219** calamenene

220 7-hydroxycalamenene

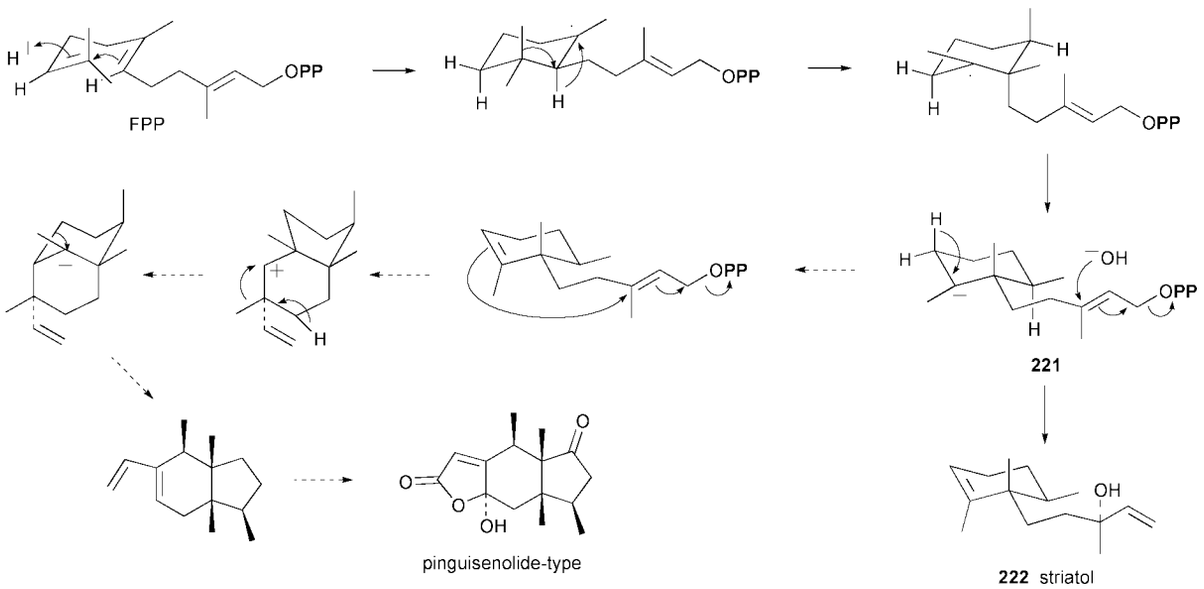
Scheme 49

unrelated to striatanes. Feeding of [2-^{13}C]mevalonate produced labelling patterns rationalized in terms of a biosynthetic sequence *via* a germacradienyl cation (Scheme 51).[197] Two further cyclizations would generate the cyclopentane and cyclopropyl rings in the alloaromadendranyl cation **223**. The production of kelsoene then requires cleavage of the cyclopropyl ring, and ring closure between C-1 and C-4. Final proton loss appears to occur equally from either of the *gem*-dimethyl groups. Prespatane **226** may arise *via* the guaianyl cation **225** (Scheme 51).

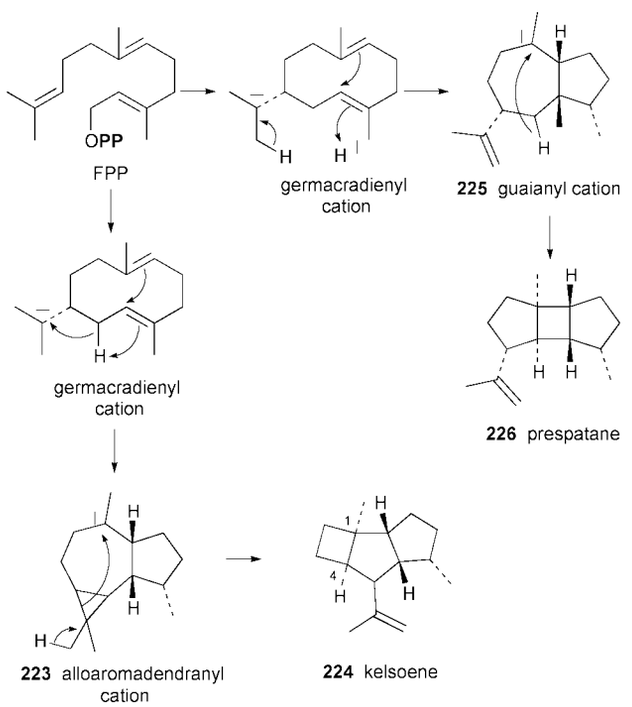
Marine molluscs belonging to the genus *Dendrobis* appear to employ sesquiterpenes of the drimane series as chemical defence agents against predators. Specimens of two species, the Mediterranean *D. limbata* and the Pacific *D. arborescens* were used in feeding experiments to demonstrate *de novo* synthesis of 7-deacetoxyolepupuane **227** from labelled MVA.[198] Labelling in other drimane sesquiterpenoids suggested that **227** could be the precursor of olepupuane **228** and polygodial **230**, and their acetoxy derivatives **229** and **231** (Scheme 52).

Biosynthetic studies on the phytotoxin sorokinianin **233** in the phytopathogen *Bipolaris sorokiniana* have confirmed that it is fundamentally a sesquiterpene with an additional C_3 portion derived from acetate.[199] It has the same carbon skeleton as prehelminthosporol **232**, a sesquiterpene that is the major metabolite of this organism, and **232** is proposed to be the precursor of sorokinianin. Thus, [1,2-$^{13}C_2$]acetate produced the same labelling patterns in the C_{15} skeleton of **233** as it did in **232**, and a further intact C_2 unit was incorporated into the lactone portion. Addition of prehelminthosporol to the cultures increased production of sorokinianin, as did feeding of succinic and fumaric acids. Intact incorporation of [2,3-$^{13}C_2$]succinic acid into sorokinianin at positions C-1′–C-2′ confirmed the TCA cycle origin of the lactone portion. A proposed biosynthetic pathway is shown in Scheme 53.

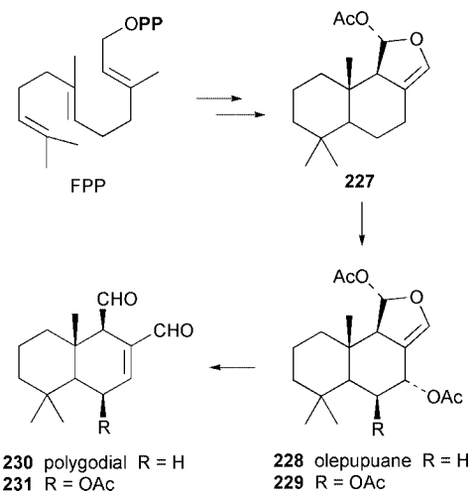
The plant growth regulator abscisic acid (ABA) **43** is produced from FPP by two main routes, which involve either direct cyclization of the C_{15} precursor or alternatively initial formation of a C_{40} carotenoid followed by oxidative metabolism. The former direct pathway is utilized by several species of fungi. Plants produce ABA by the indirect route, cleaving carotenoids with a suitable ring system through the polyene chain to xanthoxin **236**, which is then modified to ABA *via* the aldehyde **237** (Scheme 54). A gene from bean (*Phaseolus vulgaris*) has been cloned, and shown to encode the cleavage enzyme 9-*cis*-epoxycarotenoid dioxygenase.[200] The recombinant protein catalysed cleavage of both 9-*cis*-violaxanthin **234** and 9′-*cis*-neoxanthin **235**. This enzyme appears to play a key regulatory role in induced ABA synthesis. The first step in ABA catabolism is catalysed by ABA 8′-hydroxylase, also a key enzyme in regulating ABA levels. This enzyme, which converts ABA



Scheme 50



Scheme 51



Scheme 52

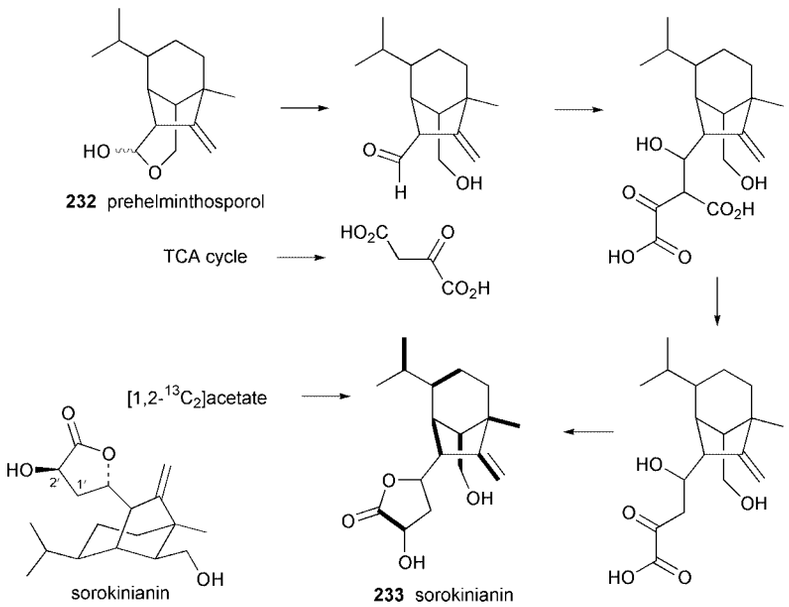
into 8′-hydroxy-ABA **238**, has been isolated from a microsomal fraction in suspension cultured maize (*Zea mays*) and shown to have the characteristics of a cytochrome P450 monooxygenase.[201]

A group of so-called homoterpenes containing eleven carbons are, in fact, more correctly considered as degraded sesquiterpenes, since they are formed by oxidative cleavage of four carbon atoms. Thus, 4,8-dimethylnona-1,3(*E*),7-triene **241** is known to originate in a range of plants from nerolidol **239**. Cucumber (*Cucumis sativus*) and lima bean (*Phaseolus lunatus*) both produce **241** in response to herbivore attack. It has now been shown that both cucumber and lima bean infested with spider mite contain a sesquiterpene synthase activity catalysing the formation of (3*S*)-(*E*)-nerolidol **239** from FPP.[202] The enzyme activity is not present in uninfested cucumber leaves, and is only weakly active in uninfested lima bean leaves, but is strongly induced by spider mite in both species. The activity correlated well with release of **241** and seems to play a regulatory role in the release of this metabolite. In independent studies,[203] maize (*Zea mays*) leaves were similarly shown to produce a (3*S*)-(*E*)-nerolidol synthase enzyme activity inducible as a result of feeding by the larvae of beet armyworm. Labelled nerolidol was incorporated into **241**, and again there was a close correlation between increase in nerolidol synthase activity and homoterpene emission.

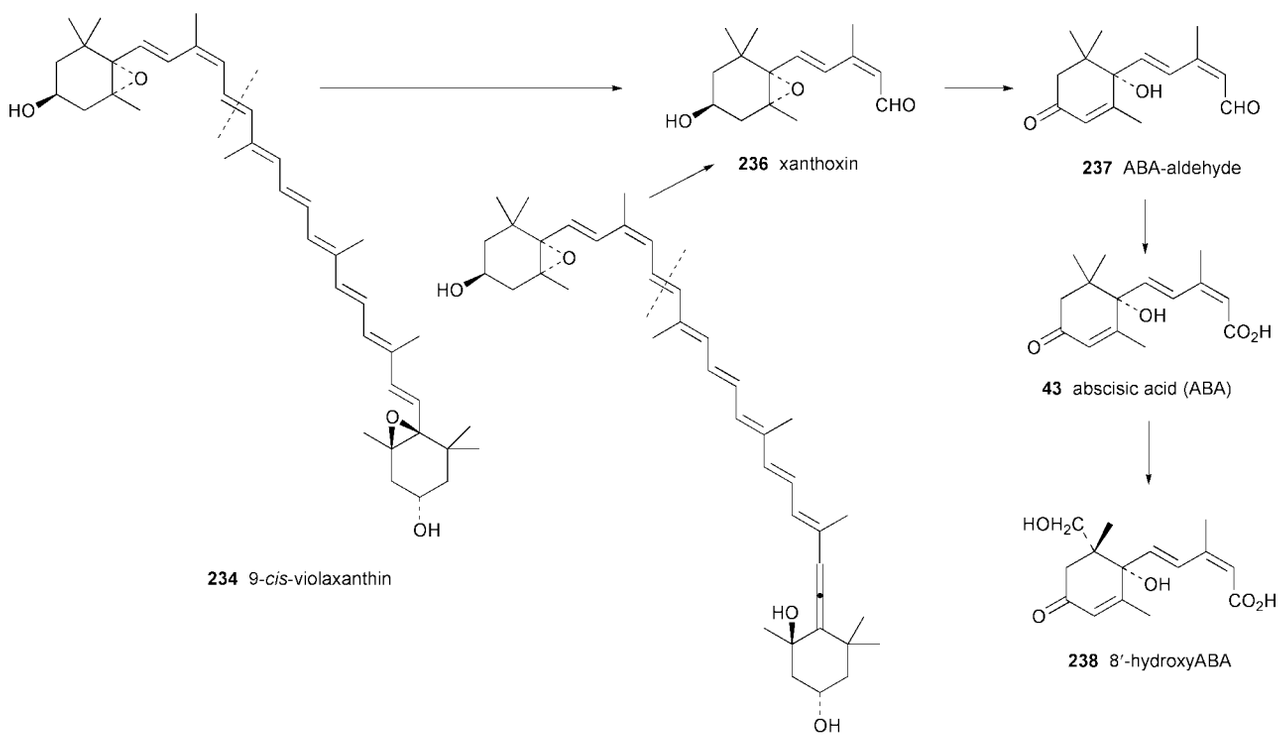
The oxidative degradation of nerolidol to **241** appears to proceed *via* geranylacetone **240** and with exclusive loss of H-5*S* from nerolidol (H-4*S* from geranylacetone). The oxidative C–C bond cleavage appears to resemble processes seen in steroid dealkylation or furocoumarin formation. The stereochemical course of bond cleavage in **241** biosynthesis has been probed by feeding chirally labelled nerolidol and geranylacetone precursors to flowers of *Magnolia liliiflora nigra* or leaves of *Phaseolus lunatus*.[204] It was established with both precursors that a *syn* elimination was involved (Scheme 55). This is consistent with a cytochrome P-450-dependent process, and a likely mechanism is presented in Scheme 56.

8 Diterpenoids

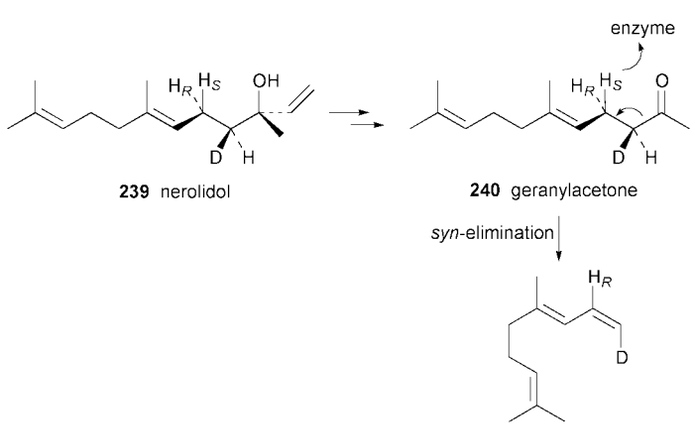
Geranylgeranyl diphosphate (GGPP), the product of GGPP synthase, is the normal progenitor of diterpenoids. Plaunotol **242** is perhaps one of the simplest diterpenoid derivatives and is found in leaves of *Croton sublyratus*. An enzyme activity involved in the biosynthesis of plaunotol has been demonstrated in *C. sublyratus* extracts, and shown to catalyse



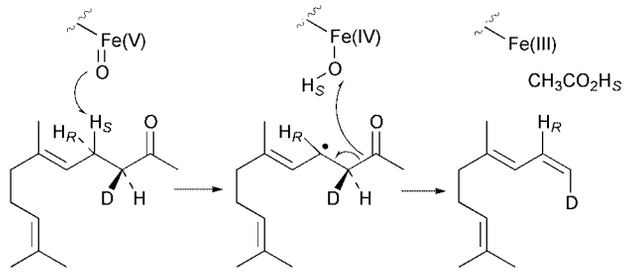
Scheme 53



Scheme 54



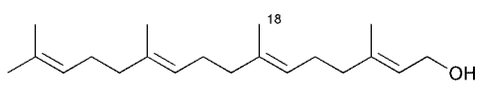
Scheme 55



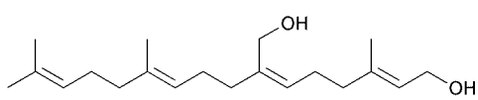
240 geranylacetone **241**

Scheme 56

18-hydroxylation of geranylgeraniol **243**.[205] The enzyme required NADPH and appeared highly specific for geranylgeraniol, since no reaction was observed with either geraniol or farnesol. The enzyme extracts also contained a phosphatase activity hydrolysing GGPP to geranylgeraniol.

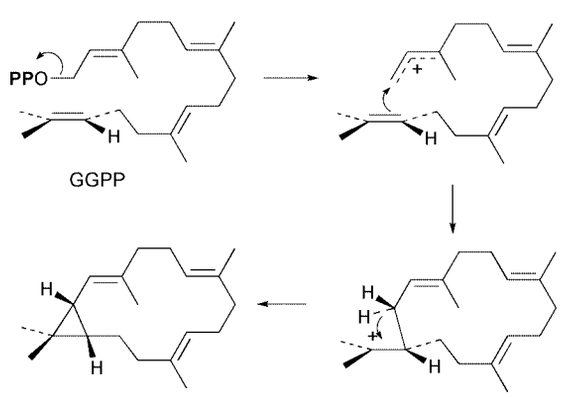


243 geranylgeraniol



242 plaunotol

Casbene **244** is a diterpene phytoalexin formed from GGPP in castor bean (*Ricinus communis*) by the action of casbene synthase (Scheme 57). Overexpression of casbene synthase as a



244 casbene

Scheme 57 *Enzyme*: casbene synthase.

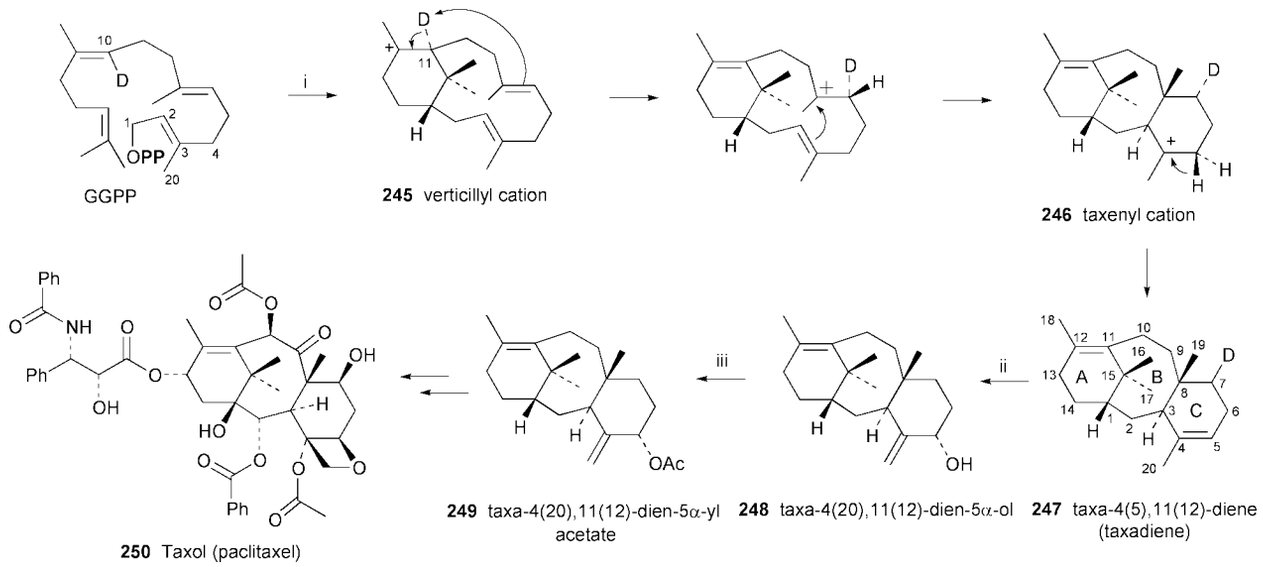
soluble fusion protein in *E. coli* has recently been reported.[206] The protein resembles several terpene cyclases, and shows the typical aspartate-rich DDTID region spanning amino acids 355–359. Seven mutants were prepared by site-specific mutagenesis, establishing the requirement for Asp-355 and Asp-356, whilst Asp-359 was not essential. In a coupled reaction using IPP isomerase, GGPP synthase, and casbene synthase, ^{13}C-labelled casbene has been synthesized in more than 80% yield from [^{13}C]-IPP.[207]

The diterpenoid portion of the important anticancer drug Taxol (paclitaxel) **250** is formed from GGPP *via* the hydrocarbon taxadiene **247**. Taxadiene synthase from *Taxus* species catalyses cyclization of GGPP to taxadiene and earlier studies have indicated the mechanism involves ionization and cyclization to a transient verticillyl intermediate **245**, which is proposed to have the 11*R* configuration to allow intramolecular transfer of the C-11 proton to C-7 to initiate transannular ring closure to the taxenyl cation **246**, followed by deprotonation (Scheme 58). The mechanism of the cyclization and of this crucial hydrogen migration have been probed further by incubation of a recombinant truncated (see below) *T. brevifolia* taxadiene synthase with specifically deuteriated substrates.[208] The stereochemistry of the C-5 deprotonation step was established to involve loss of the β proton, originally the 4-*pro*-R proton of GGPP. Incubation of the enzyme with [10-^{2}H$_1$]GGPP showed that essentially all this label was transferred to C-7, consistent with the previously observed transfer of hydride from C-11 of the verticillyl cation intermediate. The label was deduced by 1D DPFGSE-TOCSY NMR to reside in the 7α position. Thus, the proton migration occurs from the same face of the substrate. Modelling studies then suggested that an unassisted intramolecular transfer of this proton is the most plausible mechanism for the final ring closure step. Thus, the C-7/C-8 olefine serves as the Bronsted base that quenches the carbocation at C-12, and no active site enzyme base needs to be invoked for the process. To achieve this conversion of the verticill-12-yl cation into the isomeric verticill-8-yl cation, a conformational twist in the 12-membered ring is required, which also brings the C-3/C-4 double bond to the vicinity of the C-8 cation for the final ring formation.

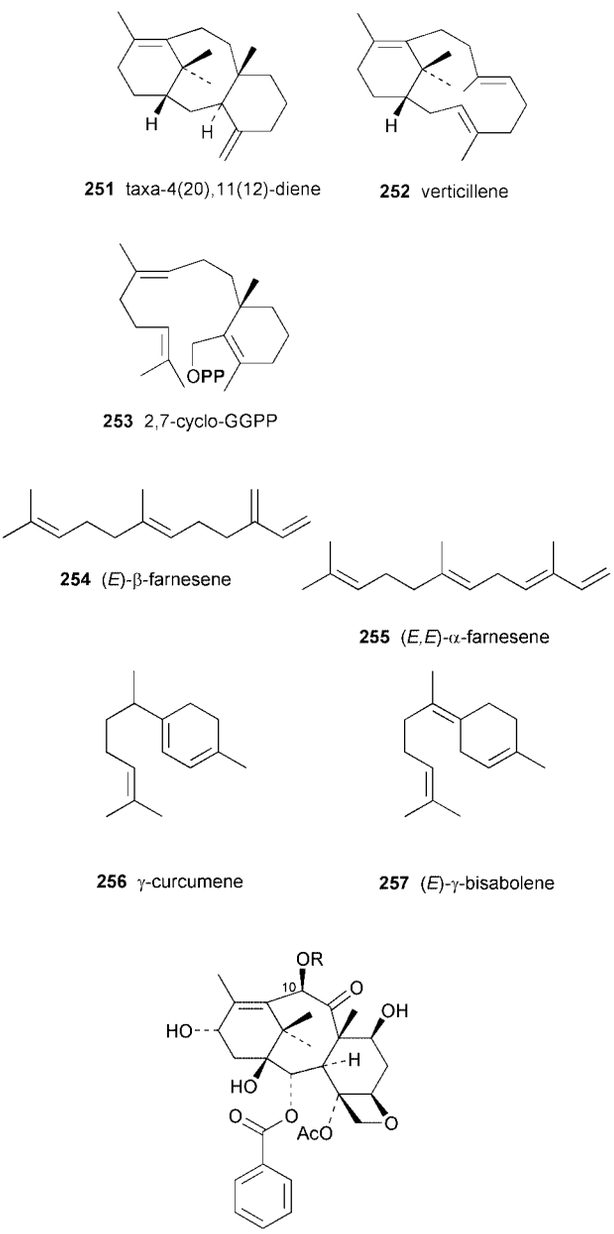
These experiments were achievable because of the availability of sufficient amounts of recombinant enzyme in a truncated form. Taxadiene synthase is translated as a preprotein bearing an *N*-terminal targeting sequence for localization to and processing in the plasmids. However, in high level expression systems, this transit peptide causes purification problems, and can also lead to catalytic impairment. In the absence of definite information about the transit peptide–mature enzyme cleavage site, a series of *N*-terminally truncated enzymes was prepared by expression of the corresponding cDNAs.[209] Deletion of up to 79 residues yielded functional protein, whereas deletion of 93 or more residues eliminated activity completely. A 60 amino acid deletion proved the most effective. This enzyme produced taxadiene as the major product (94%) along with small amounts of the isomeric taxa-4(20),11(12)-diene **251** (5%) and verticillene **252** (1%). The enzyme failed to convert 2,7-cyclogeranylgeranyl diphosphate **253**, supporting macrocyclization to the verticillyl intermediate, rather than an alternative initial formation of the C ring. In an independent study,[210] a truncated taxadiene synthase 78 residues shorter than the wild-type protein was found to produce taxadiene **247** (87%) and the isomer **251** (13%) from GGPP. However, this protein would also utilize FPP, giving a mixture of four sesquiterpenes, characterized as (*E*)-β-farnesene **254**, (*E,E*)-α-farnesene **255**, γ-curcumene **256**, and (*E*)-γ-bisabolene **257**. Homology in the *C*-terminal domain with (*E*)-α-bisabolene synthase from *Abies grandis* may partly explain the cross reactivity towards GGPP and FPP.

In the biosynthesis of taxol, taxadiene is hydroxylated at position 5α by a cytochrome P450-dependent hydroxylase giving taxa-4(20),11(12)-dien-5α-ol **248**, followed by acetylation of this hydroxy group giving **249** prior to further modifications (Scheme 58). A soluble *O*-acetyltransferase has been demonstrated in extracts of *Taxus canadensis* and *T. cuspidata* cells induced with methyl jasmonate to produce taxol, and partially purified.[211] The enzyme showed high selectivity and affinity for the taxadienol and acetyl-CoA substrates, and would not acetylate more adavanced taxol precursors such as 10-deacetylbaccatin III **258** or baccatin III **259**. A full length cDNA encoding this transferase has been isolated and expressed in *E. coli* to yield a functional enzyme.[212] This enzyme shares considerable homology with other known acyl transferases of plant origin.

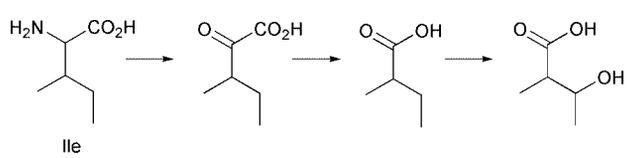
Oxygenation reactions and esterifications during the later stages of taxol biosynthesis have yet to be clarified. During formation of the related taxoids taxuyunnanine C **260** and yunnanxane **261** in *Taxus chinensis* cell cultures it has been demonstrated by feeding [^{13}C]glucose and $^{18}O_2$ that all oxygen atoms attached to the taxoid ring system are derived from



Scheme 58 *Enzymes*: i, taxadiene synthase; ii, taxadiene hydroxylase; iii, taxadienol *O*-acetyltransferase.



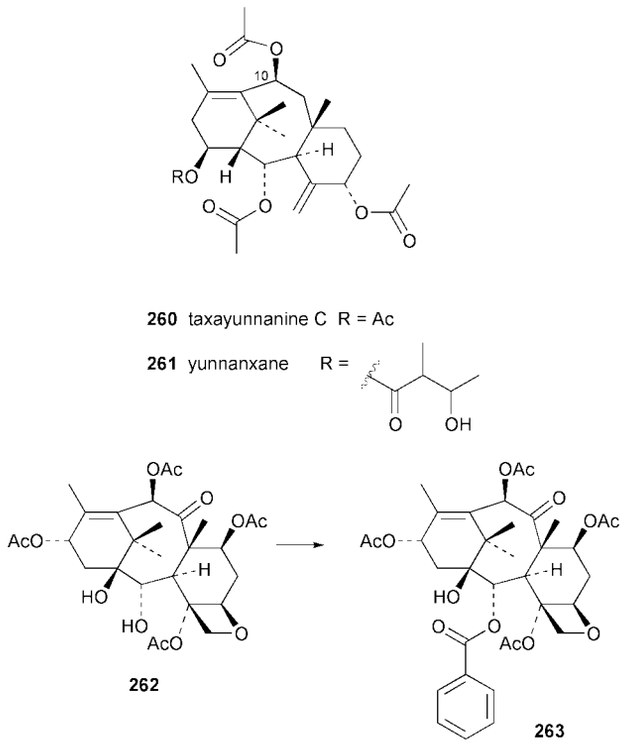
molecular oxygen.[213] The introduction of oxygens is thus likely to be catalysed by cytochrome P450-type monooxygenases. In addition, the acyl side-chain at position 14 of yunnanxane **261** also carried a hydroxy group derived from molecular oxygen. This leads to the conclusion that the 2-methyl-3-hydroxybutyryl ester group of **261** is probably derived from 2-methybutyric acid, which is generally formed in plants *via* isoleucine (Scheme 59). An *O*-acetyltransferase enzyme from a cytosolic fraction of



Scheme 59

Taxus chinensis cell suspension cultures has been purified to apparent homogeneity.[214] The purified protein *O*-acetylated 10-deacetyltaxuyunnanine C, giving taxuyunnanine C **260**, and was highly regio- and stereo-specific towards the 10β-hydroxy group of a number of taxane substrates. It was also active towards 10-deacetylbaccatin III **258** and congeners, which have significant structural differences to the *T. chinensis* metabolites. A partially purified *O*-acetyltransferase which acetylates the 10-hydroxy group of 10-deacetylbaccatin III **258** yielding baccatin III **259** has been obtained from leaves and cell suspension cultures of *Taxus cuspidata*.[215] The enzyme did not significantly catalyse acetylation of 10-deacetyltaxol to taxol. A cDNA clone for the *Taxus cuspidata O*-acetyltransferase has been isolated and expressed in *E. coli*[216] The recombinant acetyltransferase is regiospecific toward the 10-hydroxy group of taxanes, and does not acetylate any of the other three hydroxyls, nor does it acetylate the hydroxy group of taxa-4(20),11(12)-dien-5α-ol **248**. Amino acid sequence comparison of this enzyme with taxadienol-5-*O*-acetyltransferase and other known acyltransferases of plant origin indicates a significant degree of homology (80% and 64–67% respectively).

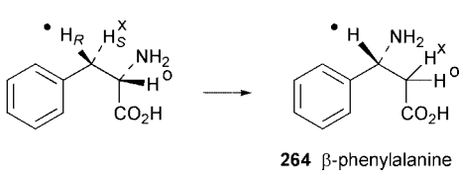
A cDNA clone encoding a taxane 2α-*O*-benzoyltransferase has been isolated from *Taxus cuspidata*, and used to produce a recombinant protein.[217] This enzyme catalyses the benzoylation of 2-debenzoyl-7,13-diacetylbaccatin III **262** to 7,13-diacetylbaccatin III **263** using benzoyl-CoA as acyl donor (Scheme 60). At present, the natural substrate for this enzyme has not been established, but it would appear to function in a late step in the taxol biosynthetic pathway. It does not benzoylate any of the hydroxyl groups in 10-deacetylbaccatin III **258**, nor either of



Scheme 60

the hydroxy groups in the less elaborated taxa-4(20),11(12)-dien-5α-ol **248**.

The phenylisoserine side-chain of taxol is known to be formed from phenylalanine *via* β-phenylalanine **264**. A phenylalanine aminomutase enzyme catalysing this conversion has now been detected in cell-free extracts of *Taxus brevifolia*.[218] The product was established to have the *R*-configuration, the same as in taxol, though *T. brevifolia* tissues are known to contain both isomers. Labelling studies showed the nitrogen migration is strictly intramolecular, the *pro-R* hydrogen of *S*-phenylalanine remains at C-3 of β-phenylalanine, whilst the *pro*-3*S* hydrogen migrates to C-2 but this migration is partly intermolecular (Scheme 61). The substrate also undergoes some

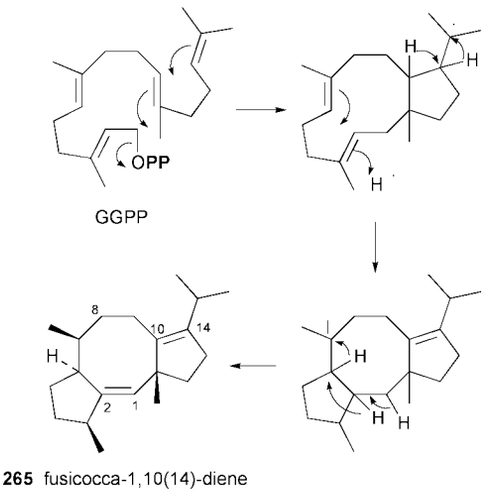


Scheme 61 *Enzyme*: phenylalanine aminomutase.

hydrogen exchange at C-2, probably through an α-amino acid racemase. Therefore, the departing *pro-S* hydrogen is replaced by the migrating nitrogen with retention of configuration at C-3, suggesting that the mechanism of action of this plant enzyme is different from that of the known microbial aminomutases.

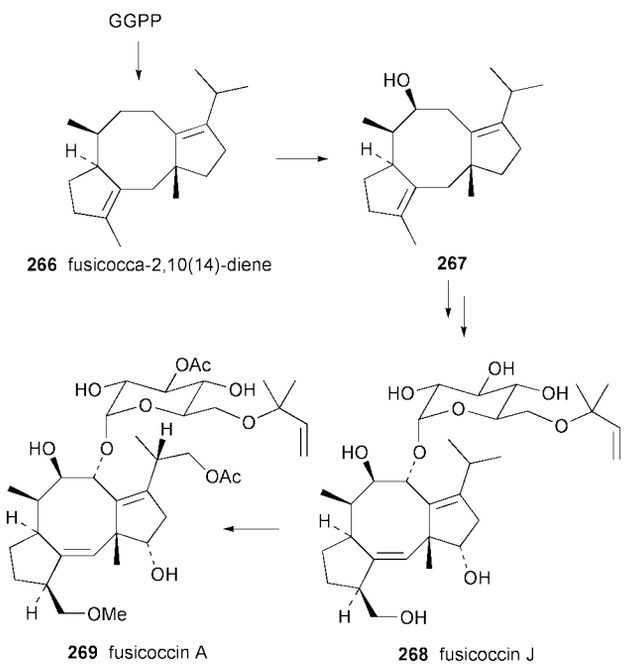
The biosynthesis of taxol and related compounds has been reviewed in recent articles.[219,220]

Earlier studies on fusicoccin biosynthesis had postulated fusicocca-1,10(14)-diene **265** to be a hydrocarbon intermediate, involving hydride shifts as shown (Scheme 62). However, the more recent isolation of fusicocca-2,10(14)-diene **266** from *Phomopsis* (*Fusicoccum*) *amygdali* led to consideration of this isomer as an intermediate, and this now appears to be the hydrocarbon involved.[221] Thus cultures of *P. amygdali* were able to transform the hydrophilic hydroxylated derivative **270** to the triol **271**, whereas the corresponding 1,2 alkene was not metabolized. Further, the 8β-hydroxy derivative **267** was a satisfactory precursor of fusicoccin J **268**, and suggested that 8β-hydroxylation might be the first step in the conversion. In



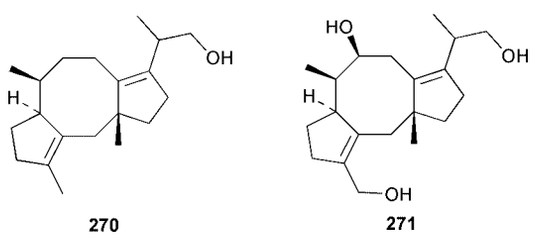
Scheme 62

additional experiments, **267** was isolated from *P. amygdali* and labelled fusicocca-2,10(14)-diene **266** was converted by the fungus into both **268** and fusicoccin A **269**.[222] A pathway from GGPP to **266**, followed by non-allylic 8β-hydroxylation to **267**, is now postulated as the early part of the pathway (Scheme 63).

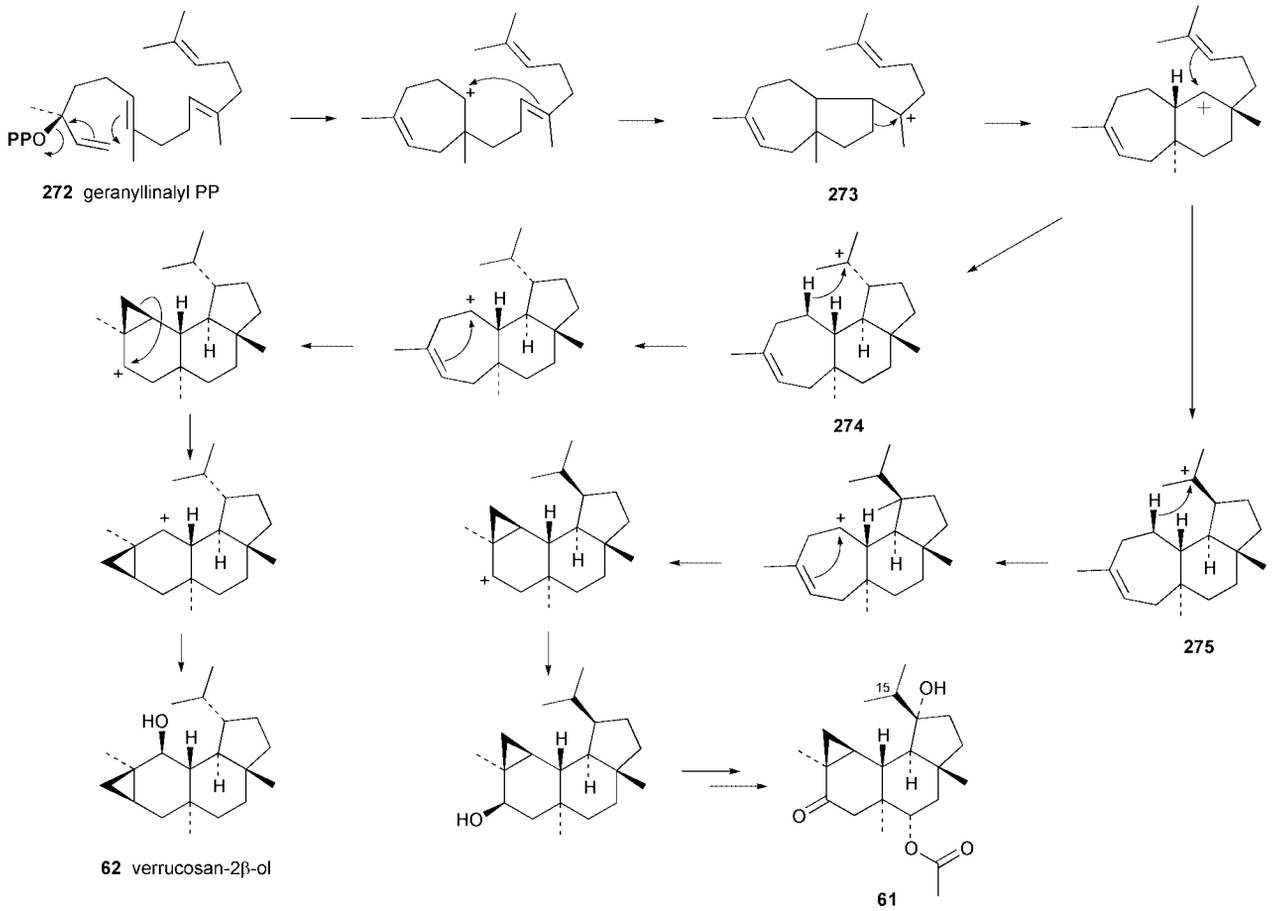


Scheme 63

This revision of the double bond position in the intermediate hydrocarbon is reminiscent of a similar occasion when the taxadiene intermediate in taxol biosynthesis was shown to be different from that postulated according to structural similarity with the final product.



The diterpene verrucosan-2β-ol **262** from the green phototrophic eubacterium *Chloroflexus aurantiacus* is biosynthesized *via* mevalonate.[88] In a retrobiosynthetic analysis, labelling

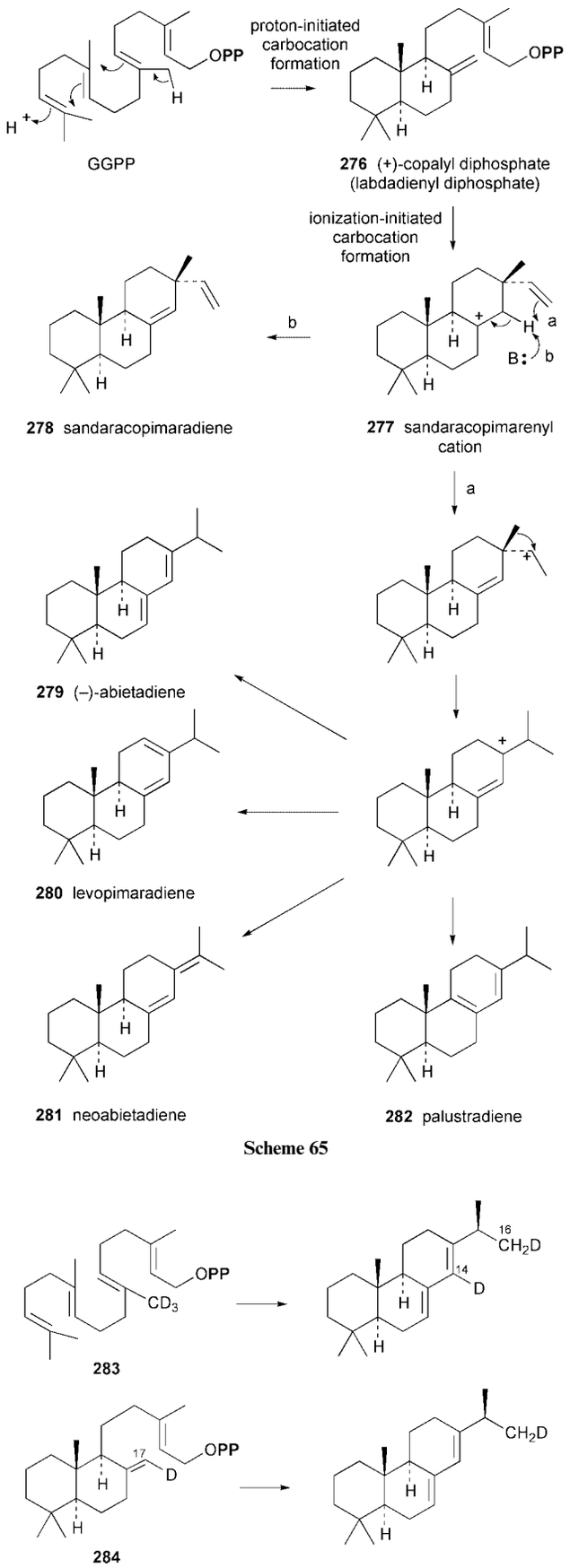


Scheme 64

patterns in verrucosan-2β-ol formed from single- and double-labelled [^{13}C]acetate precursors were compared with labelling patterns of metabolic intermediates such as acetyl-CoA, pyruvate, and glyceraldehyde 3-phosphate, which could be deduced from patterns observed in protein-derived amino acids. By this means, mevalonate rather than DXP was shown to be a precursor, and, based on the labelling, a possible pathway (Scheme 64) was proposed. The cyclization process is initiated by solvolysis of geranyllinalyl diphosphate **272** leading to a bicyclic intermediate **273**, which undergoes a 1,2-rearrangement, to then generate a tricyclic system **274**. Subsequent transformations are accommodated by a 1,5-hydride transfer, then formation of a cyclopropane ring. A rearrangement of the first-formed cyclopropylcarbinyl ion allows production of the required carbon skeleton. In marked contrast to the biosynthesis of **62** from mevalonate, similar retrobiosynthetic studies on the formation of the structurally related 8α-acetoxy-13α-hydroxy-5-oxo-13-*epi*-neoverrucosane **61** in the liverwort *Fossombronia alaskana* have shown it to be deoxyxylulose phosphate-derived.[87] The proposed pathway (Scheme 64) shares the early steps of the verrucosan-2β-ol pathway, with the *epi*-configuration at C-13 being generated during formation of the tricyclic system **275**. The neoverrucosane skeleton is analogous to the first-formed cyclopropylcarbinyl ion of the verrucosan-2β-ol sequence. The postulated 1,5-hydride shift was confirmed by an incorporation experiment using [6,6-$^{2}H_2$]glucose. This resulted in a product with a migrated deuterium at position 15.

Resin acids such as abietic acid are major components of the oleoresin synthesized by grand fir (*Abies grandis*) as a defensive secretion against insect and pathogen attack. The diterpene (−)-abietadiene **279**, obtained from cyclization of GGPP by the action of abietadiene synthase, is the first formed product on the pathway to abietic acid and related structures. A cDNA encoding abietadiene synthase has been isolated from grand fir and the heterologously expressed bifunctional enzyme shown to catalyse both the protonation-initiated cyclization of GGPP to the intermediate (+)-copalyl diphosphate **276** and the ionization-dependent cyclization of (+)-copalyl PP, *via* a pimaraenyl cation **277**, to olefin end-products (see ref. 1). Since this recombinant protein proved unsuitable for detailed study, a truncation series has been constructed to delete the targeting sequence and prepare a pseudomature form of the enzyme.[223] A pseudomature synthase having 84 residues deleted from the preprotein converted GGPP and (+)-copalyl PP into a nearly equal mixture of abietadiene **279**, levopimaradiene **280**, and neoabietadiene **281**, as indicated in Scheme 65, as well as three minor products, including sandaracopimaradiene **278** and palustradiene **282**. (−)-Copalyl PP was not utilized. Kinetic evaluation with GGPP and (+)-copalyl PP provided evidence for two functionally distinct active sites, one for the cyclization of GGPP to (+)-copalyl PP, and a second for the cyclization of (+)-copalyl PP to diterpene end products. The second cyclization proves to be the rate limiting step. The mechanism of the cyclizations catalysed by the recombinant enzyme has been examined further by the use of deuteriated substrates.[224] These demonstrated that [19-$^{2}H_3$]GGPP **283** was transformed with the retention of two labels, located at positions 14 and 16, and label from (*E*)-[17-$^{2}H_1$]copalyl PP **284** was subsequently located at position 16 (Scheme 66). Thus, abietadiene synthase effects intramolecular proton transfer from position 17 of copalyl PP to the terminal carbon of the vinyl group which becomes the *pro-S* methyl, and the methyl migration occurs to the *si* face at C-15 of a putative pimara-8(14),15-diene **285** intermediate. Two plausible mechanisms for this novel cyclization–rearrangement are shown in Scheme 67, involving either intramolecular hydrogen transfer (path A), or enzyme-mediated proton elimination, followed by reincorporation of this proton (path B).

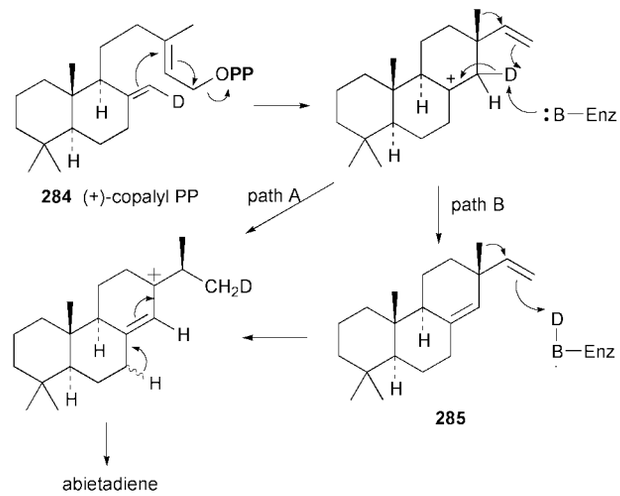
The aphidicolane skeleton of aphidicolin **290** is the result of further cyclization of a 9β-labdadienyl system **286** with additional rearrangement as shown in Scheme 68. A sequence



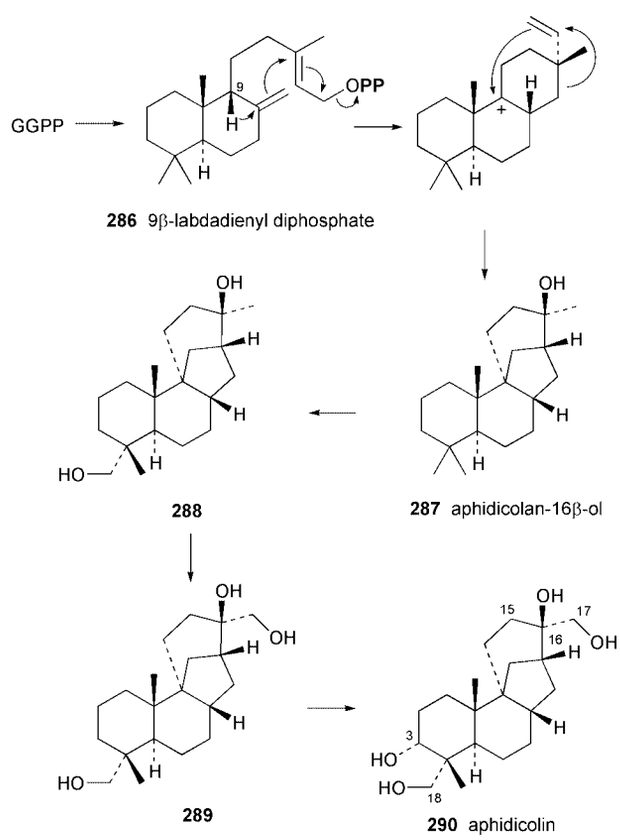
Scheme 65

Scheme 66

of hydroxylations then converts aphidicolan-16β-ol **287** into aphidicolin. Treatment of cultures of the aphidicolin producer *Phoma betae* with inhibitors of cytochrome P-450 enzymes caused accumulation of hydroxylated intermediates **287**, **288**, and **289**.[225] Further experiments demonstrated that ^{14}C-labelled



Scheme 67

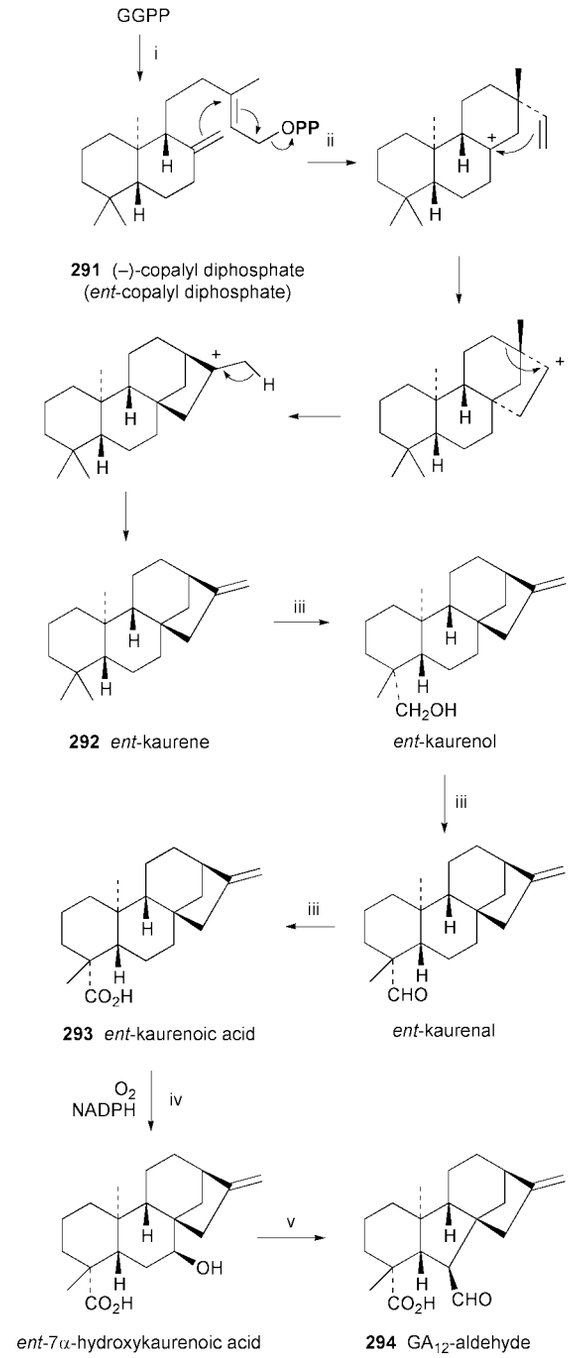


Scheme 68

samples of **287** were converted into aphidicolin by the cultures, or into **289** in the presence of a P-450 inhibitor.

Studies on the biosynthesis and metabolism of the gibberellins (GAs) always account for a considerable proportion of the diterpenoid research literature. These compounds, with over 120 different structures now known, play a significant role as plant growth hormones, and have their origins in *ent*-kaurene **292**. *ent*-Kaurene is produced from GGPP by the action of two enzymes, firstly copalyl PP synthase (*ent*-kaurene synthase A), which cyclizes the substrate to (−)-copalyl PP **291** in a protonation-initiated cyclization, and then kaurene synthase (*ent*-kaurene synthase B), which accomplishes the further cyclization and subsequent rearrangement initiated by loss of diphosphate (Scheme 69).

In pumpkin (*Cucurbita maxima*), two cDNAs encoding copalyl PP synthase have been identified.[226] However, in the GA-producing fungus *Phaeosphaeria* sp. L487, a bifunctional *ent*-kaurene synthase catalyses the two-step cyclization. Kinetic



Scheme 69 *Enzymes*: i, copalyl diphosphate synthase (*ent*-kaurene synthase A); ii, kaurene synthase (*ent*-kaurene synthase B); iii, *ent*-kaurene oxidase; iv, *ent*-kaurenoic acid 7β-hydroxylase; v, GA_{12}-aldehyde synthase.

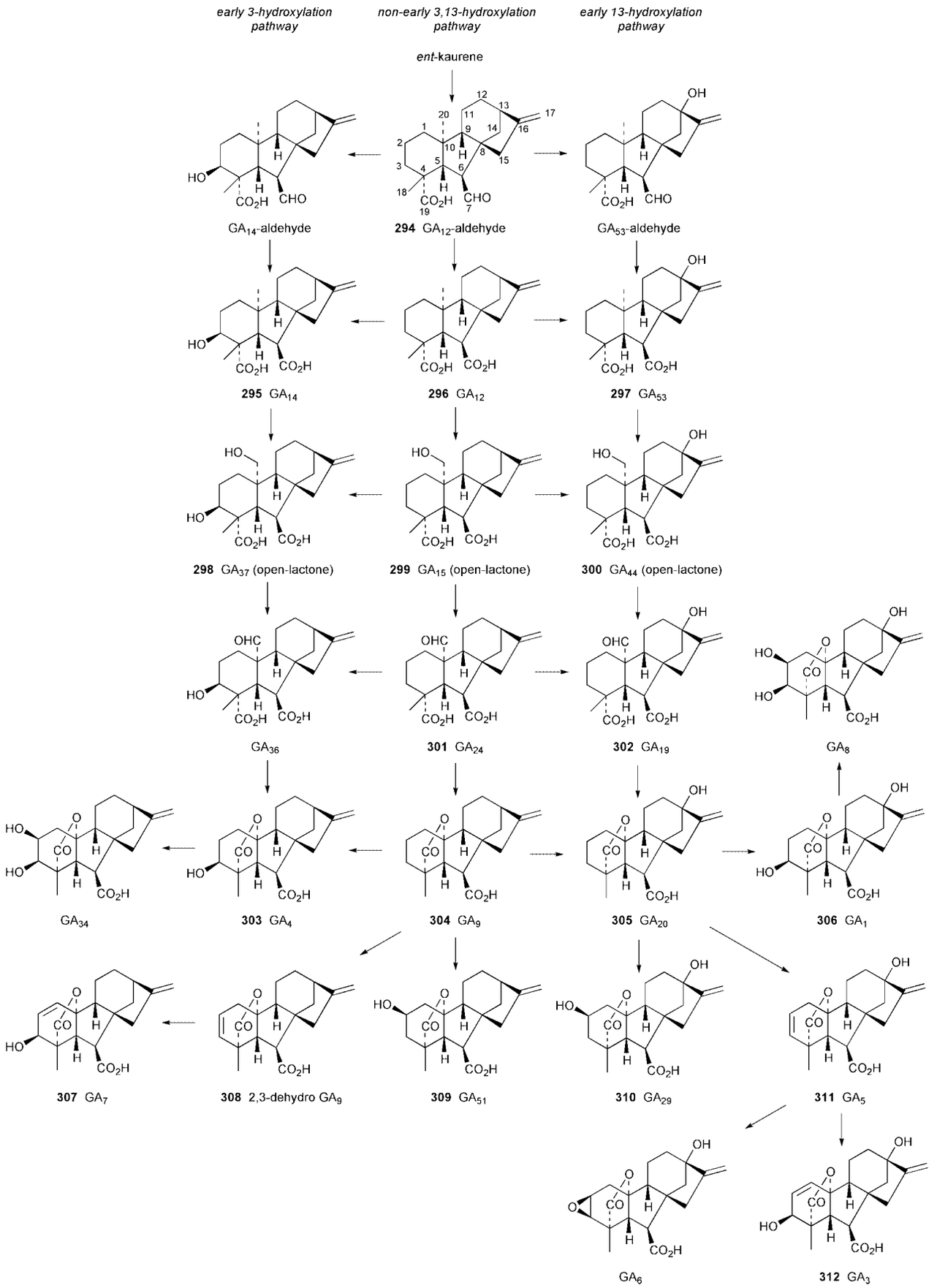
analysis of a recombinant protein indicated it had a higher affinity for copalyl PP than for GGPP, and that *ent*-kaurene production may be limited by the KS activity.[227] Site-directed mutagenesis of aspartate-rich motifs showed that the ^{318}DVDD motif near the *N*-terminus and the ^{656}DEFFE motif near the *C*-terminus may be part of the active site for the copalyl PP synthase and KS reactions, respectively. Another aspartate-rich motif ^{132}DDVLD near the *N*-terminus is thought to be involved in both reactions. Functional analysis of truncated mutants showed that an *N*-terminal 59 kDa polypeptide catalysed the copalyl PP synthase reaction, and a *C*-terminal 66 kDa polypeptide the KS reaction. A full-length cDNA encoding a bifunctional *ent*-kaurene synthase has also been isolated from *Gibberella fujikuroi* and subsequently expressed in *E. coli*.[228] The deduced amino acid sequences for the *Gibberella* and *Phaeosphaeria* proteins showed high (45%) similarity.

The known sequence from *ent*-kaurene **292** to GA_{12}-aldehyde **294** is shown in Scheme 69, and involves cytochrome P-450-dependent enzymes. The first of these is *ent*-kaurene oxidase, and studies in *Arabidopsis thaliana* have identified the corresponding gene *GA3*.[229] The deduced amino acid sequence of the protein suggest this is from a new class of cytochrome P-450 enzymes, sharing at best 28% identity with known P-450 sequences. The gene was subsequently expressed in *Saccharomyces cerevisiae* and the transformed yeast cells possessed the ability to metabolize *ent*-kaurene to *ent*-kaurenoic acid **293**, demonstrating that the single enzyme *ent*-kaurene oxidase catalyses the three oxidation steps converting the methyl into a carboxyl.[230]

Scheme 70 shows the usual interrelationships of the most commonly encountered plant gibberellins as established from various experimental data. The stepwise conversion of GA_{12}-aldehyde **294** may proceed along three main routes: the early 13-hydroxylation pathway *via* GA_{53} **297** and GA_{44} **300**, the early 3-hydroxylation pathway through GA_{14} **295** and GA_{37} **298**, or by the non-early 3,13-hydroxylation pathway which involves GA_{12} **296** and GA_{15} **299**. However, many variations to this generalization are encountered. Studies in seedlings of rice (*Oryza sativa*) have established the involvement of the early 13-hydroxylation pathway.[231] Feeding experiments with labelled GA_{53} **297**, GA_{44} **300** and GA_{19} **302** implicated the sequence $GA_{53} \longrightarrow GA_{44} \longrightarrow GA_{19} \longrightarrow GA_{20}$ **305** and the presence of these as endogenous metabolites was established. Previous metabolic studies had also indicated the $GA_{20} \longrightarrow GA_1$ **306** transformation. Administration of GA_9 **304** to seedlings of normal and dwarf maize (*Zea mays*) showed the major metabolite to be GA_{20} **305**.[232] A number of minor metabolites were also identified, but the $GA_9 \longrightarrow GA_{20}$ transformation demonstrates the possibility of late convergence of the biosynthetic branches, the early 13-hydroxylation pathway and the non-early 3,13-hydroxylation pathway. Further evidence for pathway convergence *via* a metabolic grid in maize was obtained from feeding experiments with other labelled substrates.[233] Major metabolites from GA_{15} **299** were GA_{44} **300**, GA_{19} **302**, and GA_{20} **305**, whilst GA_{24} **301** gave GA_{19} **302**, and GA_{20} **305**. GA_3 **312** was one of several metabolites from GA_7 **307**, and 2,3-dehydro-GA_9 **308** gave GA_5 **311**. However, the inability to detect the sequence $GA_{12} \longrightarrow GA_{15} \longrightarrow GA_{24} \longrightarrow GA_9$ indicates the non-early 3,13-hydroxylation pathway probably plays a minor role in maize. cDNA corresponding to the *GA4* gene of *Arabidopsis thaliana* has been expressed in *E. coli* to give protein that converted GA_9 **304** into GA_4 **303** and GA_{20} **305** into GA_1 **306**.[234] GA_9 was the preferred substrate. This 3β-hydroxylase was regiospecific, with no indication of 2β-hydroxylation or of 2,3-desaturation. Other gibberellins were also hydroxylated, preferred substrates containing a polar bridge between C-4 and C-10, and 13-deoxy GAs being preferred to 13-hydroxylated compounds.

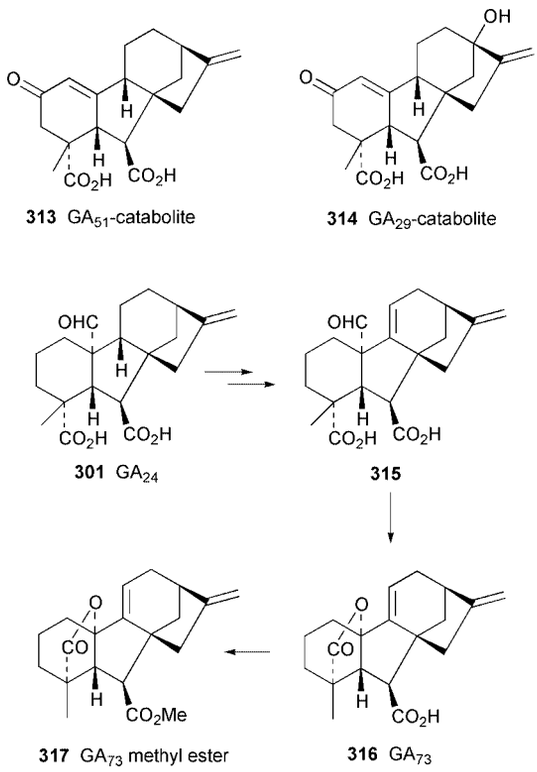
In gibberellin biosynthesis, a 20-oxidase catalyses the oxidation and elimination of C-20 to give the biologically active C_{19} GAs. A cDNA encoding 20-oxidase has been isolated from *Arabidopsis* seedlings, and overexpressed in transgenic *Arabidopsis* plants.[235] The transgenic plants displayed physical symptoms of GA overproduction, with increased levels of endogenous GA_1 **306**, GA_9 **304** and GA_{20} **305** being detected. Levels of GA_4 **303**, the predominantly active GA in *Arabidopsis*, were not affected. A cDNA clone from water-melon (*Citrullus lanatus*) that shows significant amino acid homology with GA 20-oxidases has been isolated and expressed in *E. coli* as a fusion protein.[236] This protein oxidized GA_{12} **296** to GA_9 **304**.

A major catabolic pathway for GAs is initiated by 2β-hydroxylation, a reaction catalysed by a 2-oxoglutarate-dependent dioxygenase. A 2β-hydroxylase cDNA clone has been isolated from runner bean (*Phaseolus coccineus*) and expressed in *E. coli*.[237] The expressed protein catalysed the GA_9 **304** $\longrightarrow GA_{51}$ **309** conversion, with additional production of a GA_{51}-catabolite **313** having further oxidation at C-2. The



Scheme 70

enzyme is thus multifunctional and is best described as a 2-oxidase. Three related cDNAs also encoded GA 2-oxidases. A GA 2-oxidase cDNA has also been cloned from pea (*Pisum sativum*) seeds.[238] Bacterial cultures expressing the protein converted GA_1 **306**, GA_4 **303**, GA_9 **304**, and GA_{20} **305** into their corresponding 2β-hydroxy derivatives. GA_9 **304** and GA_{20} **305** also yielded GA_{51}-catabolite **313** and GA_{29}-catabolite **314** respectively.



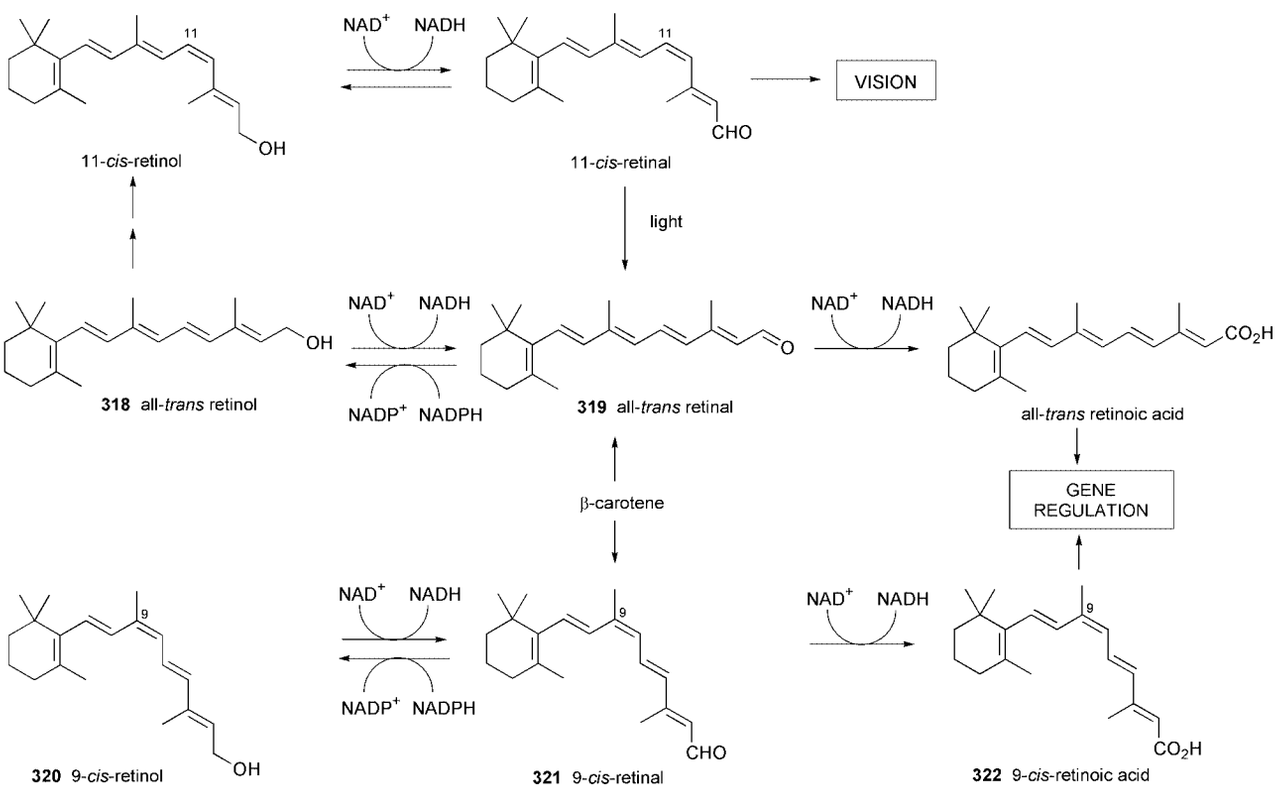
Scheme 71

Several fern species produce gibberellin-like substances termed antheridiogens. Earlier studies in *Lygodium* ferns had shown that GA_{24} **301** is a biosynthetic precursor of GA_{73} methyl ester **317** (see ref. 1) (Scheme 71). A realistic intermediate in this process is 9,11-didehydro-GA_{24} **315**, and this compound has been synthesized in labelled form and tested as a precursor of GA_{73} methyl ester in prothalli of *L. flexuosum* and *L. circinnatum*.[239] In both species, substantial incorporation was obtained. The absence of the methyl ester of **315** after the feedings supports a pathway in which the carboxyl group is methylated after conversion of GA_{24} **301** into GA_{73} **316** (Scheme 71).

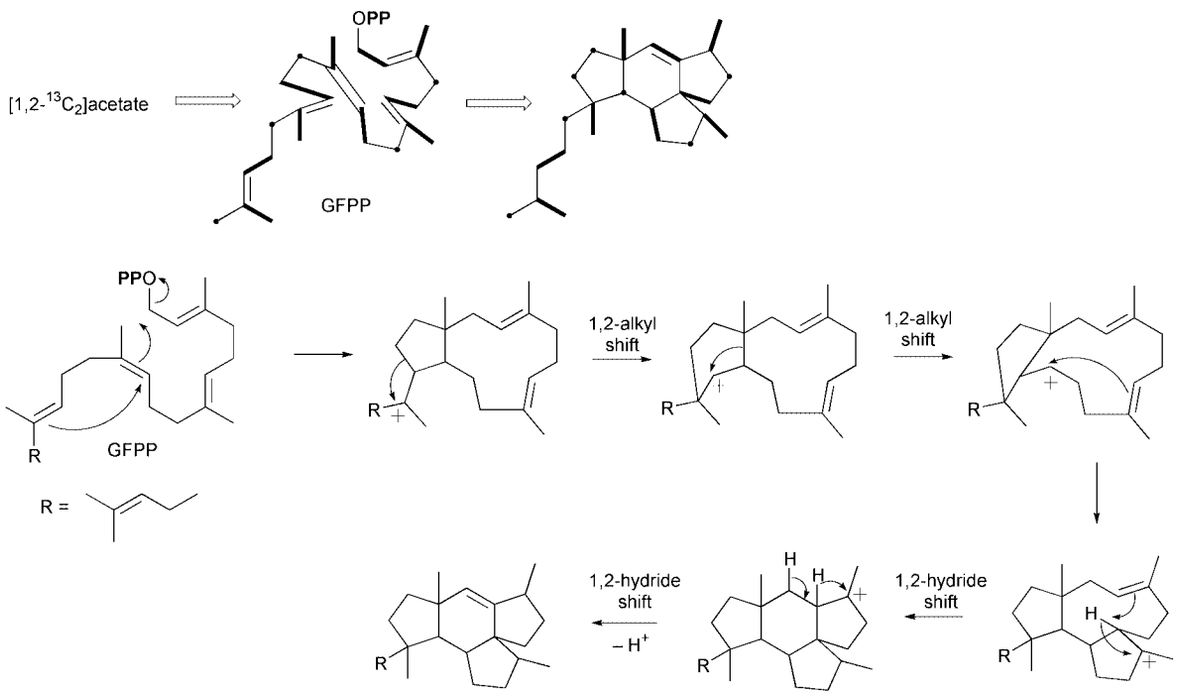
Two useful reviews on the molecular biology of gibberellin biosynthesis have been published.[240,241]

Retinoids possess a diterpenoid skeleton, but are actually derived by oxidative cleavage of a C_{40} carotenoid, primarily β-carotene. Retinoids play two main functions in biology, retinal being involved in vision as the chromophore bound to opsins, whilst retinoic acid is the activating ligand of nuclear transcription factors. Identified metabolic interconversions involving double bond stereochemistry and oxidation state of the functional group are shown in Scheme 72. The families of retinoid dehydrogenases involved in these interconversions have been reviewed.[242] A stereospecific 9-*cis*-retinol dehydrogenase has been characterized in mouse embryonic tissues, catalysing oxidation of 9-*cis*-retinol **320** into 9-*cis*-retinal **321**.[243] Human hepatic cell homogenates can convert all-*trans*-retinol **318** into 9-*cis*-retinal **321**, suggesting additional pathways for 9-*cis*-retinoic acid **322** synthesis in specialized tissues.[244] Recombinant murine 11-*cis*-retinol dehydrogenase has been found capable of oxidizing and reducing 9-*cis*-, 11-*cis*-, and 13-*cis*-isomers of retinol and retinal, respectively.[245] This visual cycle enzyme is also present in non-retinal ocular and non-ocular tissues, and may therefore play some role additional to its visual one. Carotenoid-depleted fruit flies, *Drosophila melanogaster*, produce very much less (3*S*)-hydroxyretinal **323** than normal flies. Feeding studies have shown that all-*trans*-retinal **319** can serve as a precursor of this metabolite, being first converted into (3*R*)-3-hydroxyretinal **324** which is then isomerized to the 3*S*-isomer.[246] Addition of NADPH enhanced 3-hydroxyretinal production in a head homogenate, whilst CO inhibited it, suggesting the hydroxylation is cytochrome P-450-dependent.

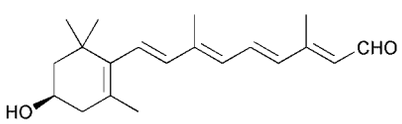
The diterpenoid crocin **327** is a digentiobiosyl ester, and the major pigment in *Crocus sativus* stigmata (saffron). The aglycone crocetin **325** is likely to be a degraded carotenoid. A glycosylating enzyme from *C. sativus* cell cultures involved in crocin biosynthesis has been partially purified.[247] The enzyme utilizes UDPglucose to form the diglucosyl ester **326**.



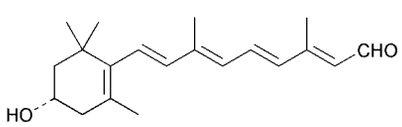
Scheme 72



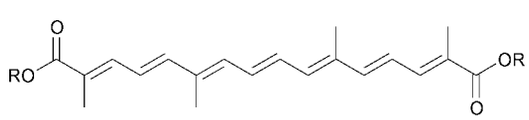
Scheme 73



323 (3*S*)-3-hydroxyretinal



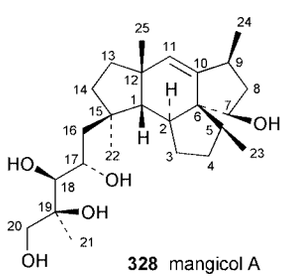
324 (3*R*)-3-hydroxyretinal



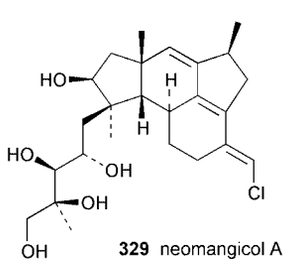
325 crocetin R = H
326 R = Glc
327 crocin R = Glc $^{6\rightarrow1}$Glc

9 Sesterterpenoids

Many examples of this least common group of natural terpenoids are known, but biosynthetic studies are rather rare. A group of structurally novel sesterterpene polyols, the mangicols, has been characterized from a marine fungus, tentatively identified as *Fusarium heterosporum*.[248] Feeding experiments with sodium [1,2-$^{13}C_2$]- and [1-^{13}C]-acetates were conducted to investigate their biosynthetic origins. Two members of the group were isolated, mangicol A **328** and neomangicol A **329**, and the results were consistent with biosynthesis from the C_{25} precursor geranylfarnesyl diphosphate. All five methyl groups in mangicol A were incorporated intact with their acetate partners, precluding any methyl migrations. Three of the isoprene units were incorporated intact. The remaining two isoprene units were rearranged such that C-1 appeared to have been inserted into an isoprene composed of C-13, C-12, C-25, C-2, and C-3. A hypothetical biosynthesis accounting for these observations is proposed (Scheme 73). The initial cyclization to give an 11-membered ring is analogous to humulene ring formation. Two 1,2-alkyl shifts account for the rearrangement in the carbon skeleton. Remaining steps are simple cation-induced ring closures and 1,2-hydride transfers. The labelling pattern in neomangicol A was nearly identical to that in mangicol A, but was not contiguous through C-6–C-8, indicating the neomangicol skeleton is derived from the mangicol skeleton. A feasible explanation is a 1,2-alkyl shift from a C-7 carbocation in the mangicol skeleton to generate a tertiary cation at C-6. Subsequent modifications include halogenation, chlorination in the case of neomangicol A, bromination for neomangicol B.



328 mangicol A



329 neomangicol A

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