The biosynthesis of C_5–C_{25} terpenoid compounds

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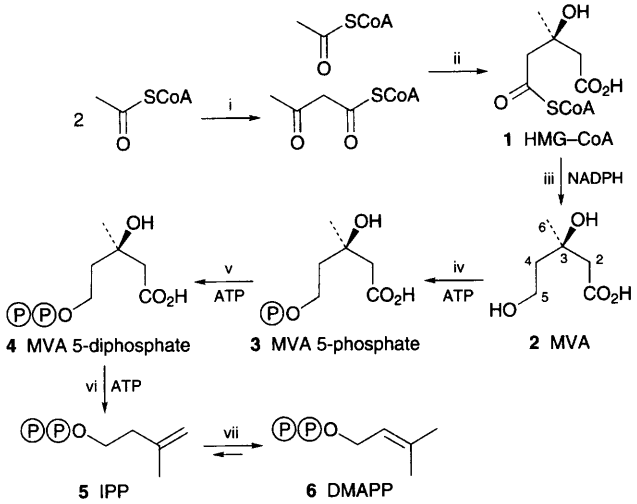
1 Introduction

This report reviews the literature that was published during the period 1993–1995 on the biosynthesis of terpenoids in the range C_5–C_{25}, and continues the coverage described in Volume 12 of *Natural Product Reports*.[1] This three-year report brings coverage effectively up to date, and hopefully, future reports will now be produced on a more regular basis. This report describes the biosynthetic pathways leading to those terpenoids smaller than C_{30} (C_{30} and larger terpenoids are covered separately under triterpenoids and steroids, and carotenoids), the enzymes and enzyme mechanisms involved, and information about genes encoding for these enzymes. A number of specific aspects more appropriate to other journals are not included. 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 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.

Some recent review articles describe general aspects of terpenoid biosynthesis and metabolism. These include: biosynthesis of mono-, sesqui- and di-terpenes,[2] new aspects of isoprenoid biosynthesis,[3,4] biochemistry and molecular biology of terpenoid metabolism,[5–7] sesquiterpene cyclases,[8] and environmental effects on isoprenoid biosynthesis.[9] Volume 9 in the series *Methods in Plant Biochemistry*[10] is devoted to 'Enzymes of Secondary Metabolism' and contains several articles of interest; prenyltransferases and cyclases,[11] cytochrome P-450 terpene hydroxylases,[12] enzymes of gibberellin biosynthesis,[13] and abscisic acid metabolism.[14] A special issue of *Plant Growth Regulation* is devoted to gibberellins.[15]

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 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 latter mitochondrial enzyme is not considered further in this report. An avian recombinant HMG-CoA synthase has been obtained by cDNA cloning and expression in *E. coli*, the soluble enzyme



Scheme 1 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; vi, IPP isomerase

accounting for more than 20% of the total cellular protein.[16] The recombinant enzyme was identical to the normal wild-type enzyme. By directed mutagenesis, it was demonstrated that a cysteine residue Cys-129 was essential for formation of a covalent acetyl–enzyme reaction intermediate. A second reactive cysteine has also been implicated, but none of those at five other possible sites was crucial for the reaction.[17] Human enzyme has also been obtained by gene cloning and expression in *E. coli*.[18] Again, mutation of Cys-129 to Ser or Ala was shown to destroy the activity by disrupting the first catalytic step, enzyme acetylation by acetyl-CoA. Two HMG-CoA synthase genes have been identified in the insect *Blattella germanica*.[19,20] Both genes have been cloned and sequenced, and shown to have similar amino acid sequences to vertebrate enzymes. The two insect proteins had 69% identity at the amino acid level, but were expressed to different extents as the insect developed.

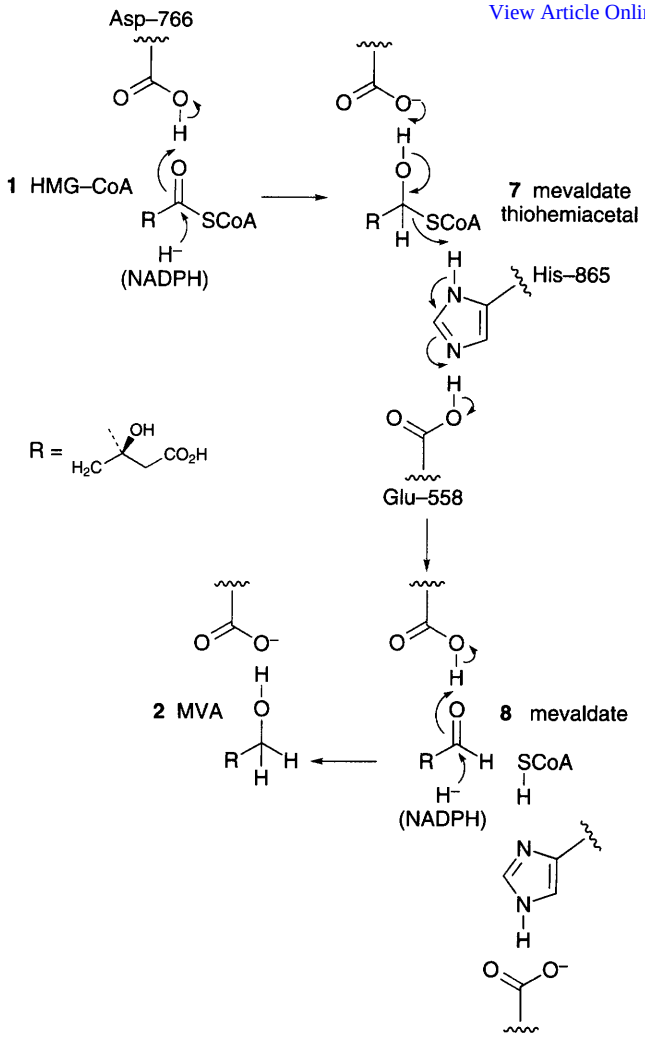
Studies of plant-derived HMG-CoA synthases have suggested that in radish (*Raphanus sativus*), both this activity and that responsible for acetoacetyl-CoA synthesis (acetoacetyl-CoA thiolase) are localized on a single polypeptide.[21,22] Thus, the soluble monomeric protein from radish membranes catalysed the two-step conversion of acetyl-CoA into HMG-CoA, and unlike yeast or animal systems, could not be resolved into separate activities. Activity was lost on purification, but could be reconstituted by the addition of Fe^{2+} chelates and quinone cofactors, especially pyrroloquinoline quinone. It is suggested that the reaction mechanism may involve radical formation by the redox couple Fe^{2+}/quinone, mimicking a natural cofactor system which has yet to be identified, and facilitating the unfavourable Claisen reaction. In *Catharanthus roseus*, a similar situation probably exists, and although the two activities acetoacetyl-CoA thiolase and HMG-CoA synthase were separately proved to be operating, they could not be separated, and a single protein may be involved.[23] A cytosolic HMG-CoA synthase preparation from rubber (*Hevea brasiliensis*) has been isolated; this preparation also utilized acetyl-CoA

without any requirement for an exogenous supply of acetoacetyl-CoA.[24] A cDNA from *Arabidopsis thaliana* that has been isolated and characterized showed a strong similarity in the deduced amino acid sequence with mammalian HMG-CoA synthase.[25] In marked contrast to the above studies, this HMG-CoA synthase was devoid of any acetoacetyl-Co thiolase activity.

The next enzyme on the mevalonic acid biosynthetic pathway is HMG-CoA reductase (HMGR) which catalyses the NADPH-dependent reduction of HMG-CoA to mevalonate (MVA) **2** (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. Recent reports on the isolation and sequencing of genes encoding HMGR include those from Syrian hamster,[26] the cockroach *Blattella germanica*,[27] fungi *Parasitella parasitica*, *Absidia glauca*, *Mucor mucedo*, *Blakeslea trispora*[28] and *Ustilago maydis*,[29] the plants wheat (*Triticum aestivum*),[30] rice (*Oryza sativa*),[31] radish (*Rhaphanus sativus*),[32] *Camptotheca acuminata*,[33] rubber (*Hevea brasiliensis*),[34] *Arabidopsis thaliana*,[35] and potato (*Solanum tuberosum*).[36] Plants tend to possess small multigene families rather than a single gene, as in animals. Wheat is reported to have four genes,[30] radish two genes,[32] rubber three genes,[34] *Arabidopsis* two genes[35] and potato seven genes.[36] The fungi *Mucor mucedo* and *Parasitella parasitica* also each contain two genes.[28] The enzyme from *Pseudomonas mevalonii* has been crystallized.[37]

HMGR is considered a key regulatory enzyme controlling isoprenoid metabolism in mammals and fungi, but the rate-limiting nature of this enzyme in plants is less well defined. Expression of one of the rubber (*Hevea brasiliensis*) HMGR genes in tobacco plants resulted in an increase in HMGR activity, and about a six-fold increase in total sterol levels, covering both intermediates and end-products.[34] Introduction of the hamster gene into tobacco gave a three- to six-fold increase in HMGR activity, a three- to ten-fold total sterol increase, but a much lower (two-fold) increase in end-product sterols.[38] Cycloartenol levels were up more than 100-fold, but levels of other isoprenoids were relatively unaffected. These results were interpreted to indicate that compartmentation, channelling or other rate-limiting enzymes must also be involved in determining relative accumulation of terpenoids. Two HMGR genes in potato are expressed at different levels by methyl jasmonate or by fungal elicitors.[39] The accumulation of *hmg1* transcripts by treatment with methyl jasmonate results in production of steroid glycoalkaloids, whilst *hmg2* transcripts that accumulate from treatment with arachidonic acid (the fungal elicitor) are responsible for the biosynthesis of sesquiterpene phytoalexins.

The mechanism of the reaction catalysed by HMGR from Syrian hamster has been probed by site-directed mutagenesis studies.[40–42] His-865, Glu-558 and Asp-766 have all been established as essential for enzyme activity. The catalytic domains of wild-type enzyme and mutants E558Q (Glu-558 replaced by Gln), H865Q and D766N were over-expressed and investigated with respect to partial reactions also catalysed by HMGR. Only the wild-type enzyme catalysed all five reactions studied: reductive deacylation of HMG-CoA to MVA, reductive deacylation of mevaldate thiohemiacetal **7** to MVA, reduction of mevaldate **8** to MVA, oxidation of mevaldate thiohemiacetal to HMG-CoA, and oxidative acylation of mevaldate to HMG-CoA. Mutant D766N was inactive for all reactions, but E558Q and H865Q catalysed various reactions at significant rates. In particular, H865Q catalysed the reduction of mevaldate to MVA at about the wild-type rate. Based on these and related data, it was deduced that Asp-766 functioned during conversion of HMG-CoA **1** into mevaldate thiohemiacetal **7** and mevaldate **8** into MVA, with Glu-558 and His-865 playing a role during the mevaldate hemithioacetal/mevaldate transformation. The suggested mechanism is shown in Scheme 2. The catalytic domain of



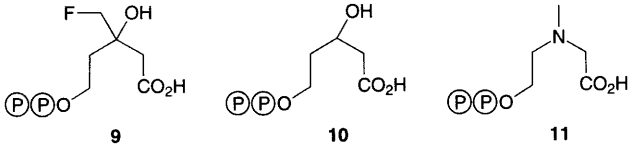
Scheme 2 Enzyme: HMG-CoA reductase

HMGR from *Arabidopsis thaliana* has been expressed in *E. coli* to yield an enzyme of extremely high specific activity.[43] The activity was reversibly inactivated by phosphorylation at a single site (Ser-577) using the enzyme HMGR kinase from *Brassica oleracea*. It is concluded that the activity of higher plant HMGR may be regulated by direct phosphorylation. A common feature of all known plant HMGR isoforms is the presence of two highly conserved hydrophobic sequences in the *N*-terminal quarter of the protein. Study of the *Arabidopsis* enzyme has led to the proposal that the two hydrophobic sequences become a membrane spanning segment, with just a short interconnecting loop exposed to the lumen. The *N*-terminal end and the *C*-terminal catalytic domain are then positioned on the cytosolic side of the membrane.[44] The role of HMGR in the biosynthesis of terpenoid phytoalexins[45] and HMGR inhibitors[46] have been reviewed.

3 Hemiterpenoids

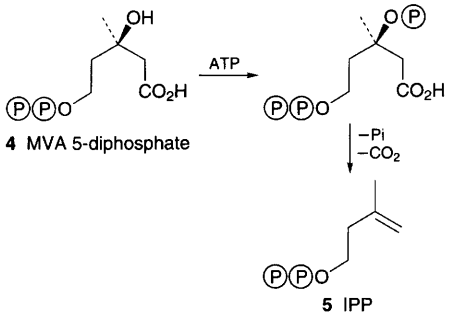
Mevalonate kinase catalyses the ATP-dependent phosphorylation of mevalonate to mevalonate 5-phosphate **3**, the first of the reactions leading up to isopentenyl diphosphate (IPP) **5** and dimethylallyl diphosphate (DMAPP) **6** (Scheme 1). The substrate and inhibitor specificity of pig liver mevalonate kinase have been studied with a range of substrate analogues.[47] Substitution of the hydroxy group and/or methyl at C-3 yielded a range of inhibitors, whilst systematic chain extension of the 3-methyl gave non-competitive inhibitors. Methylation at C-2 and C-4 produced competitive inhibitors which were

also quite good substrates. Reliable assays for the three enzymes mevalonate kinase, phosphomevalonate kinase, and mevalonate 5-diphosphate decarboxylase have been described.[48] The mechanism of action of mevalonate 5-diphosphate decarboxylase has been probed by studying its reaction with some substrate analogues.[49] The fluoromethyl analogue **9** was decarboxylated 2500-fold more slowly than **4**,



9 **10** **11**

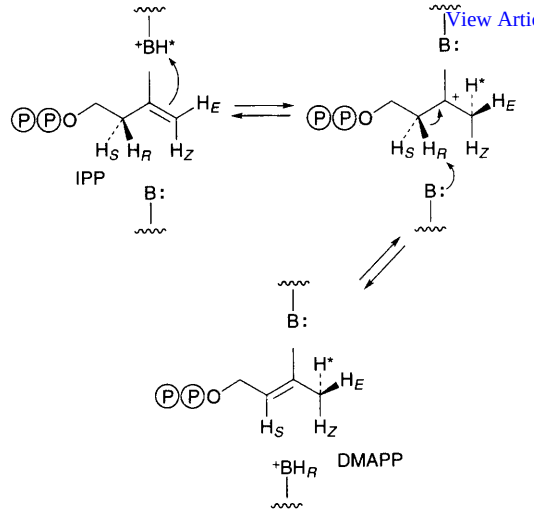
and was a competitive inhibitor of the enzyme. At saturating concentrations of **9** and ATP, nearly one equivalent of 3-phosphorylated **9** and ADP were bound to the enzyme. The 3-demethyl derivative **10** was phosphorylated at the 3-hydroxy group and released without decarboxylation. Both these analogues have decreased electron density at C-3 relative to the normal substrate, and the transition state for the decarboxylation step therefore has considerable cationic character. In confirmation, the 3-aza analogue **11** was also a good inhibitor. The results strongly support a two-step mechanism for the enzyme, in which phosphorylation of the 3-hydroxy group is followed by decarboxylation, and the transition state has carbocationic character (Scheme 3).



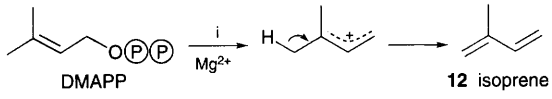
Scheme 3 Enzyme: mevalonate 5-diphosphate decarboxylase

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 4), resulting in the observed antarafacial transposition of hydrogen. Active site cysteine residues Cys-138 and Cys-139 have been identified in the yeast (*Saccharomyces cerevisiae*) enzyme over-expressed in *E. coli*. In recent studies, Glu-207 has also been found to play a role.[50] A mutant C139S (Cys-139 replaced by Ser) was active, but was a poor catalyst. When treated with 3-(fluoromethyl)but-3-enyl diphosphate, an electrophilic active-site-directed irreversible inhibitor, the enzyme became inactivated by covalent modification of Glu-207. The mutants E207Q and E207V were found to be totally inactive. The nucleotide sequence of the IPP isomerase gene from *Clarkia breweri* has been reported.[51] The deduced amino acid sequence was found to be 90% identical with that from *Arabidopsis*, but less than 50% identical with the sequence from yeast.

Isoprene **12** 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. An enzyme isoprene synthase catalyses the elimination of diphosphate from DMAPP, though an equivalent non-enzymic acid-catalysed reaction is feasible (Scheme 5). In velvet bean



Scheme 4 Enzyme: IPP isomerase

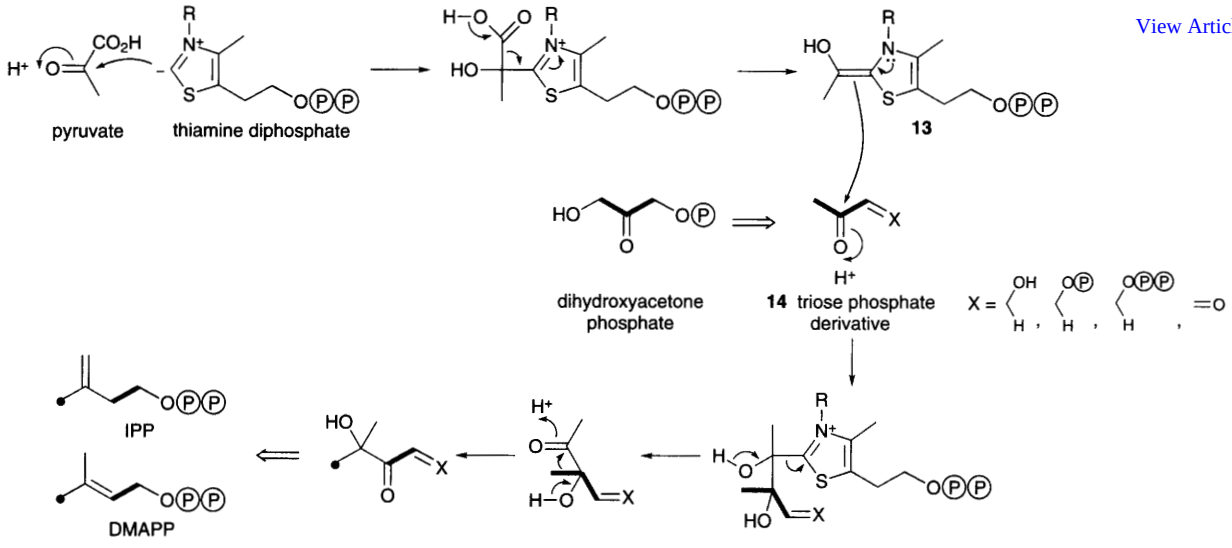


Scheme 5 Enzyme: isoprene synthase

(*Mucuna* sp.), changes in extractable isoprene synthase activity exactly parallel isoprene emission, thus supporting the enzymic derivation of this volatile hydrocarbon.[52] The enzyme from aspen (*Populus tremuloides*) has been purified and shown to be a heterodimer dependent on Mg^{2+} or Mn^{2+} for activity.[53] Partial amino acid sequences for the subunits indicated they were closely related, but they did not share strong homology with any other reported proteins.

4 The dihydroxyacetone phosphate pathway

A number of studies have provided evidence that some bacteria employ a biosynthetic pathway to terpenoids which is different from the well-established mevalonate pathway.[54,55] The origins of IPP in cell-free extracts of several bacteria were studied. Extracts from *Myxococcus fulvus*, *Staphylococcus carnosus*, *Lactobacillus plantarum* and *Halobacterium cutirubrum* all converted labelled acetyl-CoA or HMG-CoA into MVA. Also, labelled MVA, MVA 5-phosphate and MVA 5-diphosphate were all metabolized to IPP, thus showing operation of the normal pathway *via* acetoacetyl-CoA and MVA. In contrast, no intermediates of this reaction sequence could be detected using cell-free extracts of *Zygomonas mobilis* or *E. coli*. In further studies with ^{13}C-labelled glucose, acetate, pyruvate and erythrose substrates, the origins of the carbon atoms of ubiquinones and triterpenoid hopanes were investigated.[55] In *Z. mobilis*, *E. coli*, *Methylobacterium fujisawaense* and *Alicyclobacillus acidoterrestris*, the labelling patterns were consistent with a novel route for the early steps of isoprenoid biosynthesis, and explained some earlier incompatibilities noted when the biosynthesis of hopanoids had been studied. The C_5 framework of the isoprenoid units is most probably built up by condensation of a C_2 unit derived from decarboxylation of pyruvate (*i.e.* a thiamine-activated acetaldehyde **13**) onto the C-2 carbonyl of a triose phosphate derivative **14** (Scheme 6). The triose phosphate is probably formed from dihydroxyacetone phosphate and not from pyruvate, and several alternative possibilities could be considered. Lastly, there is a transposition step in which a methyl migrates from the triose C_3 unit to the C_2 unit. The whole sequence resembles that operating in L-valine biosynthesis, but this amino acid and



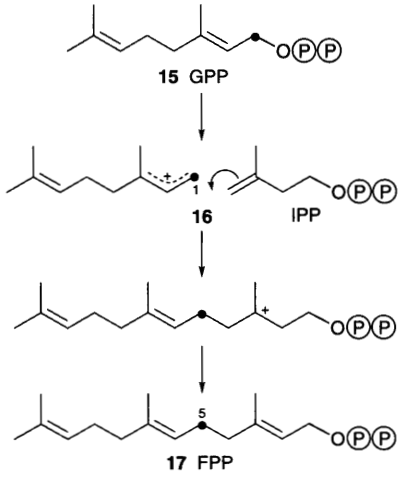
Scheme 6

its C_5 precursors were excluded as intermediates. This pathway is not operative in chloroplasts from young spinach (*Spinacia oleracea*) plants.[56] Acetate was a much better precursor of chloroplast isoprenoids than pyruvate, and the normal mevalonate pathway was utilized.

5 Prenyltransferases

Prenyltransferases are responsible for the alkylation steps involving DMAPP and one or more IPP residues, reactions which provide the polyprenyl diphosphate precursors for the various terpenoid families. Many prenyltransferase enzymes are relatively non-specific and catalyse the condensation of DMAPP with IPP units, building up polyprenyl diphosphate chains of different lengths. A geranyl diphosphate synthase purified from grape vine (*Vitis vinifera* cv. Muscat de Frontignan) cell cultures formed only geranyl diphosphate (GPP) as the product.[57] Divalent metal ions Mn^{2+} or Mg^{2+} were required as cofactors.

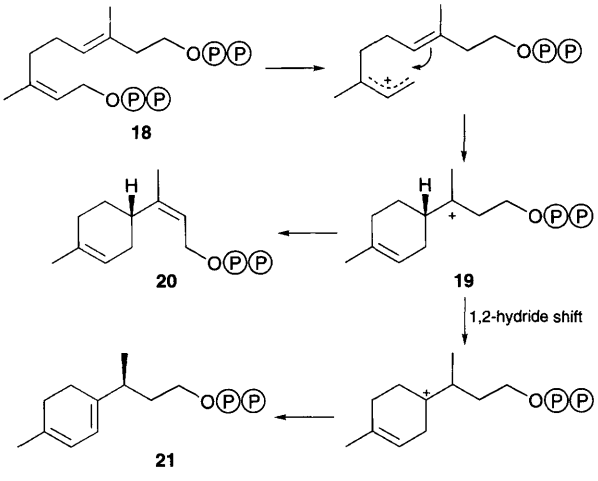
Available evidence points to the initial ionization of the chain starter unit to provide an allylic cation prior to addition of IPP extender units (Scheme 7). An allylic cation has now been detected by NMR spectrometry in the reaction of GPP **15** and IPP to farnesyl diphosphate (FPP) **17** catalysed by avian FPP synthase.[58] Incubation of [1-^{13}C]GPP and IPP with the enzyme led to detection of signals for C-1 of GPP, C-1 of geraniol, C-1 of linalool and C-5 of FPP, with a further signal assigned to and consistent with C-1 of the allylic cation **16**. This signal was also observed in the absence of IPP and was not present when heat-treated enzyme was used. The signal disappeared on addition of the inhibitor 6,6′-dinitro-3,3′-disulfanediyldibenzoic acid but this treatment did not affect either FPP or GPP signals. The new signal reappeared on further treatment with the reactivator dithiothreitol, and the FPP signal increased whilst the GPP signal decreased. A thermostable FPP synthase from *Bacillus stearothermophilus* has been obtained by molecular cloning and over-expression in *E. coli*.[59] The recombinant protein was identical to the native enzyme and was still active after 70 min at 65 °C. The deduced amino acid sequence showed 42% similarity with *E. coli* FPP synthase. In contrast to thermolabile prenyltransferases which have 4–6 cysteine residues, this thermostable enzyme carried only two, but mutagenesis studies showed neither was essential for catalytic function.[60] Three *C*-terminal amino acids, Arg-295, Asp-296 and His-297, were similarly shown to be non-essential for activity, despite the arginine residue being completely conserved throughout FPP synthases of prokaryotes and eukaryotes.[61] A single gene encodes for FPP synthase in *Arabidopsis thaliana*, and the deduced amino acid sequence of the enzyme showed *ca.* 50% identity with that known for yeast, rat and human enzymes.[62] The *Arabidopsis* gene was able to functionally complement a yeast mutant deficient in the enzyme. Two cDNA clones encoding FPP synthase were isolated from white lupin (*Lupinus albus*) and expressed in *E. coli*.[63, 64] The deduced amino acid sequences were 90% identical, *ca.* 80% identical to the *Arabidopsis* enzyme, 51% to the yeast enzyme and 44% to rat and human systems. One enzyme displayed both GPP synthase and FPP synthase activities. Polyprenyl transferases from a variety of organisms contain several highly conserved amino acids, including aspartic acid rich domains. Site-directed mutagenesis studies with rat FPP synthase have established that Asp-104, Asp-107, Arg-112, Arg-113 and Asp-243 are essential for catalytic activity, and are presumably involved in either the condensation or release steps.[65] Similar experimentation using yeast enzyme has indicated that mutations affecting Asp-100, Asp-101, Asp-104, Arg-109, Arg-110, Asp-240 and Asp-241 dramatically lowered catalytic activity.[66] The crystal structure of avian liver FPP synthase has been reported.[67] Two aspartate-rich sequences were found on opposite walls of a large central cavity, suggesting these residues participate in substrate binding or catalysis.



Scheme 7 Enzyme: farnesyl diphosphate synthase

The catalytic versatility of avian liver FPP synthase has been demonstrated by incubating the enzyme with substrate

analogues containing both DMAPP and IPP moieties joined by a one-carbon bridge in a single molecule.[68, 69] Thus, **18** was transformed into **20** and **21**, suggesting a sequence in which ionization of the DMAPP portion then allowed alkylation by the IPP portion, but in doing so, generating a cyclic cationic product **19** (Scheme 8). A 1,2-hydride shift, established by labelling studies, occurs in the formation of **21**. The products were optically active as shown, indicating strict control of the substrate conformation. Pig liver FPP synthase will accept a wide range of substrate analogues with modified hydrocarbon chains, or with ether linkages.[70, 71] The enzyme from *Bacillus stearothermophilus* was more stringent in its specificity, and analogues with oxygen-containing chains were poor substrates.[72]



Scheme 8

Geranylgeranyl diphosphate (GGPP) synthase from bovine brain has been purified and shown to be specific for the condensation step between FPP and IPP.[73, 74] A separate FPP synthase enzyme utilizing DMAPP and IPP was also purified, indicating the synthesis of GGPP **22** from C_5 precursors requires the action of two enzymes. GGPP synthase was a homooligomer with 4–5 subunits whereas FPP synthase was a homodimer. Two different GGPP synthase activities giving different products have been detected in rat tissues.[75] A membrane associated activity synthesized (*E*,*E*,*Z*)-GGPP and is involved in the biosynthesis of long chain polyprenols, whilst a cytosolic activity producing (*E*,*E*,*E*)-GGPP is concerned with protein prenylation reactions. Most eubacteria and eukaryotes seem to possess distinct FPP and GGPP synthases. In contrast, bifunctional FPP/GGPP synthases from the archaebacteria *Methanobacterium thermoformicicum*[76] and *M. thermoautotrophicum*[77] have been isolated and purified. These enzymes are thermostable with optimal activity at 65 °C, require Mg^{2+} or Mn^{2+} as cofactor and catalyse prenyl transfer of IPP to all three substrates DMAPP, GPP and FPP. It thus appears that only one prenyltransferase enzyme is involved in the biosynthesis of squalene and other polyprenyl lipids. The gene encoding the *M. thermoautotrophicum* enzyme has been isolated and sequenced.[78] The deduced amino acid sequence for the protein contained five conserved regions as found in eubacterial and eukaryotic FPP and GGPP synthases, including aspartate-rich motifs commonly found in prenyltransferases.[79] An *Erwinia uredovora* enzyme produced by overexpression of the gene in *E. coli* would accept both FPP and GPP as substrates, but not DMAPP.[80] Gene sequences encoding for GGPP synthase have been characterized from a variety of sources, including pepper (*Capsicum annuum*),[81] *Arabidopsis thaliana*[82, 83] and white lupin (*Lupinus albus*).[84] The procedure for obtaining a thermostable GGPP synthase from *Sulfolobus acidocaldarius* by gene cloning has been patented.[85]

Activities for three key enzymes in the biosynthesis of membrane lipids, namely IPP isomerase, GGPP synthase and geranylgeranylglyceryl phosphate (GGGP) synthase, were found in cytosolic fractions from the strict anaerobe *Methanobacterium thermoautotrophicum* and the extreme halophile *Halobacterium halobium*.[86] The membrane lipids consist of glyceryl ethers bearing saturated isoprenoid side-chains, *e.g.* **26**, and in *M. thermoautotrophicum*, the diethers are then joined covalently at the ends of the isoprene chains to form dimeric membrane spanning tetraethers, *e.g.* **27** (Scheme 9). In both organisms, (*S*)-glyceryl phosphate **23** was the preferred acceptor for the prenyltransferase reaction catalysed by GGGP synthase, though both GGPP and phytyl PP could be utilized. Two enzyme activities were subsequently separated, a cytosolic enzyme catalysing the alkylation of (*S*)-glyceryl phosphate by GGPP to give the monoether **24**, and a microsomal enzyme catalysing a second alkylation to produce **25**.[87] The cytosolic enzyme was purified and shown to require a divalent metal ion as the cofactor, with Mg^{2+} preferred.[88]

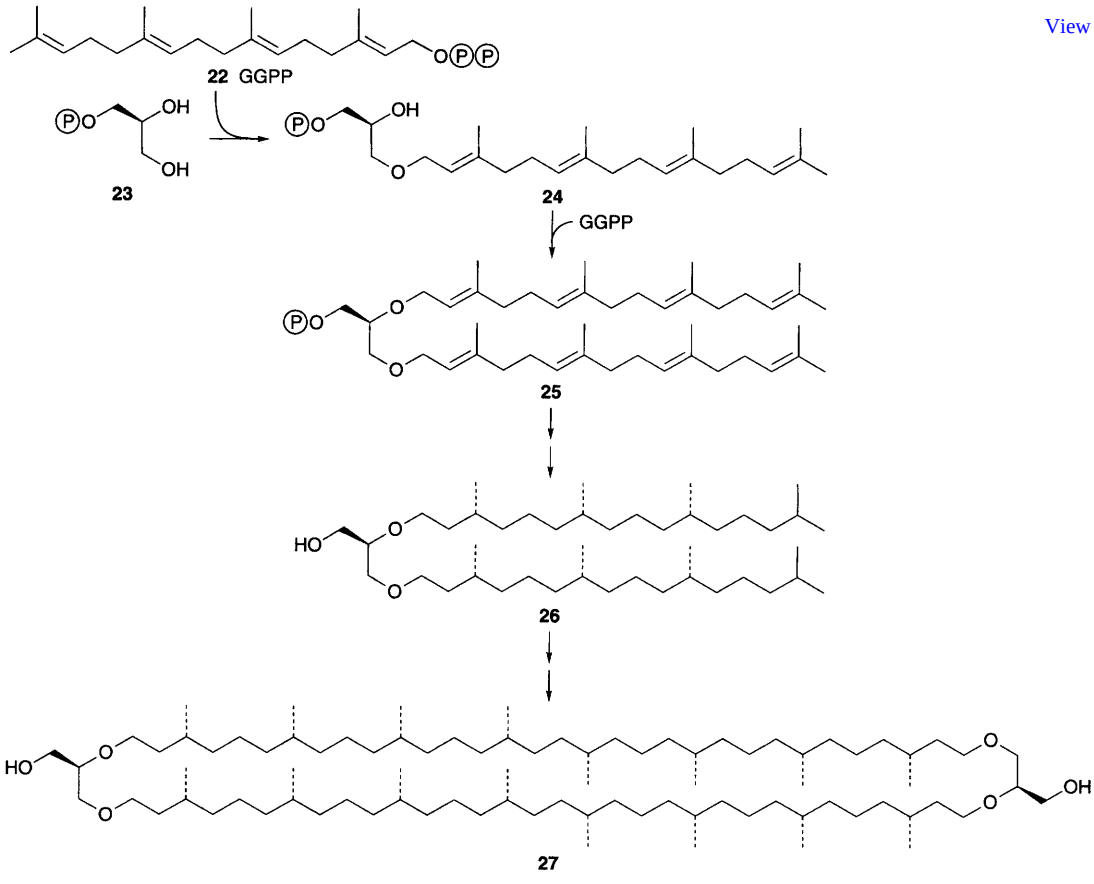
A novel farnesylgeranyl diphosphate (FGPP) synthase from the haloalkaliphile *Natronobacterium pharaonis* has been separated from GGPP synthase, which is the major prenyltransferase in this organism.[89] This enzyme had highest activity with GGPP, but also accepted DMAPP, GPP and FPP. The GGPP synthase was most active with GPP, whilst DMAPP was preferred to FPP. The enzyme is believed to be involved in the biosynthesis of C_{25}-containing diether lipids, such as **28**, and the FGPP precursor may thus be formed by the action of just two activities, GGPP synthase and FGPP synthase, the former enzyme probably utilizing DMAPP.

A review discussing the role of prenyltransferases in the biosynthesis of a range of isoprenoid systems has been published.[90]

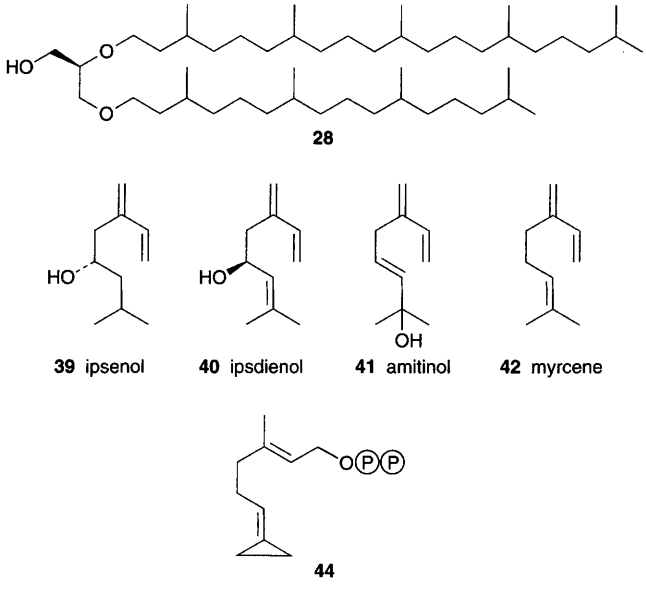
6 Monoterpenoids

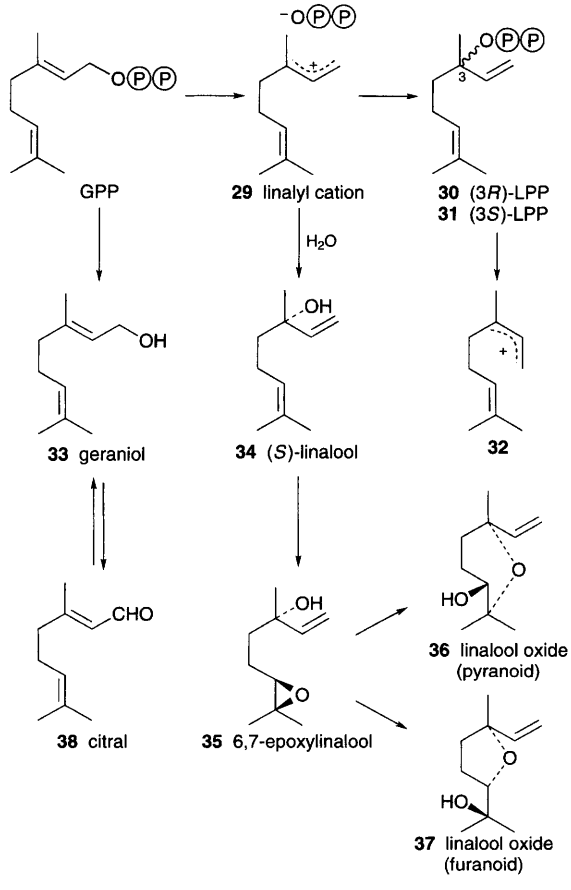
The conversion of geranyl diphosphate (GPP) into simple cyclic monoterpenes involves initial isomerization to (3*R*)-(−)- or (3*S*)-(+)-linalyl diphosphate (LPP) (**29** and **33**). GPP is typically bound to the enzyme as a complex with a divalent metal ion, and undergoes ionization to the allylic linalyl cation **29**, which allows formation of LPP, and the opportunity for cyclization *via* the stereochemically favourable cation **32** (Scheme 10). A series of cation–diphosphate ion pairs participates in the sequence, and both the isomerization and cyclization reactions are catalysed by a single enzyme. Interestingly, the biosynthesis of linalool **34** in the flowers of *Clarkia breweri* does not proceed as far as LPP, but involves quenching of the linalyl cation with water.[91] An enzyme isolated from the freshly opened flowers converted GPP into (*S*)-linalool, but would accept neither enantiomer of LPP as substrate. The enzyme showed a preference for Mn^{2+} over Mg^{2+} as cofactor. Linalool is one of the volatiles in the floral scent of *C. breweri*, and is subsequently transformed into the other characteristic monoterpenes, 6,7-epoxylinalool **35**, and the linalool oxides (**36** and **37**).[92] Linalool synthase activity is highest in the petals, stigma and style, and no such activity was detected in leaves, which merely hydrolysed GPP to geraniol **33**. Geraniol is the precursor of citral **38** in leaves of lemongrass (*Cymbopogon flexuosus*), and an $NADP^+$-dependent geraniol dehydrogenase has been characterized.[93] The reaction catalysed was reversible and the enzyme activity was distinct from alcohol dehydrogenase.

Acyclic monoterpene alcohols ipsenol **39**, ipsdienol **40**, and amitinol **41** are components of the aggregation pheremones produced by the pine bark beetles *Ips paraconfusus* and *Ips pini*. In previous studies it had been established that these were derived from the olefine myrcene **42**, and since this is a component of the pine trees colonized by the beetles, it was



Scheme 9



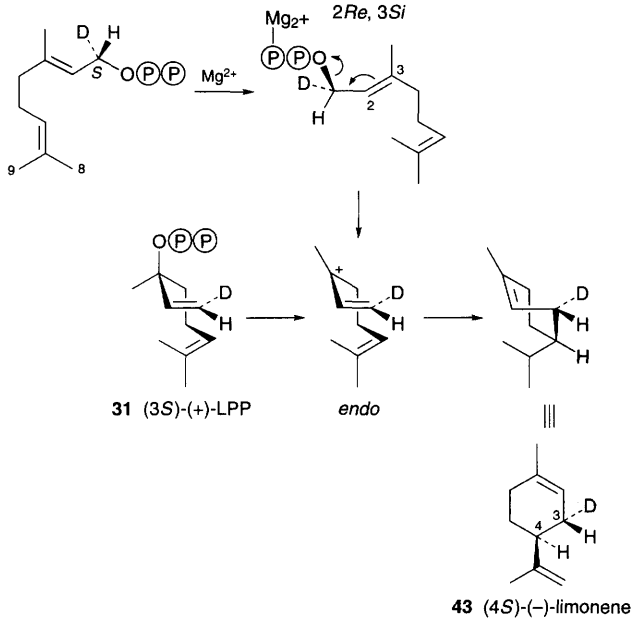


Scheme 10

thought the alcohols were biotransformation products. In recent studies,[94] feeding experiments in male beetles using labelled acetate demonstrated *de novo* biosynthesis without any requirement for plant-derived myrcene; female beetles did not produce any pheremone components.

The enzyme limonene synthase catalyses the isomerization–cyclization of GPP to limonene, the chirality in the product being determined by the enzyme and its source. Thus, limonene synthase from spearmint (*Mentha spicata*) yields (4*S*)-(−)-limonene **43**, whilst that from unshiu orange (*Citrus*

unshiu) produces (4*R*)-(+)-limonene. How the different stereochemistries are generated has been studied using deuterium-labelled GPP and LPP substrates with partially-purified enzyme systems from the two sources.[95] Thus, (1*S*)-[1-^{2}H]GPP with the *Mentha* enzyme gave (−)-limonene with deuterium label *cis* to the 4-hydrogen. Only (3*S*)-(+)-LPP **31** was utilized, and the [1*Z*-^{2}H]-labelled substrate also gave (−)-limonene with deuterium label *cis* to the 4-hydrogen. Of the various stereochemical sequences possible, only one is accommodated by these findings. This requires elimination of the diphosphate group from the (2*Re*,3*Si*)-face of GPP, and isomerization to a cyclizable linalyl cation having an *endo*-spatial arrangement (Scheme 11). Complementary studies with the (+)-limonene



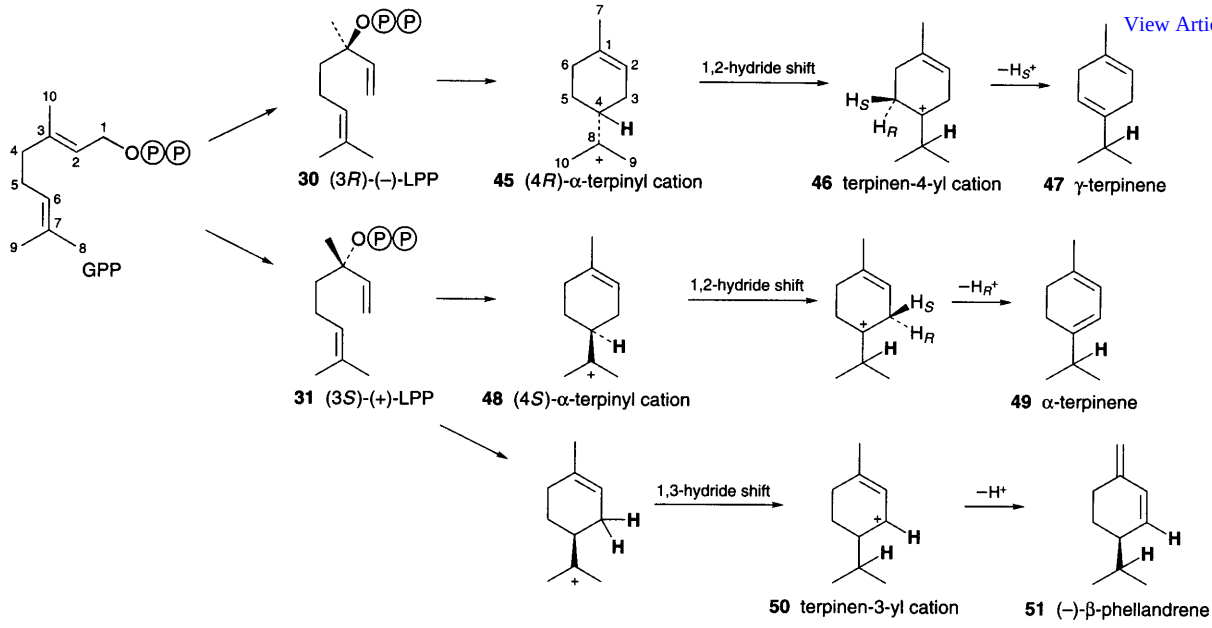
Scheme 11 Enzyme: limonene synthase

synthase from *Citrus* established the same restrictions, though in the enantiomeric series. Since the linalyl cations formed in the two limonene synthase systems are mirror image related, the active sites of the enzymes must also be enantiomeric. In the later stages of the sequence to limonene, a proton is lost from one of the methyl groups, thus generating the propenyl side-chain. It has now been established in enzyme systems from both *Citrus sinensis* [(+)-limonene synthase] and *Perilla frutescens* [(−)-limonene synthase] that the proton lost is almost exclusively provided by the *cis* terminal methyl group (C-9).[96] This contrasts with the (+)- and (−)-pinene synthases from *Salvia officinalis* where eliminations occur from both the *cis* (55–65%) and *trans* (35–45%) methyls in GPP. The cyclopropyl analogue **44** of GPP has been demonstrated to be a potent inhibitor of (−)-limonene synthase from *Mentha spicata*, and of several other monoterpene cyclases.[97] It formed a 1:1 stoichiometric complex with the enzyme, but became irreversibly bound, probably by undergoing ionization and cyclization steps to an allylic cation that then alkylated the protein. Several limonene synthase genes are known to be present in the genome of *Mentha spicata*.[98] Three cDNA isolates were obtained and functionally expressed in *E. coli* to give a catalytically active protein which elaborated a product mixture identical to that of the native enzyme. This contains predominantly limonene with small amounts of α-pinene, β-pinene and myrcene. Sequence comparisons showed a significant degree of similarity with the sesquiterpene cyclase *epi*-aristolochene synthase from tobacco, and the diterpene cyclase casbene synthase from castor bean. Antibodies raised against the *M. spicata* enzyme were highly specific for the denatured protein, but did not recognize the native protein.[99] However, they did cross-react with the cyclase expressed in *E. coli*.

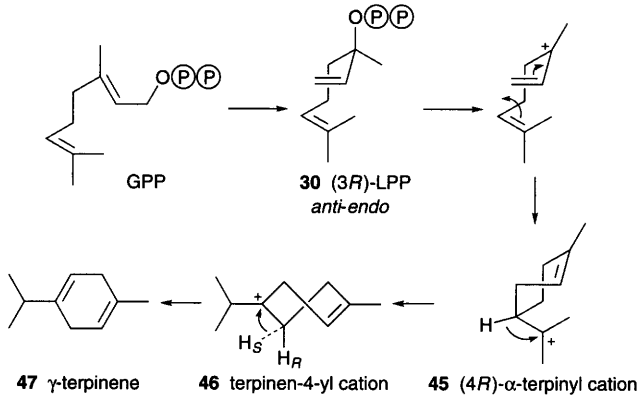
The formation of cyclic monoterpenes is usually *via* the intermediacy of the α-terpinyl cation which may be produced in either 4*R* **45** or 4*S* **48** configurations depending on whether the LPP produced from GPP has the 3*R* or 3*S* configuration, respectively. Biosynthesis of many other monoterpenes then requires migration of the positive charge from the side-chain into the ring, and this may be achieved by hydride shifts. The precise nature of such shifts in the formation of γ-terpinene **47** in thyme (*Thymus vulgaris*), of α-terpinene **49** in American wormseed (*Chenopodium ambrosioides*), and of (−)-β-phellandrene **51** in lodgepole pine (*Pinus contorta*) has been investigated using cell-free enzyme systems and specifically-labelled GPP substrates.[100] It was established that a 1,2-hydride shift from C-4 to C-8 of the α-terpinyl cation thus generating the terpinen-4-yl cation **46** was occurring during γ-terpinene formation, and that the *pro-S* hydrogen from C-5 of GPP was subsequently lost (Scheme 12). The reaction rate with (3*R*)-(−)-LPP **30** was higher than with (3*S*)-(+)-LPP **31**, although both enantiomers were utilized. Utilization of both enantiomers of LPP by monoterpene cyclases is not unusual, but the disfavoured enantiomer often gives aberrant product mixtures, as was noted here with (3*S*)-LPP. The stereochemical features of the transformation, assuming a suprafacial isomerization step and an *anti-endo* conformation for the cyclizing intermediate are shown in Scheme 13. α-Terpinene **49** formation also involved the same 1,2-hydride shift, and a similar proton loss, this time from C-3 of the terpinen-4-yl cation (Scheme 12). The proton lost was originally the 1-*pro-S* of GPP. However, for α-terpinene, (3*S*)-LPP was the preferred substrate, and therefore the isomerization and cyclization steps are antipodal to the reactions seen with γ-terpinene. For (−)-β-phellandrene **51**, a 1,3-hydride shift from C-3 to C-8 of the α-terpinyl cation giving the terpin-3-yl cation **50** and the intermediacy of (3*S*)-LPP was established (Scheme 12).

The biosynthesis of (+)-3-carene **52**, a major component of turpentine, has been studied previously, but the results from feeding experiments with labelled MVA precursors led to a hypothesis which now seems untenable in the light of more recent enzymic studies of monoterpene cyclases. In particular, the double bond appeared to have been transposed during the biosynthesis. Accordingly, the formation of (+)-3-carene has been reinvestigated using cell-free extracts of Douglas fir (*Pseudotsuga menziesii*) and a partially purified carene synthase preparation from lodgepole pine (*Pinus contorta*), and these studies have produced results different from the earlier ones, but consistent with what might now be expected based on the growing pool of enzymic data.[101] Using both plant preparations, it was established that label from [1-^{3}H]GPP was retained at C-5 in carene, in keeping with the intermediacy of a terpinyl cation, then cyclopropane ring formation by loss of a proton from C-5 of the cation (Scheme 14). It was then shown that in lodgepole pine, the 5-*pro-R* proton of GPP was the one eliminated during ring closure. Because (3*S*)-LPP **31** was found to be the preferred substrate, ring closure and hydrogen loss must therefore be accompanied by inversion of configuration at C-5 of the (4*S*)-α-terpinyl cation **48**. To accommodate this, it is deduced that cyclopropyl ring closure most likely occurs by an *anti*-1,3-elimination from the α-terpinyl cation which needs to be in a half-chair conformation with the tertiary cationic centre in an axial position (Scheme 14). In this way, the leaving group (5-*pro-R* proton) can be coplanar with the three carbons forming the cyclopropane ring. There then needs to be a 90° rotation about the 6,7-bond (carene numbering) to allow the *anti*-1,3-elimination with inversion of configuration.

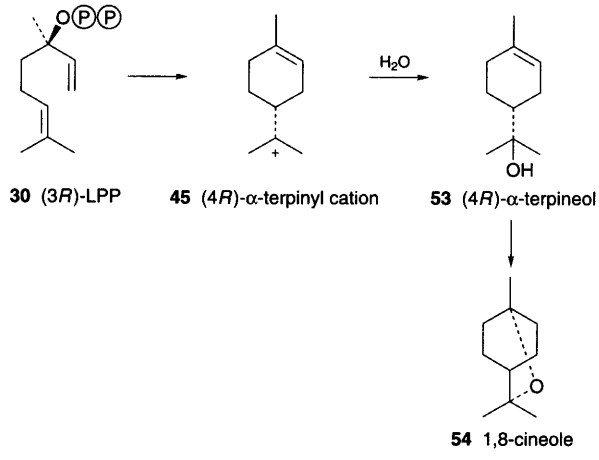
A 1,8-cineole synthase activity has been partially purified from glandular trichomes of sage (*Salvia officinalis*), converting GPP into the symmetrical monoterpene ether 1,8-cineole **54** (Scheme 15).[102] This has properties of a typical

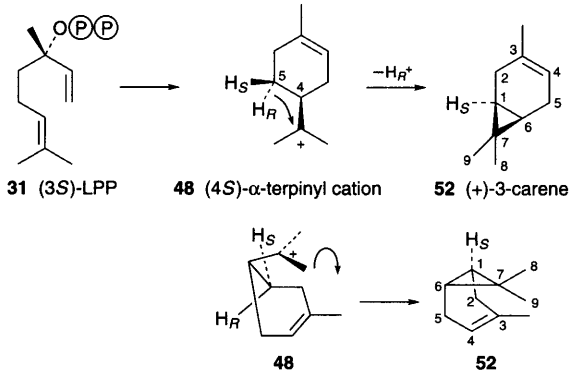


Scheme 12

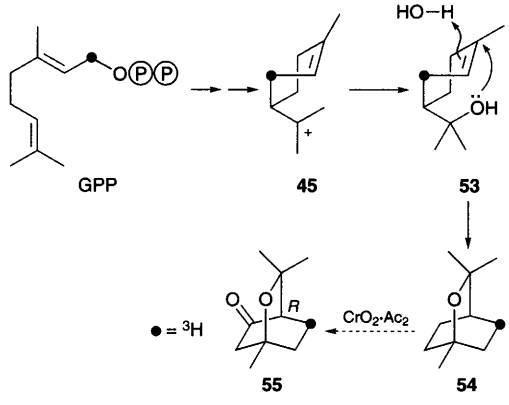


Scheme 13 Enzyme: γ-terpinene synthase



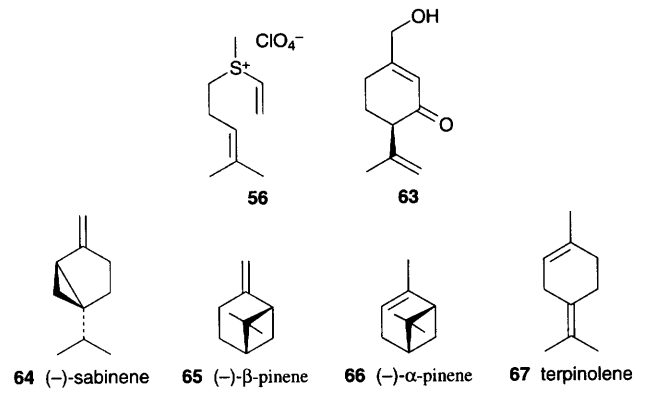


Scheme 14 Enzyme: carene synthase



Scheme 15 Enzyme: 1,8-cineole synthase

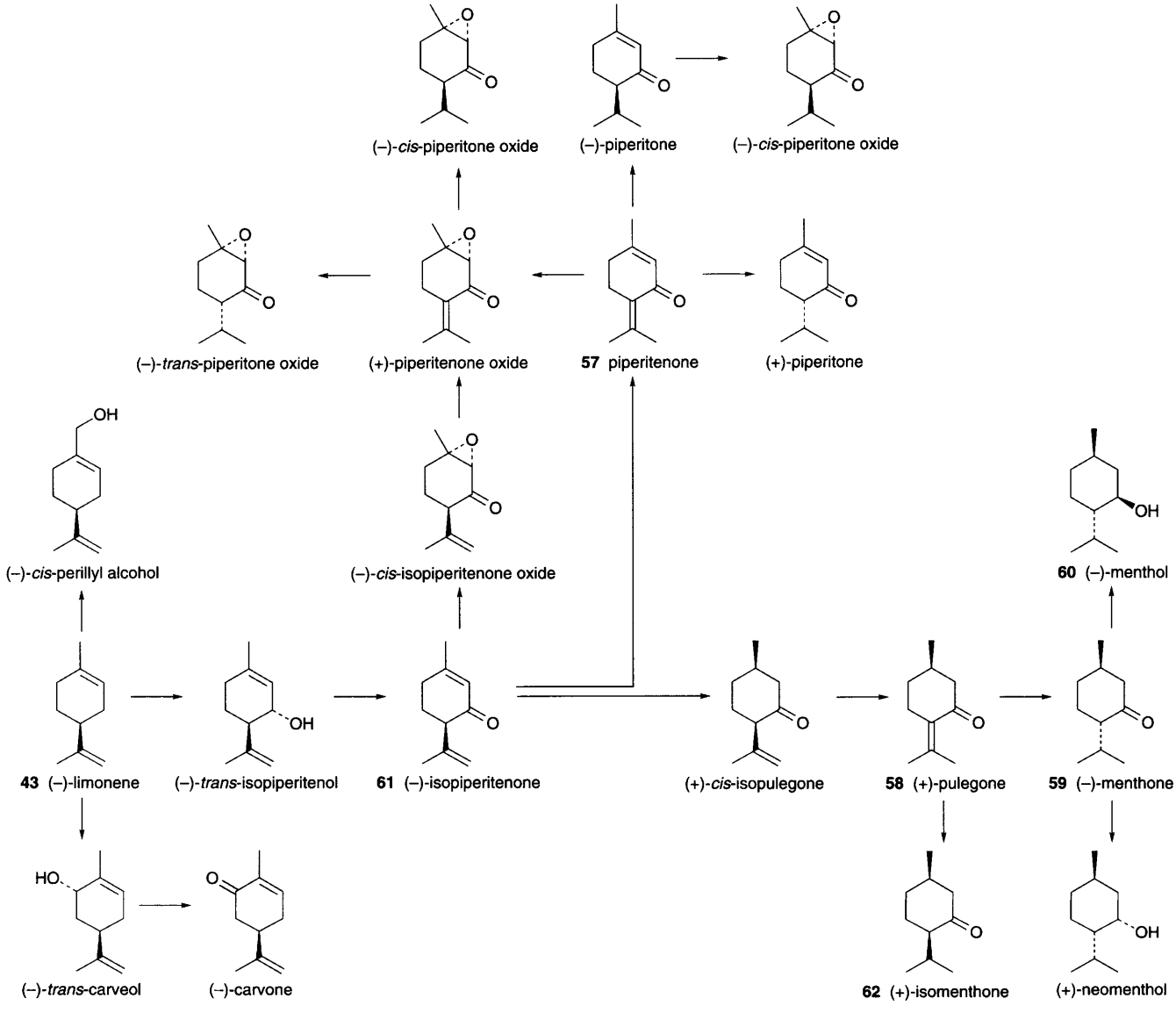
monoterpene cyclase, requiring a divalent metal ion (Mn^{2+} or Mg^{2+}), being inhibited by cysteine- and histidine-directed reagents, and being protected from inactivation by pretreatment with the substrate-M^{2+} ion complex. (3*R*)-LPP **45** was a much better substrate for the enzyme than (3*S*)-LPP, indicating its stereospecificity, and the cyclization of LPP was faster than the coupled isomerization–cyclization of GPP, showing the isomerization was the slow step, as with other monoterpene cyclases. Labelling studies showed the oxygen atom was derived from water, which also supplied one hydrogen atom. The pathway is formulated as involving capture of the α-terpinyl cation **45** by water, giving α-terpineol **53**, followed by attack of the alcohol function onto the remaining double bond (Scheme 15). However, α-terpineol was not utilized by the enzyme system, and neither were the phosphate or diphosphate esters. This may be a consequence of a protein



conformational change during the normal reaction cycle, with the enzyme thus failing to recognise intermediates. The sulfonium analogue **56** of the linalyl cation was a good reversible inhibitor, providing evidence for ion-pair intermediates. The overall stereochemistry of the reaction was also confirmed by feeding [1-^{3}H]GPP, oxidation of the cineole produced to (*R*,*S*)-3-keto-1,8-cineole **55**, and resolution *via* diastereomeric ketals. All activity resided in the *R* antipode (Scheme 15).

The pathways to the *Mentha* monoterpenes and related structures are now well characterized at the enzymic level, and were outlined in the last report.[1] The author regrets that the scheme given in that report contained errors which misplaced some of intermediates. A corrected version is now shown in Scheme 16. Some of these conversions have now been confirmed by using cell suspension cultures of *Mentha piperita*.[103] The cultures retained most transformations of the menthol pathway, although they were not able to incorporate the early precursor (−)-limonene **43**. Thus, (+)-pulegone **58** was transformed into (+)-isomenthone **62** and (−)-menthone **59**, and (−)-menthone into (−)-menthol **60**. The feeding of (−)-isopiperitenone **61** yielded (+)-pulegone, piperitenone **57**, (−)-menthone, (−)-menthol, (−)-7-hydroxyisopiperitenone **63**, plus other compounds.[104]

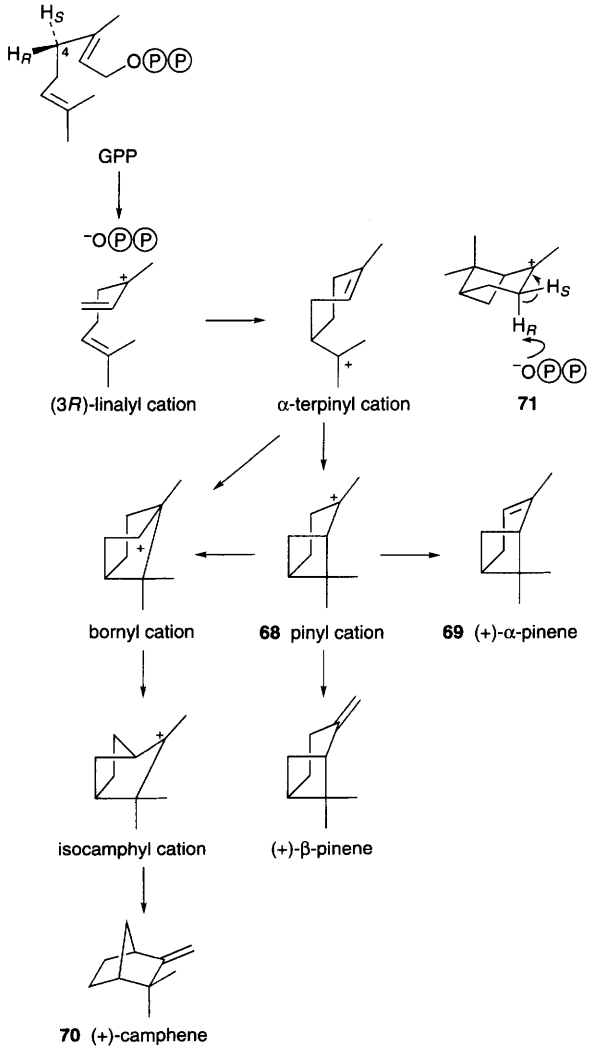
Monoterpene cyclases from gymnosperms appear to have slightly different characteristics to those from angiosperms.[105] Monoterpene cyclases from lodgepole pine (*Pinus contorta*) and grand fir (*Abies grandis*) are similar to terpene cyclases from angiosperms and fungi in respect of mass, K_m for GPP, and pI, but differ in having an alkaline pH optimum, are activated by K^+, Rb^+, Cs^+, or NH_4^+ but not Li^+ or Na^+, and for their M^{2+} cofactor require Mn^{2+} or Fe^{2+}, whilst Mg^{2+} is not effective. Furthermore, they are not protected against histidine-directed reagents by the substrate–M^{2+} complex.



Scheme 16

Four monoterpene cyclases were separated from the xylem of lodgepole pine, and each of these transformed GPP into at least some of the major products of the oleoresin, namely α-pinene **66**, β-pinene **65**, 3-carene **52**, sabinene **64** and β-phellandrene **51**, in varying proportions.[106] Like enzymes from peppermint and sage, these monoterpene cyclases were inactivated by thiol-directed reagents, but unlike the angiosperm enzymes, they were not protected against inactivation by co-incubation with the substrate and metal ion co-factor. Thus, the gymnosperm enzymes appear to have at least some catalytically important thiol residues at a non-substrate-protectable region. Similarly, the behaviour of gymnosperm and angiosperm enzymes to arginine-directed reagents was different. Conifers appear to have catalytically important arginine residues located at or near the active site, and are protected against inactivation by pretreatment with the substrate and metal cofactor, whilst angiosperms have some catalytically active arginines located at a non-substrate-protectable site. Like angiosperm enzymes, the lodgepole pine monoterpene cyclases are able to catalyse both the isomerization of GPP to LPP and the subsequent cyclization. The products formed were predominantly enantiomerically pure and derived from (3*S*)-LPP, but two of the enzymes yielded both (−)- and (+)- α-pinene, the latter which must result *via* the intermediacy of (3*R*)-LPP.

The last step in the formation of α-pinene is loss of a proton from the pinyl cation **68** and what was originally C-4 of GPP [see Scheme 17 for (+)-α-pinene; consider a mirror image scheme for (−)-α-pinene]. The stereochemistry of this proton

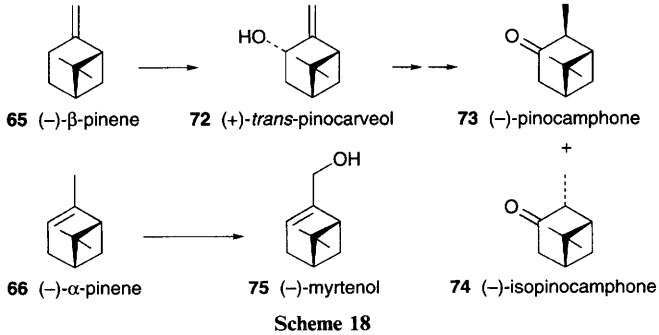


Scheme 17

elimination has been studied using three monoterpene cyclases from sage (*Salvia officinalis*).[107] Cyclases I and III which both produce (+)-α-pinene **69** were both shown to retain label from (4*S*)-[4-$^2H_1$]GPP and lose the deuterium label from (4*R*)-[4-$^2H_1$]GPP (>94% loss; a small retention is due to a primary kinetic isotope effect). Cyclase II which produced (−)-α-pinene **66** retained label from (4*R*)-[4-$^2H_1$]GPP but a higher isotope effect showed in the loss of only 78% label from (4*S*)-[4-$^2H_1$]GPP. This means that in all cases, the proton lost was the axial hydrogen (see **71**), which would represent maximum overlap of the axial sp^3 orbital with the empty *p* orbital of the cationic centre. Since the diphosphate counterion is probably located on the face opposite the *gem*-dimethyl bridge (Scheme 17), this anion may act as the base removing the proton. Samples of (+)-camphene **70** produced by cyclase I, but not (−)-camphene from cyclase II, were shown to suffer loss of deuterium label by exchange with the medium. Label at C-10 of GPP was also vulnerable to exchange. Since there was no exchange in the case of (+)-α-pinene, exchange must occur after the branch-point pinyl cation. There had also been earlier reports of the exchange of hydrogen at C-2 of GPP based on applications of natural abundance deuterium NMR, and the importance of this has been investigated.[108] Studies with two of the monoterpene cyclases from sage using [2-^{2}H, 2-^{13}C]-labelled GPP, either alone, or in admixture with unlabelled GPP, confirmed the complete retention of deuterium and ruled out any transfer between GPP molecules. (+)-α-Pinene **69**, (−)-α-pinene **66**, (+)-camphene **70**, (−)-camphene, terpinolene **67**, and 1,8-cineole **54** [1,8-cineole cyclase coeluted with (−)-α-pinene cyclase] figured amongst the products analysed for deuterium exchange. In all cases, GC–MS analysis indicated more than 98% deuterium retention.

A presumptive active site peptide from a sage monoterpene cyclase has been identified.[109] The monoterpene cyclase contained both (+)-α-pinene cyclase and (+)-bornyl diphosphate synthase, since they are difficult to separate and purify in sufficient amounts for detailed study. The protein was subjected to irreversible binding with a labelled inhibitor, the GPP analogue **44**, and then cleaved with CNBr. This gave a covalently modified peptide mixture, from which an abundant labelled 5 KDa peptide was isolated and sequenced. This was highly homologous through 22 residues with a segment of (4*S*)-(−)-limonene synthase from spearmint, the only monoterpene cyclase for which a complete amino acid sequence was known.

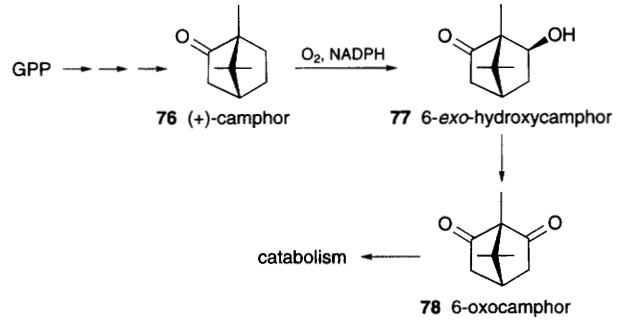
A cytochrome P-450-dependent hydroxylating enzyme has been found in a microsomal preparation from the oil glands of hyssop (*Hyssopus officinalis*) that converts (−)-β-pinene **65** into the allylic alcohol (+)-*trans*-pinocarveol **72** (Scheme 18).[110] This product is then presumably the substrate for



Scheme 18

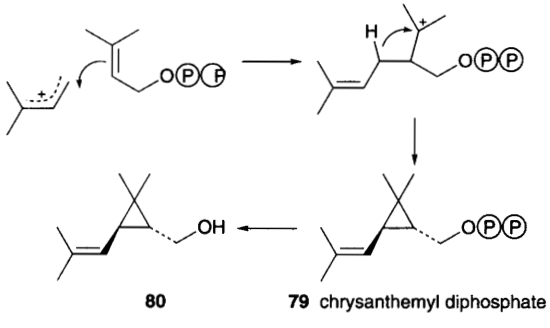
oxidation to the ketone then reduction of the double bond to give (−)-pinocamphone **73** and (−)-isopinocamphone **74**, the major components of hyssop oil. The same preparation catalysed the hydroxylation of (−)-α-pinene **66** to (−)-myrtenol

75, but at a slower rate. The major monoterpenoid of sage (*Salvia officinalis*) leaves is (+)-camphor **76**, but cell cultures accumulate only traces, due to its very rapid catabolism in the cultures. Catabolism proceeds through 6-*exo*-hydroxycamphor **77** and 6-oxocamphor **78** (Scheme 19). An NADPH–O_2 dependent camphor hydroxylase has been demonstrated in a microsomal preparation of sage cells, and this has typical characteristics of a P-450 system.[111] The protein also cross-reacted with polyclonal antibodies raised against the P-450-dependent enzymes camphor-5-*exo*-hydroxylase from *Pseudomonas putida*, and limonene-6*S*-hydroxylase from spearmint (*Mentha spicata*), suggesting it shares common properties with bacterial and other plant monoterpene hydroxylases.



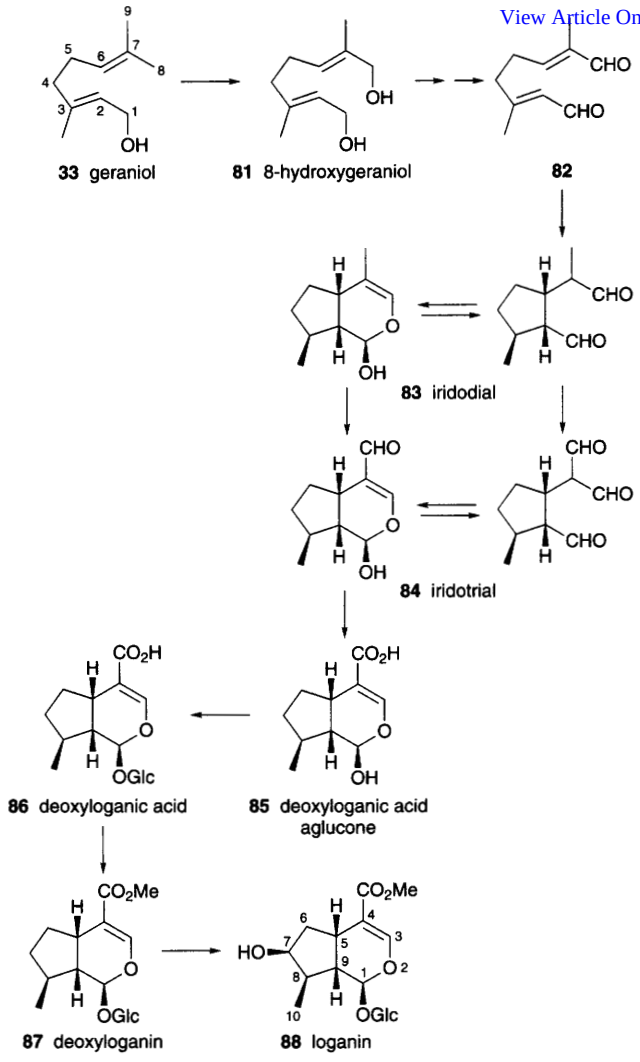
Scheme 19

The irregular monoterpene chrysanthemic acid is found in esterified form in the pyrethrins from *Chrysanthemum cinerariaefolium*, though details of its biosynthesis have been poorly defined. A US patent[112] now describes isolation of an enzyme chrysanthemyl diphosphate synthase which catalyses the conversion of DMAPP into (+)-*trans*-chrysanthemyl diphosphate **79** (Scheme 20), the gene sequence encoding for the enzyme, and expression of this gene in yeast or *E. coli*. Transformed yeast cells carrying the gene are used to produce (+)-*trans*-chrysanthemyl alcohol **80**, which may be oxidized chemically and converted into pyrethroids.



Scheme 20

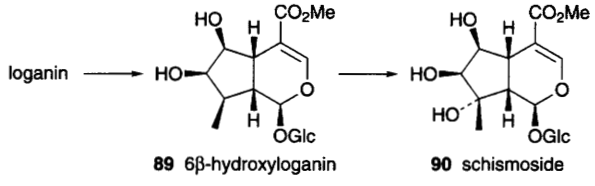
Iridoids are derived from geraniol **33** *via* early hydroxylation of the 8-methyl (Scheme 21). A specific P-450-dependent hydroxylase has been demonstrated in catmint (*Nepeta racemosa*) and shown to transform both geraniol and nerol into their 8-hydroxy derivatives.[113] Further, a soluble enzyme from the leaves would oxidise 8-hydroxygeraniol **81** at both the alcohol functions to produce the dialdehyde **82**.[114] 8-Hydroxynerol was similarly oxidised, whilst geraniol and nerol, which were actually rather better substrates, were oxidised to the corresponding monoaldehydes. The enzyme required $NADP^+$ as cofactor, with NAD^+ being ineffective, and was a dimer. A similar enzyme activity from *Rauwolfia*



Scheme 21

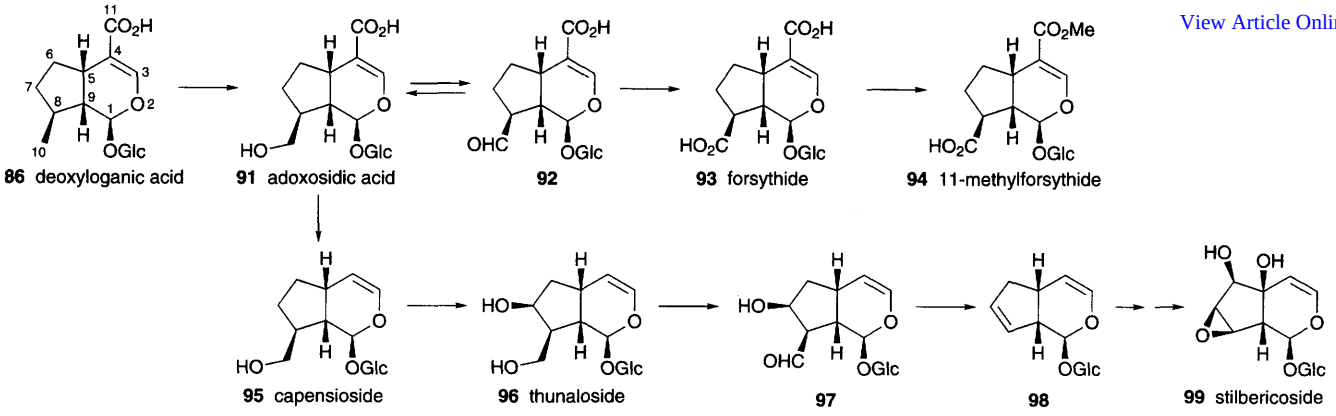
serpentina had also been shown to catalyse multiple oxidations, but was a monomer. (Note that an alternative numbering system for geraniol is employed in these papers.)

Feeding experiments with a range of ^2H-labelled potential precursors of schismoside (≡8-*epi*-lamalbid) **90** in shoots of *Schismocarpus matudai* have demonstrated good incorporations of deoxyloganin **87**, loganin **88**, and 6β-hydroxyloganin **89**, thus suggesting a sequence in which hydroxylations of loganin proceed in the order 6, 7 and then 8 (Scheme 22).[115] In

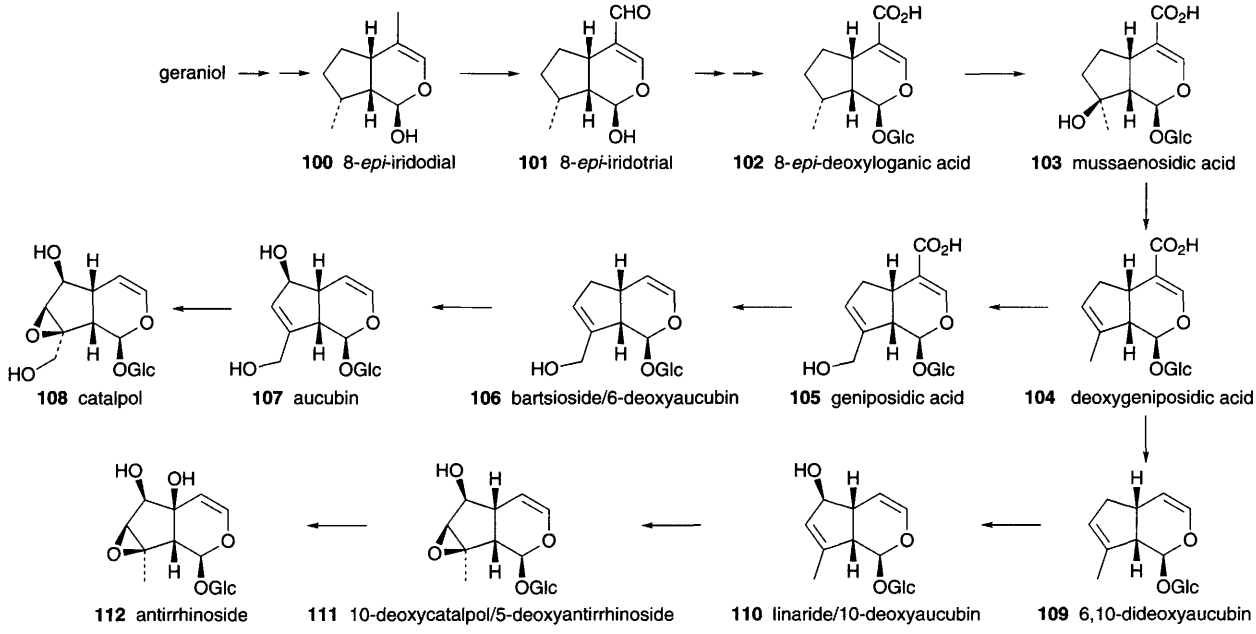


Scheme 22

the case of the *Forsythia* iridoids forsythide **93** and 11-methylforsythide **94**, modifications to the oxidation level appear to be effected on the acid rather than the ester. Thus, feeding experiments in *F. viridissima* and *F. europaea* showed good incorporations of deoxyloganic acid **86** into adoxosidic acid **91**, forsythide **93** and 11-methylforsythide **94**, and adoxosidic acid was also a good precursor of the latter two



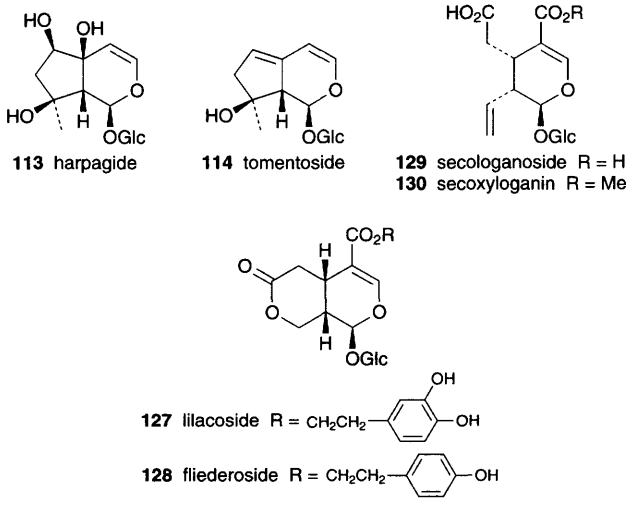
Scheme 23



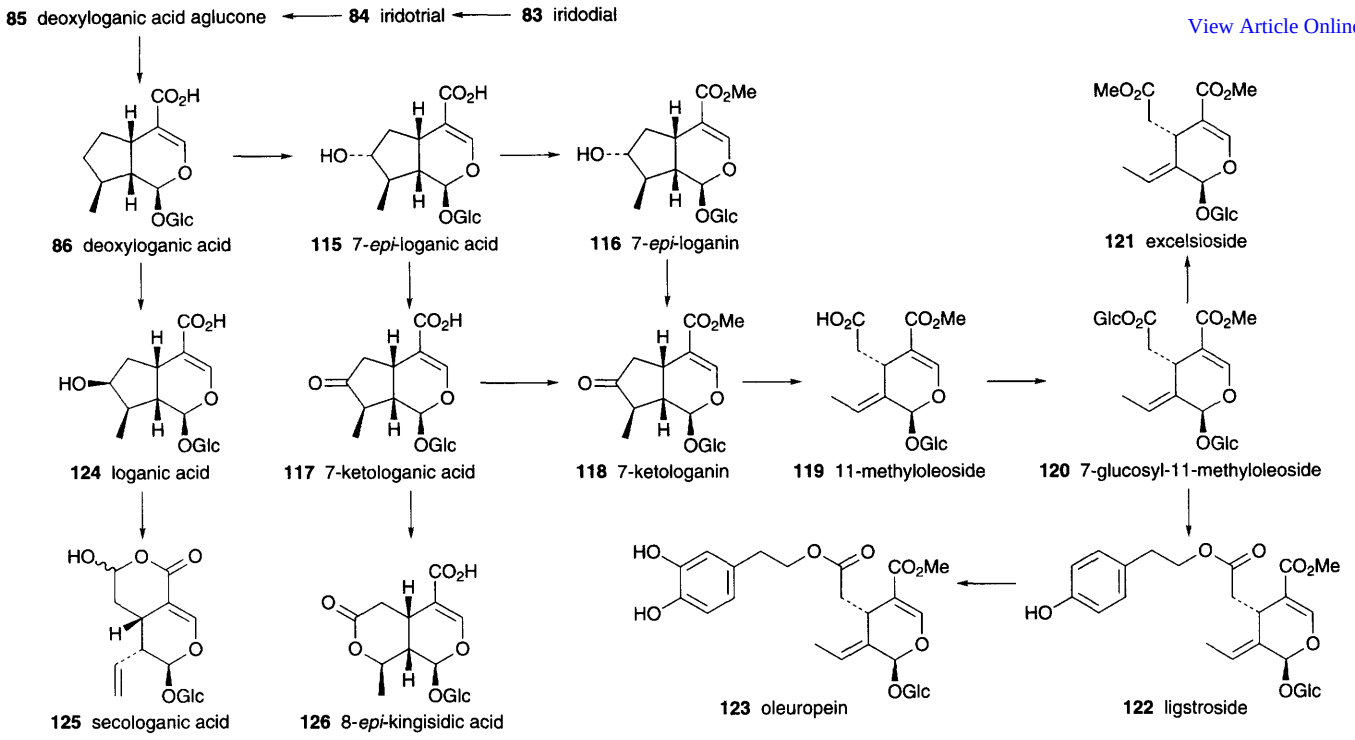
Scheme 24

metabolites.[116] This supports a sequence involving successive oxidations at position 10 of deoxyloganic acid, with methylation at the C-11 carboxy group as the last step (Scheme 23). The observed loss of some of the label from C-10 of adoxosidic acid may be the result of reversible oxidation–reduction between alcohol and aldehyde **92**. In *Thunbergia alata*, adoxosidic acid **91** is decarboxylated to capensioside **95** which serves as the precursor of stilbericoside **99** (Scheme 23).[117] Since deoxyloganic acid **86** was also efficiently incorporated into thunaloside **96** in these experiments, it is suggested that 7-hydroxylation of capensioside is likely to be the next step in the pathway, and that the aldehyde **97** and the alkene **98** may then be involved as potential intermediates. A pathway *via* these two intermediates would be consistent with the observation that label from H-6, H-7 and H-8 from deoxyloganic acid is retained during stilbericoside formation.

A further range of decarboxylated iridoid structures arise *via* 8-*epi*-iridodial **100**, 8-*epi*-iridotrial **101** and 8-*epi*-deoxyloganic acid **102** (Scheme 24). The formation of the 8-*epi*-series necessitates generation of the different stereochemistry during the cyclization of the dialdehyde **82** (Scheme 21). Feeding experiments with deuterium-labelled substrates have been used to demonstrate the incorporation of 8-*epi*-iridodial, 8-*epi*-iridotrial and 8-*epi*-deoxyloganic acid into aucubin **107** and harpagide **113** in *Scrophularia umbrosa*.[118] The glucoside of



8-*epi*-iridotrial was also well incorporated but this is likely to be hydrolysed before incorporation, and may not be a direct precursor, since in the loganin series, oxidation of the aldehyde

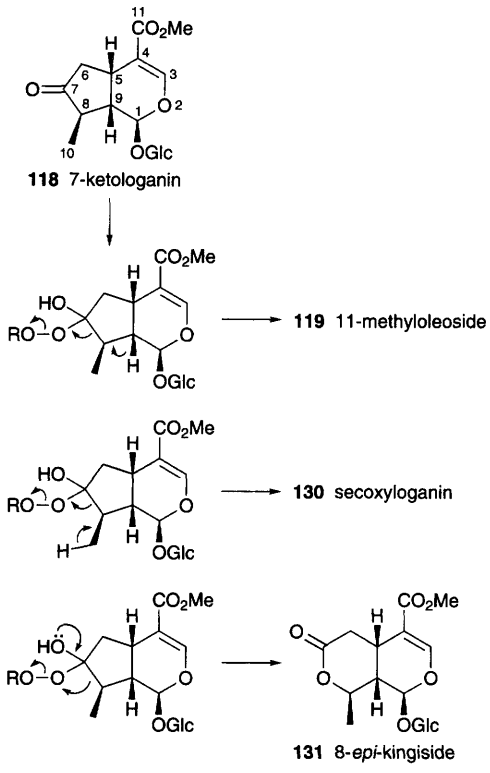


Scheme 25

group precedes glucosylation. The later stages of the pathway to aucubin have also been defined by feeding experiments.[119] These registered excellent incorporations of deoxygeniposidic acid **104**, geniposidic acid **105**, and bartsioside (6-deoxyaucubin) **106**, but not 6,10-dideoxyaucubin **109**. Dilution experiments also established the presence of geniposidic acid after feeding 8-*epi*-deoxyloganic acid. These experiments support the pathway shown in Scheme 24, with decarboxylation occurring at the geniposidic acid stage, and mussaenosidic acid **103** is postulated as an intermediate between 8-*epi*-deoxyloganic acid and deoxygeniposidic acid. In both *Scutellaria albida* and *Paulownia tomentosa*, aucubin is further transformed into the epoxide catalpol **108**.[120] 8-*epi*-Deoxyloganic acid **102** and bartsioside **106** were also shown to be excellent precursors, and the role of aucubin was supported by its isolation from dilution experiments. 8-*epi*-Deoxyloganic acid was also incorporated into tomentoside **114** in *P. tomentosa*. However, subtle variations to the catalpol pathway are observed in the biosynthesis of antirrhinoside **112** in *Antirrhinum majus* (Scheme 24). Decarboxylation occurs at the deoxygeniposidic acid **104** stage, giving 6,10-dideoxyaucubin **109**, followed by 6-hydroxylation to linaride (10-deoxyaucubin) **110**, epoxidation to 10-deoxycatalpol **111**, and finally hydoxylation at the ring junction.[121, 122] All intermediates from 8-*epi*-deoxyloganic acid onwards except for mussaenosidic acid **103** were shown to be well incorporated, and the role of **103** was supported by dilution experiments.[121]

The biosynthesis of a range of secoiridoids (oleosides) has been investigated in an extended series of feeding experiments using plants of the Oleaceae.[123–126] Deoxyloganic acid **86** was incorporated into 11-methyl oleoside **119**, ligstroside **122** and oleuropein **123** in *Syringa josikaea*, and into 7-glucosyl 11-methyl oleoside **120**, 11-methyl oleoside, ligstroside and excelsioside **121** in *Fraxinus excelsior*.[123] 7-*epi*-Loganic acid **115** also proved a good precursor of ligstroside, and the subsequent involvement of 7-ketologanic acid **117** was proposed, with the remaining compounds forming a logical sequence as shown in Scheme 25. 7-Ketologanic acid **117** was then proven to be well incorporated into excelsioside **121** in *Fraxinus excelsior*, with significant incorporations into 11-methyl oleoside **119** and ligstroside **122**.[124] Iridodial **83** was incorporated into 7-ketologanin **118**, whilst iridotrial **84**, deoxyloganic acid aglucone **85**, deoxyloganic acid **86**, 7-*epi*-loganic acid **115** and 7-ketologanin **118** were all incorporated into one or more oleosides, predominantly excelsioside. From these results, the precise sequence of the 7-oxidation and methylation steps could not be defined. The sequence postulated as in Scheme 25 was supported by further feeding experiments using *Syringa josikaea* and *S. vulgaris* tissues. It was noted that the sequence of steps between deoxyloganic acid and 7-ketologanin could vary with plant species and also season. In one series of experiments in *Syringa vulgaris*, neither deoxyloganic acid **86** nor 7-ketologanic acid **117** were incorporated into oleosides, but instead were converted into 8-*epi*-kingisidic acid **126**. At another time, deoxyloganic acid proved a good precursor of the oleosides, including oleuropein **123**, lilacoside **127**, fliederoside **128** and the dimethyl ester of oleoside.

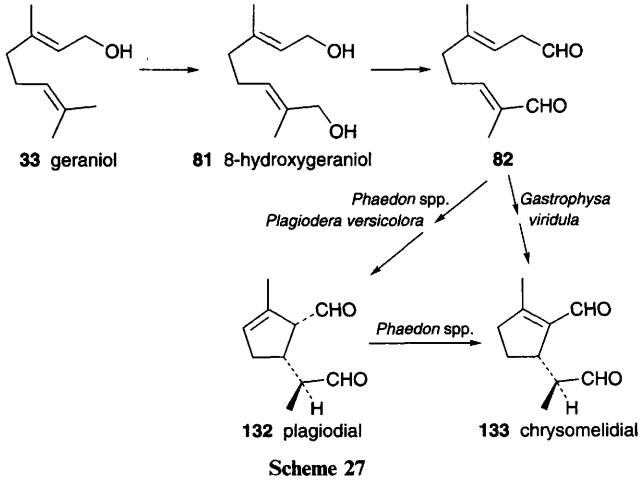
The oleosides are characterized by an 8,9-double bond and their origins appear to differ from those of the 'ordinary' secoiridoids such as secologanin by proceeding through the oxidised intermediates 7-ketologanic acid **117** or 7-ketologanin **118**. In a series of feeding experiments, the possible involvement of intermediates of the secologanin or 7-ketologanin type has been investigated.[125] Compounds such as secologanoside **129** and secoxyloganin **130** were not precursors of the *Fraxinus* and *Syringa* oleosides, though secologanin, particularly in *Syringa josikaea*, was converted into 7-glucosyl 11-methyl oleoside **120**. 11-Methyl oleoside **119** was by far the most efficient precursor of 7-glucosyl 11-methyl oleoside, ligstroside **122** and oleuropein **123**, and, in combination with the earlier results, it is concluded that 7-ketologanin **118** is likely to be the immediate precursor of 11-methyl oleoside. This conversion is possibly a Baeyer–Villiger type mechanism (Scheme 26). Rupture of the peroxide bond followed by cleavage of the 7,8-bond and abstraction of H-9 would produce 11-methyl oleoside **119**. Alternatively, a similar reaction with abstraction of H-10 would produce secoxyloganin **128**, and a Baeyer–Villiger oxidation with migration of the alkyl group would account for formation of 8-*epi*-kingiside **131**. The origins of secologanic acid **125** have been investigated in leaves of *Fontanesia fortunei* and *F. phillyreoides*.[126] This compound is



Scheme 26

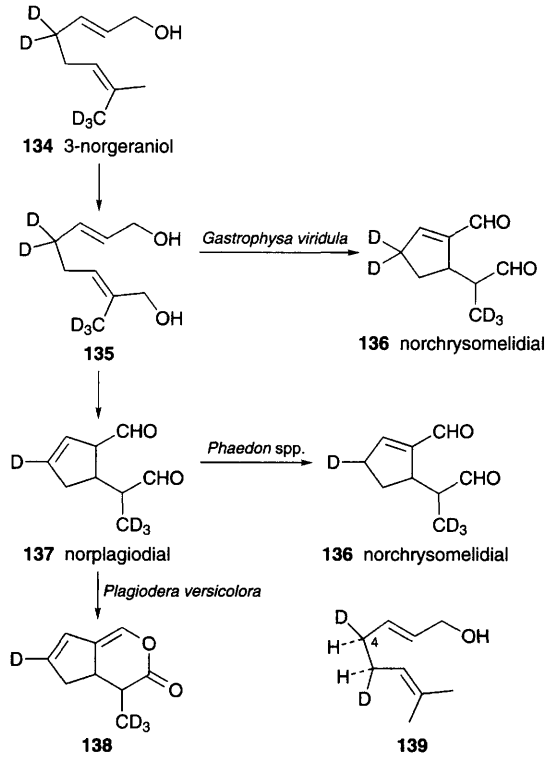
not derived from 7-*epi*-loganic acid **115** but instead from loganic acid **124** (Scheme 25). Deoxyloganic acid **86** was also a good precursor. Furthermore, deoxyloganic acid was shown to be hydroxylated at C-7 with retention of configuration, and H-7 from loganic acid was retained during transformation to secologanic acid. This must therefore exclude involvement of 7-ketologanic acid.

Larvae of the leaf beetles *Phaedon amoraciae*, *P. cochleariae*, *Gastrophysa viridula*, and *Plagiodera versicolora* synthesize iridials chrysomelidial **133** and/or plagiodial **132** as defensive secretions. Feeding experiments have shown these arise from geraniol **33** *via* 8-hydroxygeraniol **81** and the dialdehyde 8-oxocitral **82**, with the early part of this pathway (Scheme 27)



Scheme 27

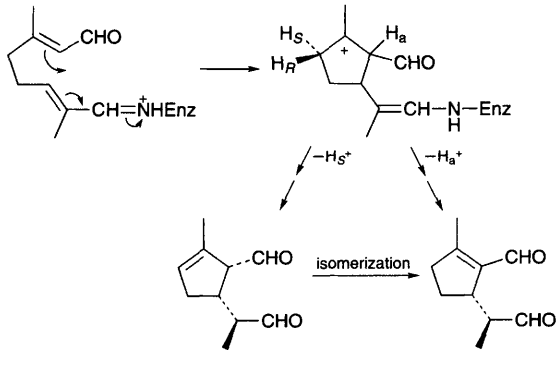
resembling the plant sequence to iridoids (Scheme 21).[127, 128] Since the insects were able to transform 3-norgeraniol **134** and 8-hydroxy-3-norgeraniol **135** into norchrysomelidial **136**, this substrate rather than the natural precursor was used as a probe. It was established that in *G. viridula*, [$^2H_1$]-8-hydroxy-3-norgeraniol **134** was incorporated with no loss of label. On the other hand, the *Phaedon* species transformed the substrate with loss of one H-4 deuterium, suggesting that they first synthesize 1-norplagiodial **137**, and that this is then isomerized to the more stable conjugated 1-norchrysomelidial (Scheme 28). In the case of *Plagiodera versicolora*, the insect produced



Scheme 28

1-norplagiodial and a further metabolite, 2-norplagiolactone **138**, each with four deuterium labels. Feeding experiments in *Phaedon amoraciae* with the chirally labelled [$^2H_2$]-3-norgeraniol **139** and its enantiomer established that the *pro-S* hydrogen was the one eliminated from position 4. Cyclization and proton loss are accommodated by the mechanism shown in Scheme 29, the process probably being initiated by Schiff base formation with the enzyme.

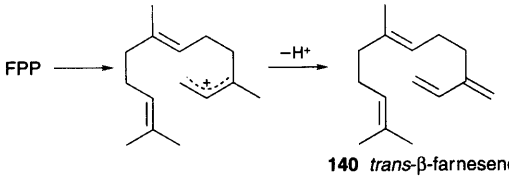
Reviews describing the biosynthesis of thujane monoterpenes,[129] genetic control of *Mentha* monoterpene biosynthesis,[130] the biosynthesis of monoterpenes[131–133] and the role of geraniol 8-hydroxylase in iridoid biosynthesis[134] have been published.



Scheme 29

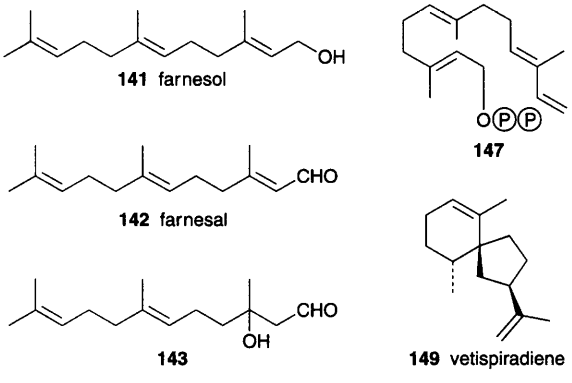
7 Sesquiterpenoids

The simple acyclic sesquiterpene *trans*-β-farnesene **140** is elaborated from FPP by an enzyme purified from maritime pine (*Pinus pinaster*).[135] The enzyme requires a divalent metal ion, with Mg^{2+} being preferred over Mn^{2+}. The reaction presumably involves cation formation and proton loss (Scheme 30). Farnesol **141** is transformed into farnesal **142** and

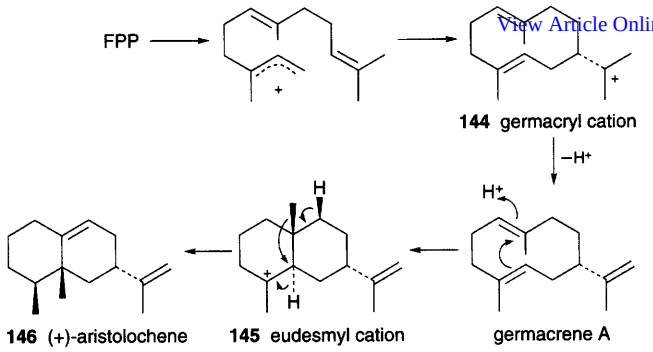


Scheme 30

3-hydroxy-2,3-dihydrofarnesal **143** by protoplasts of the microalga *Botryococcus braunii*, and both compounds were then efficiently incorporated into the triterpenoid hydrocarbons, the botryococcenes.[136] Fatty acid esters of farnesol, such as oleate, palmitate, palmitoleate and linolenate could be formed when fractions from homogenized cells were incubated with farnesol.[137] Farnesol was incorporated into squalene and botryococcenes by cultures of the algae, whereas the supernatant from cell homogenates converted FPP into squalene, but not into the botryococcenes.[138] The pellet fraction was able to phosphorylate farnesol to give mono- and di-phosphate esters utilizing CTP as the phosphate donor. ATP was less effective than CTP, and neither GTP nor UTP would participate. It is suggested that farnesol can be phosphorylated by the cultures to give FPP which is then incorporated into squalene, but the botryococcenes appear to be derived from farnesol rather than FPP. The farnesol-phosphorylating enzyme did not transform other isoprenoid alcohols such as geraniol and geranylgeraniol, and furthermore, would not phosphorylate farnesyl monophosphate.[139] This may indicate the membranes are permeable to farnesol but not its monophosphate.

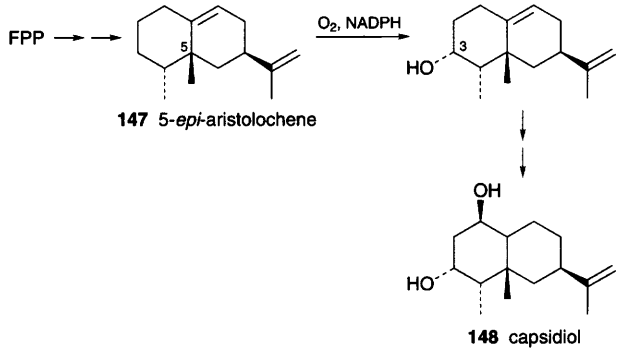


Aristolochene synthase accomplishes the cyclization of FPP to the germacryl cation **144** and its subsequent rearrangement *via* the eudesmyl cation **145** to (+)-aristolochene **146** (Scheme 31). The gene coding for this enzyme has now been isolated from *Penicillium roquefortii*, and expressed in *E. coli* as a fusion protein which has the appropriate sesquiterpene cyclase activity.[140] The aristolochene synthase coding sequence has since been amplified, and this has allowed overproduction of the protein to the level of about 40% of the bacterium's soluble protein.[141] The 12-methylidene analogue **147** of FPP has proved to be an effective mechanism-based inhibitor of aristolochene synthase.[142] This substrate analogue may probably be cyclized by the enzyme, but it could then undergo



Scheme 31 Enzyme: aristolochene synthase

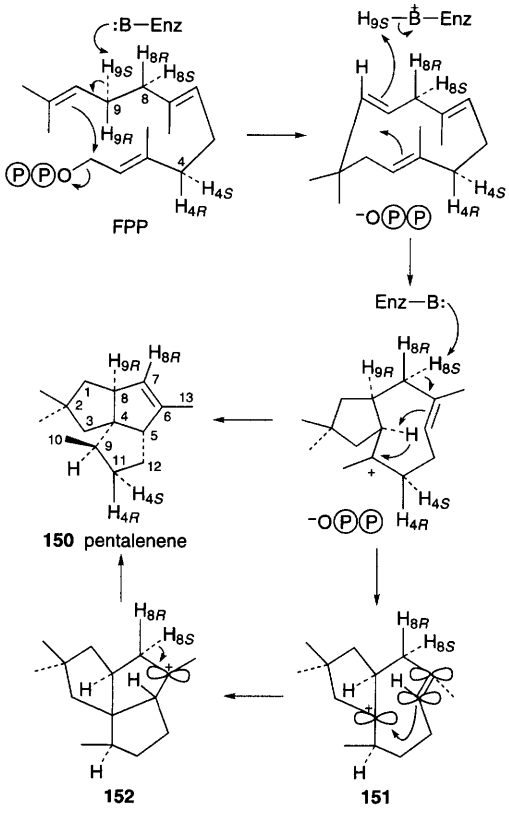
rearrangement or delocalization to place the positive charge in a region of the protein that does not normally encounter such charge, perhaps resulting in alkylation of the protein. 5-*epi*-Aristolochene **146a** is produced in tobacco (*Nicotiana tabacum*) and pepper (*Capsicum annuum*) as part of a sequence to sesquiterpene phytoalexins when the plant tissues are challenged by fungi. Its formation presumably requires similar skeletal rearrangements as in the aristolochene pathway, but a different conformation for the cyclizing FPP will be needed to attain the correct stereochemistry. In tobacco, the 5-*epi*-aristolochene synthase enzyme appears to be encoded by a complex gene family. A full length cDNA sequence has been cloned into *E. coli* to allow production of the enzyme at a level of 5–8% of total soluble protein.[143] Enzymic reaction products from the recombinant protein were identical to those from the native enzyme. 5-*epi*-Aristolochene **146a** is a good precursor of capsidiol **148** in arachidonic acid-treated green pepper seedlings,[144] and a 3-hydroxylase enzyme catalysing the first step in this pathway (Scheme 32) has been isolated.[145] This enzyme



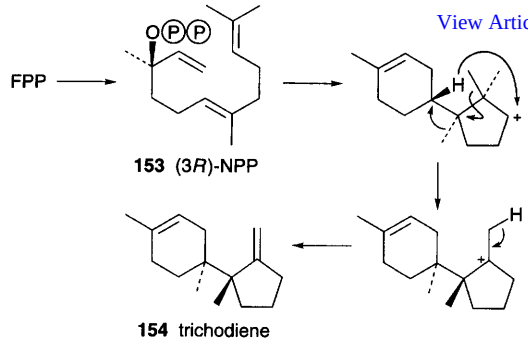
Scheme 32

was localized in the microsomes of elicitor-treated fruit and found to be a P-450-dependent system, requiring O_2 and NADPH cofactors. Alternative modifications to a eudesmyl cation can lead to vetispiradiene **149** and related compounds. Vetispiradiene synthase from *Hyoscyamus muticus* is encoded by a gene family of some 6–8 genes.[146] Genomic and cDNA clones have been isolated from cell cultures treated with a fungal elicitor preparation, and bacterial expression of three of these showed they all coded for enzymes giving the same single reaction product. The deduced amino acid sequence was 77% identical to that of 5-*epi*-aristolochene synthase from tobacco, though there were conspicuous differences, suggesting several partial reactions were common, but others were unique.

Earlier studies have established that during the biosynthesis of pentalenene **150**, the 9-*pro*-*S* hydrogen of FPP undergoes an intramolecular migration to the adjacent carbon, probably by transfer to a basic group on the enzyme, followed by its



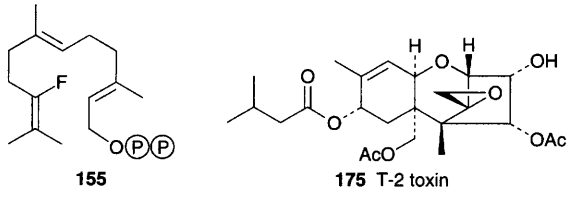
Scheme 33 Enzyme: pentalenene synthase



Scheme 34 Enzyme: trichodiene synthase

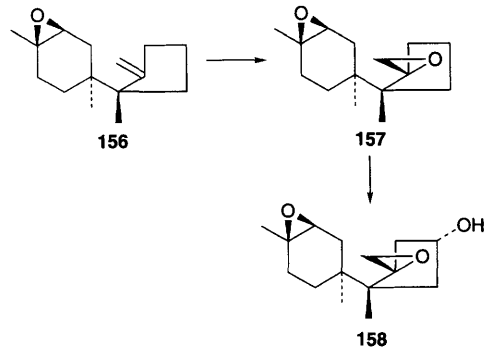
subsequent reincorporation, becoming the 1-*pro-S* hydrogen in pentalenene (Scheme 33). In more recent experiments, the stereochemical features of the electrophilic allylic addition–elimination ($S_{E'}$) reaction in which the C-4–C-5 bond of pentalenene is formed have been investigated.[147] Incubation of (4*R*,8*R*)-[4,8-$^{3}H_2$, 4,8-$^{14}C_2$]FPP with a pentalenene synthase preparation from *Streptomyces* UC5319 gave pentalenene retaining some 95% of the ^{3}H label, whilst only 53% of tritium was retained from the corresponding (4*S*,8*S*)-precursor. Thus, the 8-*pro-S* hydrogen from FPP is lost. The implications of this are as follows. Cyclization of the cation **151** involves attack on the *si* face of the *trans*-double bond, and the *p*-orbitals need to be aligned in a parallel orientation. If H_{8R} were then to be removed in the final deprotonation step, this would require a 30° rotation of the *p* orbital in the pentalenyl cation **152** to get the necessary alignment for double bond formation. Since H_{8S} is actually lost, the *p* orbital must therefore undergo a 90° rotation prior to deprotonation. It is hypothesized that a single base at the enzyme's active site could be responsible for deprotonation at C-9, reprotonation at C-10, and deprotonation at C-8 of the original FPP. Pentalenene synthase from *Streptomyces* has now been purified, cloned and sequenced.[148] Expression of the gene in *E. coli* has allowed production of a recombinant protein with properties identical to those of the native enzyme and at levels accounting for more than 10% of the soluble protein. The recombinant protein has also been crystallized and studied by X-ray diffraction analysis.[149]

The biosynthesis of trichothecene mycotoxins proceeds from FPP *via* the intermediate hydrocarbon trichodiene **154**, and its origin through preliminary isomerization to nerolidyl diphosphate (NPP) **153**, then cyclization and subsequent rearrangements, has been well investigated (Scheme 34). The trichodiene synthase gene from *Fusarium sporotrichioides* has previously been cloned and expressed at low levels in *E. coli*, and now, amplification of the coding sequence has allowed the protein to be expressed at much higher levels, accounting for some 20–30% of the total soluble protein.[150] The substrate specificities of the recombinant and native enzymes have been compared and found to be identical.[151] Although NPP is known to be produced initially from FPP by allylic isomerization, kinetic parameters show it to be a less attractive substrate for the enzyme. This contrasts with monoterpene cyclases, where LPP is typically 2–7 times more effective than GPP as substrate. In the case of trichodiene synthase, this may be the consequence of a slow conformational change to the enzyme after it has bound NPP. Several FPP analogues were found to act as competitive inhibitors of trichodiene synthase, with 10-fluoro-FPP **155** being the most



effective. Reaction with thiol-directed reagents has suggested the presence of two cysteine residues at the active site of the *F. sporotrichioides* enzyme.[152] The construction of mutants by site-directed mutagenesis has shown that C190A mutant (Cys replaced by Arg) retained partial activity, whilst C146A was essentially inactive. A hybrid enzyme constructed from amino acids 1–309 of *F. sporotrichioides*, and amino acids 301–383 of *F. sambucinum* had almost wild-type activity, and allowed construction of a further series of mutants *via* the base-rich region 302–306. Three such mutants were overexpressed and purified. Two of these, R304K and Y305T, when incubated with FPP, were found to accumulate both trichodiene and additional unidentified sesquiterpene hydrocarbons.

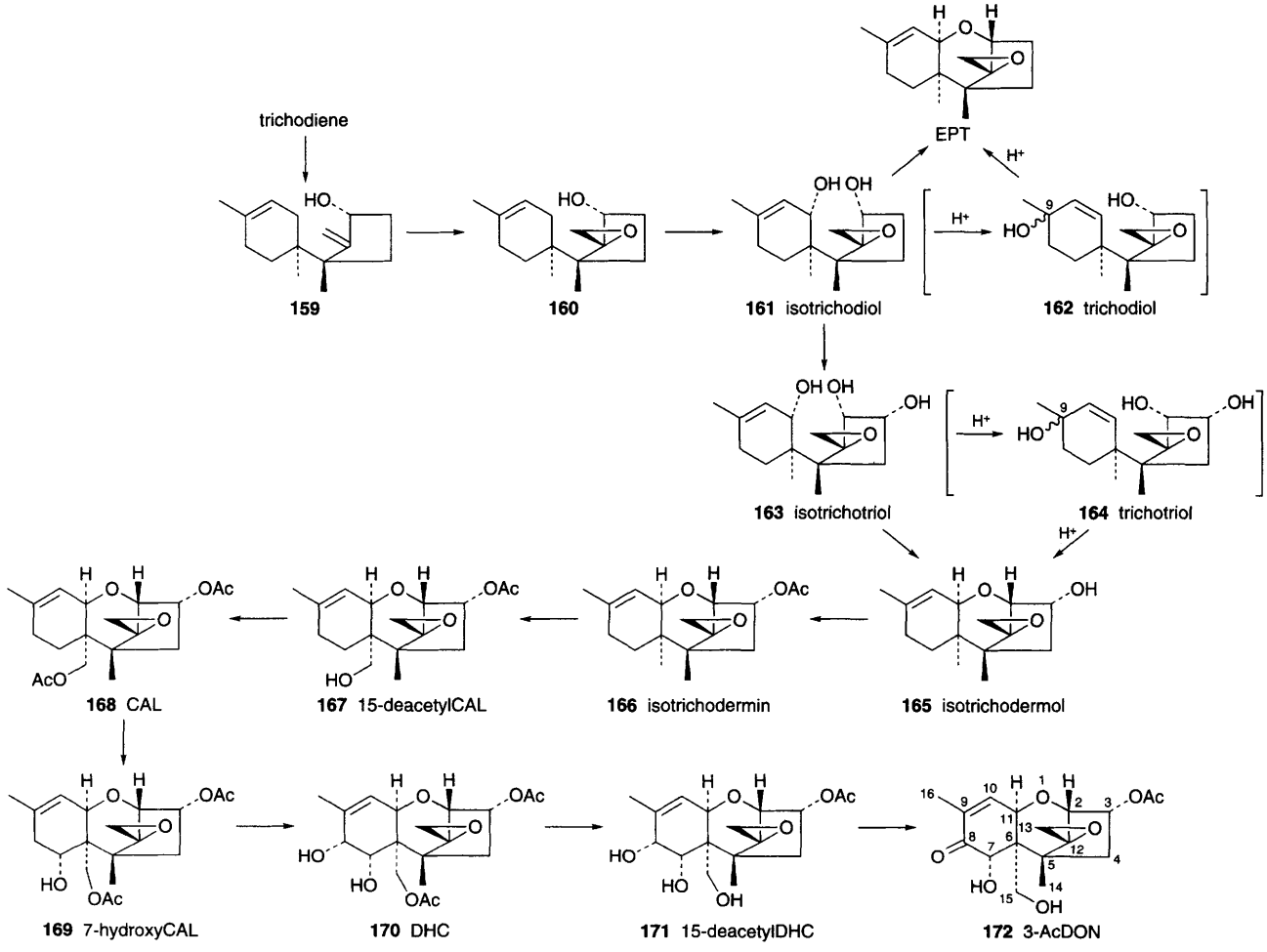
During formation of the trichothecene skeleton, trichodiene must be oxygenated at several positions, and subjected to further cyclization. Isotrichodiol **161** is a proven intermediate, and is suggested to be elaborated by the sequence shown in Scheme 36. Evidence supporting this comes from the observation that the trichodiene epoxide **156** was partially transformed by cultures of *Fusarium culmorum* into the diepoxide **157** and the hydroxylated product **158** (Scheme 35).[153] The epoxidase activity appears to be an O_2–NADPH dependent P-450 system, and is probably the same enzyme which introduces the epoxide function in the normal pathway. Since trichodiene is not transformed by a cell-free extract, and the 2-hydroxy-12,13-epoxy derivative (trichothecene numbering) **159** had previously been shown to be a trichothecene precursor, the sequence is likely to be **159**→**160**→**161**, which thus features two allylic oxidations (Scheme 36). For many years, trichodiol **162** had been postulated as an intermediate in the



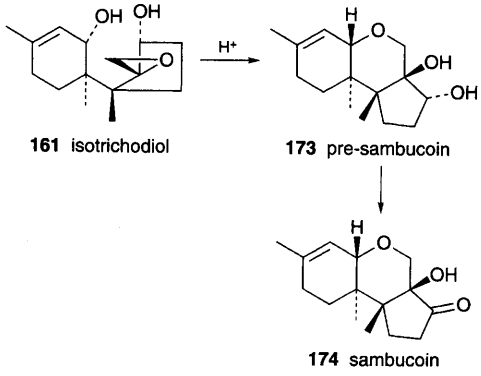
Scheme 35

biosynthesis of trichothecenes and more recently, trichotriol **164** was added to the sequence. These compounds are now shown to be formed as artefacts from isotrichodiol **161** and isotrichotriol **163**, respectively, and **161** and **163** are proposed as true intermediates.[154] Both 9-epimeric forms of trichodiol can be isolated from *Trichothecium roseum* and of trichotriol from *F. culmorum*. Such epimers equilibrate on treatment with acid, with cyclization then occurring if a 2-hydroxy group is present. All studies suggest trichodiol and trichotriol result from acid-catalysed isomerization of isotrichodiol and isotrichotriol, respectively, *via* the allylic cation. Isotrichodiol was a much better precursor of the trichothecenes 3-acetyldeoxynivalenol (3-AcDON) **172** and dihydroxycalonectrin (DHC) **170** in *F. culmorum* than trichotriol, the latter probably being incorporated after non-enzymic cyclization to isotrichodermol **165**. In competitive feeding experiments, the incorporation of isotrichodiol was not influenced by simultaneous administration of trichodiol, and only marginally by trichotriol, whereas isotrichotriol did repress incorporations. In later parts of the pathway, DHC **170** and 15-deacetylDHC **171** were shown to be good precursors of 3-AcDON **172**, indicating the last two steps are 15-deacetylation and then 8-oxidation.[153] Based on these and earlier studies, the complete pathway is now believed to be as shown in Scheme 36. Pre-sambucoin **173**, one of the products from acid-catalysed cyclization of isotrichodiol **161**, was shown to be efficiently incorporated into sambucoin **174**, a trichothecene-related metabolite in *F. culmorum* (Scheme 37).[154]

Two or more genes which are involved in the biosynthetic pathway to trichothecenes in *Fusarium sporotrichioides* appear to be linked with the trichodiene synthase gene *Tri5* (≡*Tox5*) in a gene cluster.[155] One of these (*Tri3*) is assigned to a transacetylase which acetylates 15-deacetylCAL **167** to CAL **168**, whilst *Tri4* is suggested to encode a P-450 enzyme, perhaps for the first oxygenation step in the pathway after trichodiene.[156] Transformants lacking a functional *Tri4* gene were unable to synthesize trichothecenes but could convert isotrichotriol into T-2 toxin **175**, suggesting all later enzymes were present. *Tri6* from the gene cluster specifies a protein similar to Cys_2His_2 zinc finger proteins, and disruption destroys the ability of *Fusarium sporotrichioides* to synthesize trichothecenes.[157] However, none of a variety of trichothecene precursors could be transformed into T-2 toxin, and transcription of both *Tri4* and *Tri5* was greatly reduced relative to the wild-type fungus. It is believed that *Tri6* encodes a protein involved in transcriptional regulation of trichothecene biosynthetic enzymes.



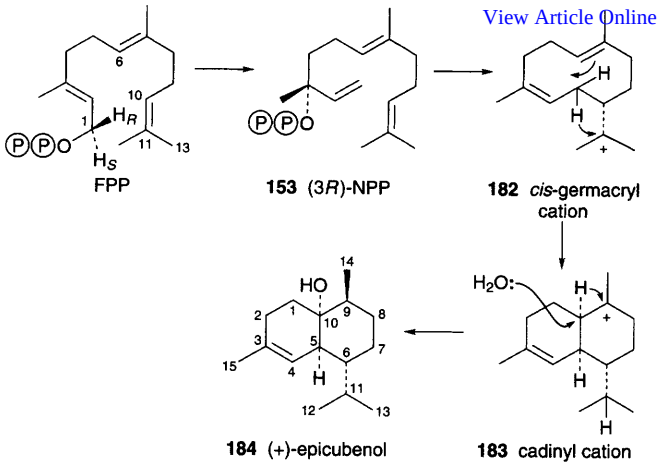
Scheme 36

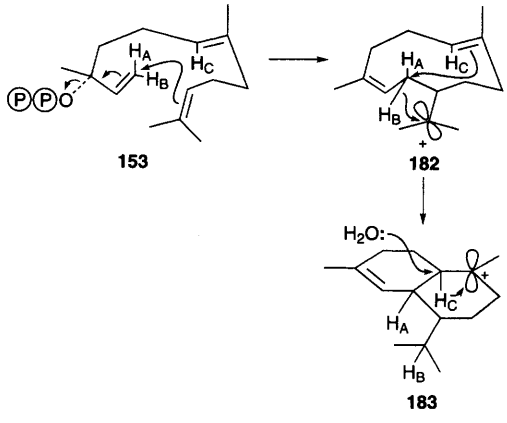


Scheme 37

Feeding of the deuteriated mevalonates [4-$^2H_2$]-, [5-$^2H_2$]- and [2-$^2H_2$]-MVA to callus cultures of *Perilla frutescens* var. *crispa* f. *purpurea* has exposed a number of hydride shifts in the biosynthesis of the constituent sesquiterpenes.[158] The pathways share a common FPP-derived bisabolyl cation **176** (Scheme 38).The labelling patterns in cuparene **179** demonstrate two concurrent pathways from **177**, one involving a 1,4-hydride shift to **178** (compare trichodiene above), whilst the other features two 1,3-hydride shifts. Aromatic ring formation then involves loss of one hydrogen from each of the methylenes in **178**. For α-curcumene **180**, a 1,2-hydride shift is demonstrated. β-Bisabolene **181** on paper should be formed merely by loss of a proton. Whilst this proton loss is confirmed, an unexpected change occurs in the cyclohexene ring, implicating partial migration of the double bond from C-2/3 to C-3/4. As a consequence, there is loss of a proton from C-4, and incorporation of a proton from the medium.

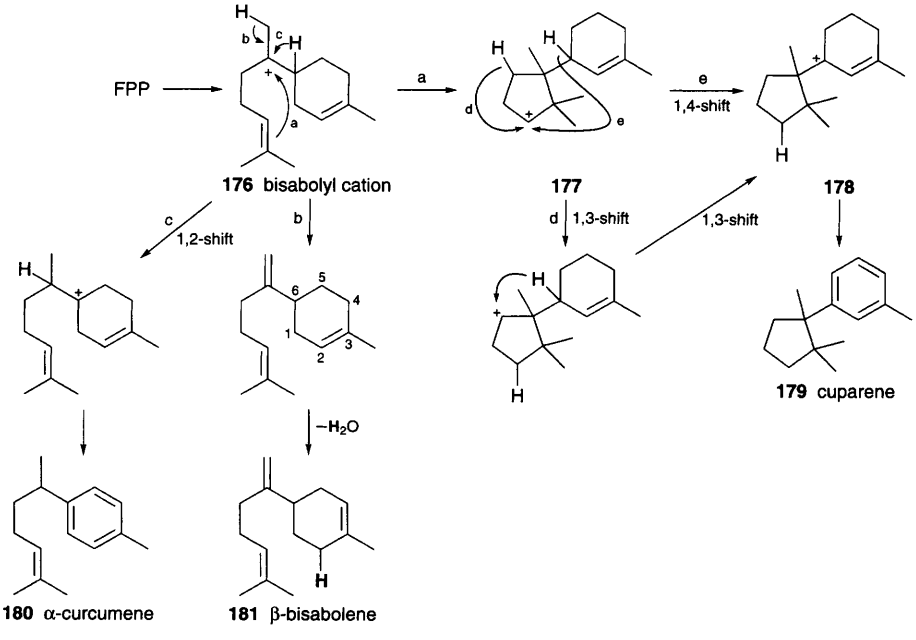
Sesquiterpenes of the cadinane series arise by cyclization of NPP to a ten-membered ring, followed by the formation of the decalin system with an accompanying 1,3-hydride shift (Scheme 39). A study of (+)-epicubenol **184** biosynthesis in a cell-free extract of *Streptomyces* sp. LL-B7 from [1-$^2H_2$]FPP confirmed the 1,3-hydride shift by the labelling of **184** at C-5 and C-11.[159] [13-$^2H_3$]FPP produced [13-$^2H_3$]labelled epicubenol. Incubation of [6-^{2}H]FPP with the epicubenol synthase preparation was then used to confirm the predicted 1,2-hydride shift from C-10 to C-9 (Scheme 39).[160] Continuing these





Scheme 39 Enzyme: epicubenol synthase

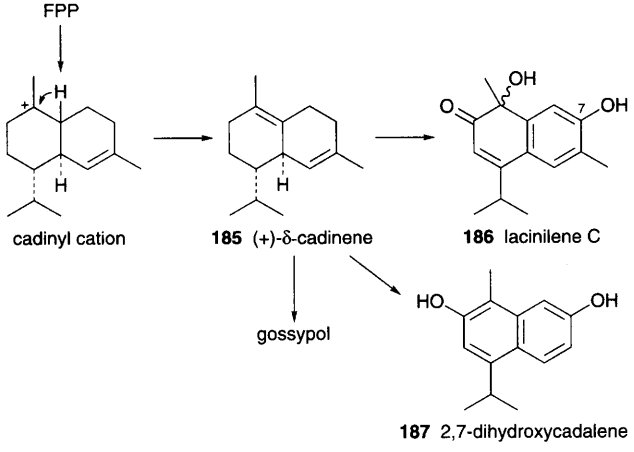
studies, it was then established that the migrating hydride from C-1 of FPP was the 1-*pro-S* hydrogen, the 1-*pro-R* hydrogen residing at C-5 in epicubenol.[161] The intermediate role of NPP was explored by feeding both (3*R*)- and (3*S*)-(1*Z*)-[1-^{3}H]NPP, with only the former giving a labelled product. Both (3*R*)-(1*Z*)-[1-^{2}H]NPP and (1*R*)-[1-^{2}H]FPP gave essentially the same ^{2}H



Scheme 38

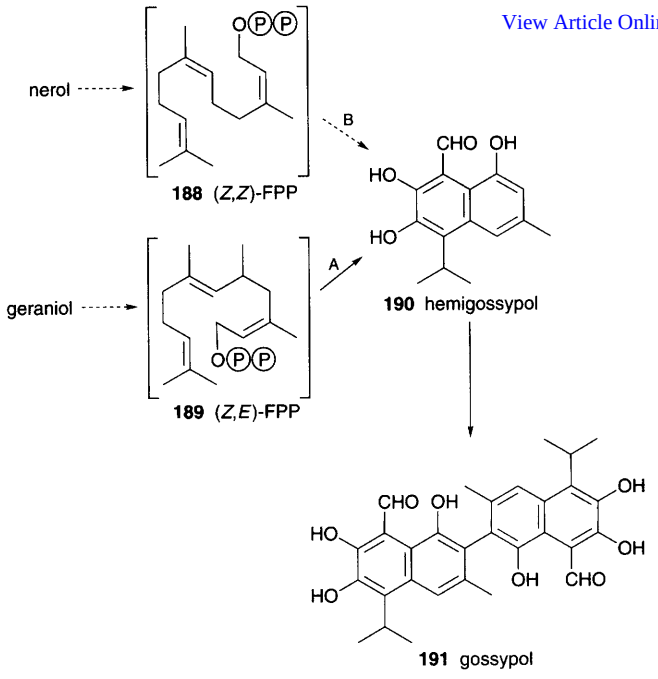
distribution as analysed by GC–MS, confirming the position of labelling as C-5. Thus, initial isomerization of FPP to NPP must occur with suprafacial stereochemistry as seen with trichodiene synthase, and also in the GPP to LPP isomerizations for monoterpene cyclases. Attack of the ionized NPP by the C-10–C-11 double bond will be on the *re* face, to generate the *cis*-germacryl cation **182**, and the original 1-*pro-S* hydrogen of FPP will be able to align with the vacant *p* orbital of the cationic centre. A second electrophilic cyclization gives the cadinyl cation **183**, and finally, capture of hydroxy group from water is in a *syn* sense with respect to the migrating hydride (Scheme 39).

Enzyme preparations from upland cotton (*Gossypium hirsutum*) tissues infected with *Xanthomonas campestris* pv. *malvacearum* are able to convert labelled FPP into (+)-δ-cadinene **185** (Scheme 40).[162] In the plant, this material is then



Scheme 40

incorporated into lacinilene C **186**, its 7-methyl ether and 2,7-dihydroxycadalene **187**, and may also be an early precursor of the cotton phytoalexins such as gossypol. A similar enzyme activity has been identified in extracts from *G. barbadense* infected with *Verticillium dahliae*.[163] By using [1-^{2}H]-labelled FPP as substrate, the formation of [5-^{2}H]- and [1-^{2}H]-labelled δ-cadinenes could be demonstrated, thus supporting the 1,3-hydride shift. Challenging cell suspension cultures of *G. arboreum* with a preparation from *V. dahliae* initiates production of gossypol, and this is accompanied by increased mRNA levels. This has led to the isolation of two cDNA clones, and the encoded proteins from these showed a significant degree of sequence identity with known plant terpene cyclases.[164] The resultant protein was expressed in *E. coli* and shown to cyclize FPP into (+)-δ-cadinene. Again, the conversion of this sesquiterpene into lacinilene C and 2,7-dihydroxycadalene by cotton plants was demonstrated. Understanding of the biosynthesis of the binaphthyl *bis*-sesquiterpene gossypol **191** in *Gossypium* species is confused because of conflicting experimental data. Thus, one earlier study had shown that the folding of FPP generating the aromatic sesquiterpene hemigossypol **190** was in accord with the involvement of (*Z*,*E*)-FPP **189** (route A), whilst another had justified folding consistent with the use of (*Z*,*Z*)-FPP **188** (route B) (Scheme 41). In an effort to clarify this dichotomy, gossypol biosynthesis in *Thespesia populnea* has been probed using a number of potential precursors labelled with ^{3}H and ^{14}C.[165] In this plant, feedings with [2-^{14}C, 5-$^3H_2$]MVA gave gossypol labelled in accord with route A, and consistent with the 1,3-hydride migration typical of cadinane systems. The incorporations of [1-^{14}C,1-$^3H_2$]geraniol, [1-^{14}C,1-$^3H_2$]farnesol and [2-^{14}C,4-$^3H_1$]MVA also fitted route A, but not route B, and inferred that GPP is not isomerized to NPP, nor is (*Z*,*E*)-FPP

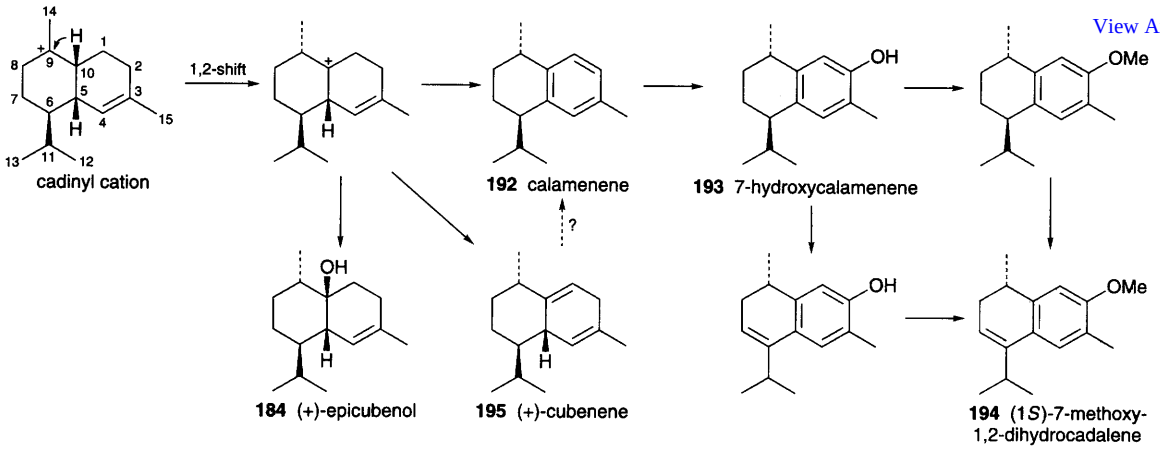


Scheme 41

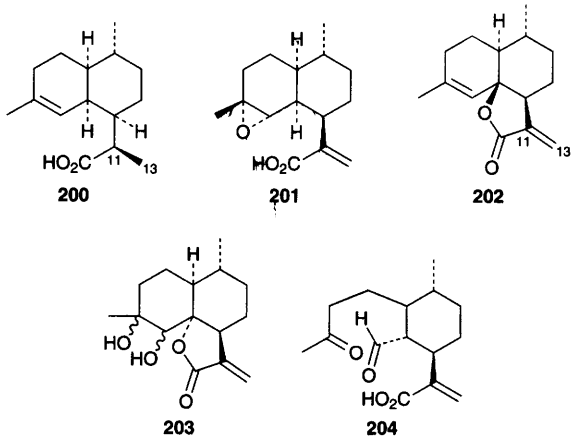
isomerized to (*Z*,*Z*)-FPP. However, when [1-^{14}C,1-$^3H_2$]nerol was fed, cyclization occurred according to route B. Accordingly, this indicates that the normal route from GPP is *via* FPP (and then presumably NPP) to the cadinyl cation, whereas if nerol is fed, this is chain extended and can participate *via* route B. Gossypol is ultimately derived by oxidative coupling of two hemigossypol residues (or similar), but no details are yet available.

Cultured cells of the liverwort *Heteroscyphus planus* accumulate a number of cadinane sesquiterpenes including 7-hydroxycalamenene **193** and 7-methoxy-1,2-dihydrocadalene **194**. The biosynthesis of these metabolites has been studied using a variety of ^{2}H- and ^{13}C-labelled MVAs.[166–168] The labelling patterns are consistent with a 1,3-hydride shift in the formation of the cadinyl cation, then a 1,2-hydride shift prior to aromatization to calamenene **192** (Scheme 42). The hydroxylation of calamenene must occur without any NIH shift. The conversion of 7-hydroxycalamenene **193** into 7-methoxy-1,2-dihydrocadalene **194** could occur by either of the two routes shown in Scheme 42, since the labelling patterns are consistent with either. The formation of (+)-epicubenol **184** and (+)-cubenene **195** in cell-free extracts from *H. planus* cultures has also been reported.[168] These experiments demonstrated the incorporation of (*E*,*E*)-[1-$^2H_2$]FPP but not (*Z*,*E*)-[1-$^2H_2$]FPP, with both hydrogen labels retained, though one was involved in the 1,3-hydride shift. Label from [6-$^2H_2$]FPP was incorporated into C-9, providing evidence for the 1,2-hydride shift from C-10 (Scheme 42). Whether or not cubenene is a precursor of calamenene and other metabolites has not been established.

Artemisinin (qinghaosu) **199** is a sesquiterpene derivative containing a peroxide linkage that has been shown to be crucial for the antimalarial activity displayed by this compound. Artemisinin is a modified cadinane derivative, and both arteannuic acid (≡artemisinic acid) **196** and arteannuin B **197** are shown to be precursors. Labelled arteannuic acid was incorporated into arteannuin-B and artemisinin in both *Artemisia annua* plants, and in a cell-free system from *A. annua* leaves.[169] Use of oxidizing cofactors Fe^{2+}–2-oxoglutarate, or peroxidase–H_2O_2 enhanced incorporations *in vitro*. Arteannuin B was converted into artemisinin using a crude extract from *Artemisia annua* leaves, as well as in a semi-purified cell-free extract.[170] A possible mechanistic sequence has been proposed, in which the last step is the reduction of the



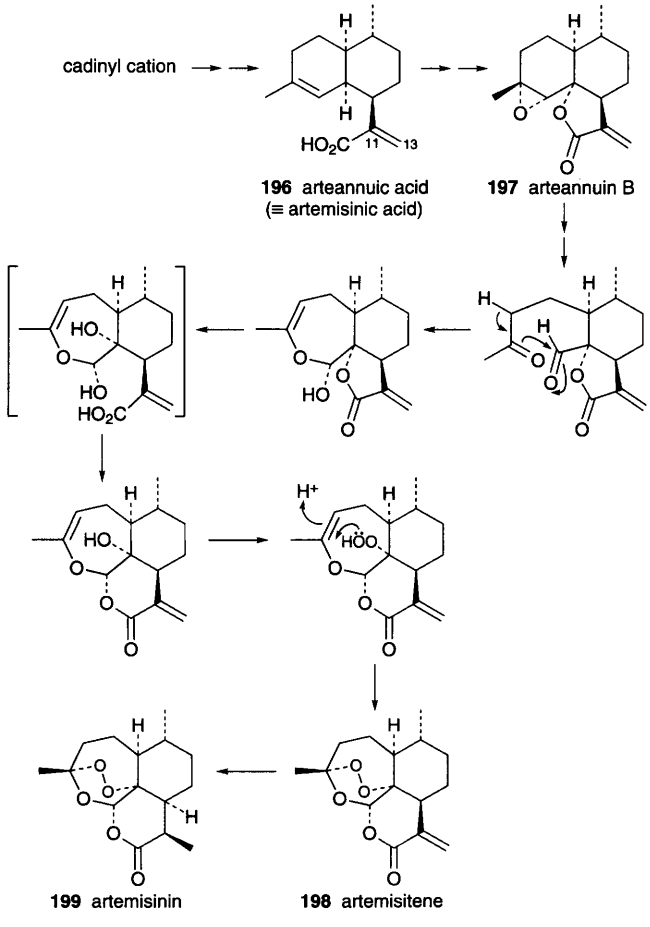
Scheme 42



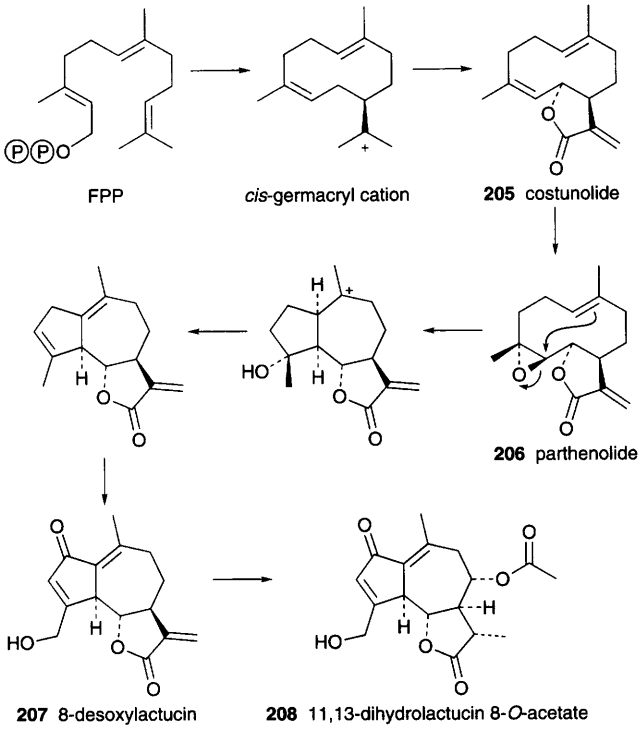
11,13-double bond in artemisitene **198** (Scheme 43). In other studies, 11,13-dihydroarteannuic acid **200** has been employed as a precursor of artemisinin.[171,172] Arteannuic acid and dihydroarteannuic acid were reported to be precursors of artemisinin in leaf homogenates of *A. annua*, and arteannuic acid and epoxyarteannuic acid **201** the precursors of arteannuin B.[172] In another report, 6-*epi*-deoxyarteannuin B **202** and 11,13-dihydro-6-*epi*-deoxyarteannuin B could be used as precursors of artemisinin.[173] Oxygen was necessary for formation of artemisinin from the latter lactone by the leaf homogenates. The isolation of the cadinanolide **203** and the secocadinane **204** from *A. annua* may implicate these compounds as post-arteannuin B intermediates in the biosynthesis of artemisinin, and a variant on the pathway shown in Scheme 43 has been put forward.[174]

The biosynthesis of guaianolide sesquiterpenes in hairy root cultures of blue-flowered lettuce (*Lactuca floridana*) has been investigated by feeding ^{13}C-labelled acetate (single- and double-labelled) and MVA.[175] The labelling patterns in the carbon skeletons of 8-desoxylactucin **207** and 11,13-dihydrolactucin 8-*O*-acetate **208** were entirely consistent with the pathway shown in Scheme 44, and the germacranolides costunolide **205** and parthenolide **206** are suggested as intermediates.

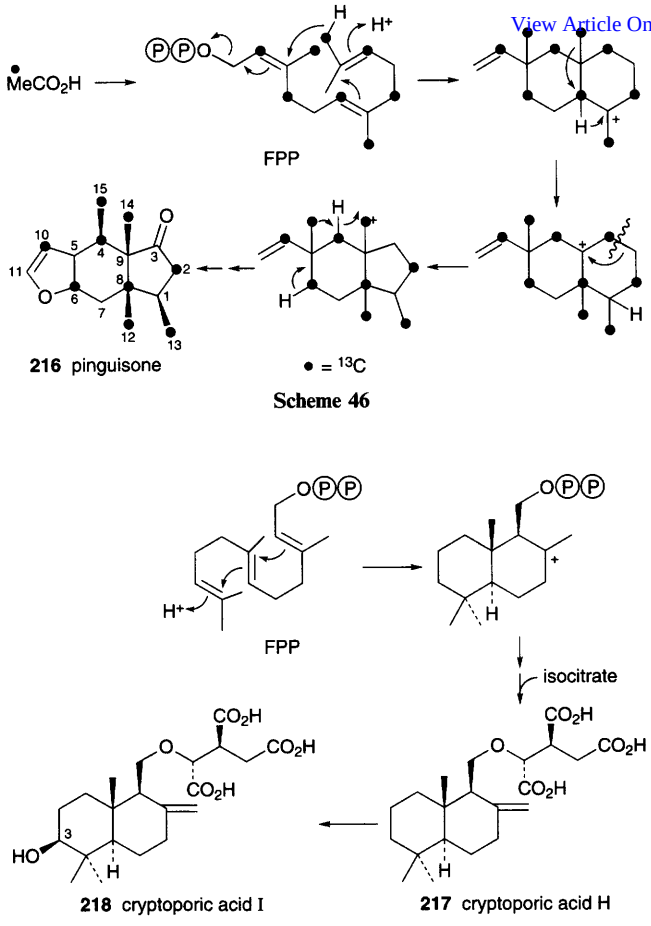
Fatty acid esters of the marasmane sesquiterpene velutinal **209** in the fruit bodies of the fungus *Lactarius vellereus* are converted into dialdehyde derivatives, *e.g.* isovelleral **214** and the lactarane velleral **211**, when the fungus is damaged. These dialdehydes act as a chemical defence system to protect against parasites but are then subsequently reduced to inactive alcohols, *e.g.* isovellerol **215** and vellerol **212**. [12-^{2}H$_3$]Velutinal proved to be efficiently transformed into both isovellerol **215** and vellerol **212** when fed to freshly ground fruit bodies.[176] Velleral **211** is believed to arise from velutinal **209** by elimination of a water equivalent (Scheme 45). The ring expansion process involved in the formation of velleral **211** was probed by reconstituting freeze-dried fruit bodies in $^{2}H_2O$, but this did not lead to any deuterium labelling. This suggests the proton at C-3 required during the cyclopropane ring opening originates from velutinal and is not introduced from water. This is accommodated by a mechanism in which a C-4 proton migrates to C-3 as shown in Scheme 45. Involvement of the enol acetal **210** would also account for the formation of other identified metabolites such as piperdial and *epi*-piperdial **213**.



Scheme 43



Scheme 44

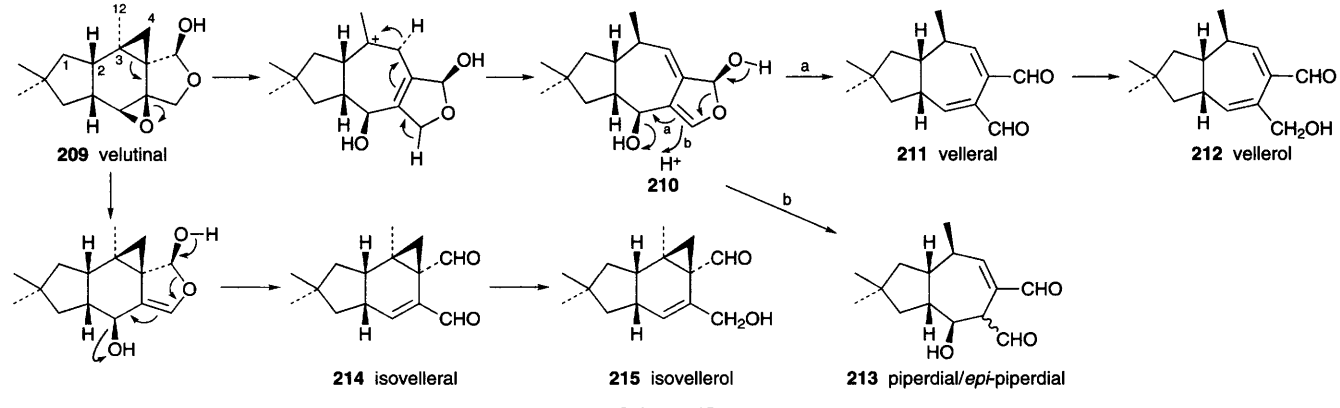


Scheme 47

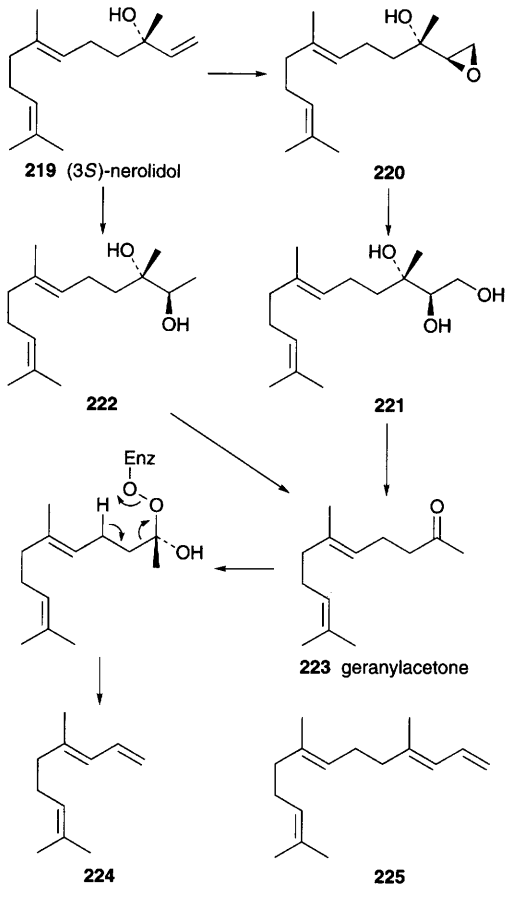
Feeding experiments using [2-^{13}C]acetate have been used to outline the biosynthetic origins of pinguisone **216** in cultures of the liverwort *Aneura pinguis*.[177] The labelling pattern observed is consistent with the pathway outlined in Scheme 46. ^{13}C–^{13}C couplings for C-4/C-15 and C-8/C-12 indicated that two methyl migrations had occurred, and the labellings also showed that bond cleavage of the main FPP chain was responsible for production of the indane system *via* a decalin skeleton. Hydride shifts as indicated will also be required in the proposed sequence.

The drimane sesquiterpenoid cryptoporic acid I **218** from the fungus *Ganoderma neojaponicum* has a hydroxy group at C-3 and could in principle be derived by cyclization of epoxyFPP by a mechanism similar to that of steroid biosynthesis, or alternatively may be formed by direct hydroxylation of cryptoporic acid H **217** (Scheme 47). The latter pathway is operative as demonstrated by the good incorporation of cryptoporic acid H into **218**.[178] Furthermore, incorporation of **217** was inhibited when the P-450 inhibitor ancymidol was also employed.

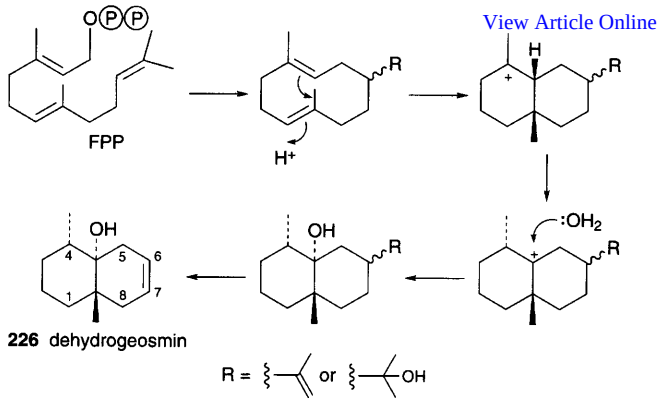
A group of so-called homoterpenes containing eleven carbons are, in fact, more correctly considered as degraded sesquiterpenes, since they are formed by oxidative cleavage of four carbons. Thus, 4,8-dimethylnona-1,3,7-triene **224** is known to originate in a range of plants from nerolidol **219**. In a series of feeding experiments to explore the oxidative processes, ^{2}H-labelled precursors were fed to lima bean (*Phaseolus lunatus*) plantlets.[179] Nerolidol **219**, geranylacetone **223**, the epoxide **220**, the diol **222** and the triol **221** were all converted into **224**, indicative of two possible sequences as shown in Scheme 48. The chirality of the putative intermediates was established by feeding 1:1 racemic mixtures where the enantiomers had different labelling patterns, and a high degree of enantioselectivity was observed. This sequence exhibits a formal analogy to the P-450 linked processes which are responsible for cleaving the side-chain of steroids, *e.g.* cholesterol→pregnenolone→androstanes. The degree of enantioselectivity



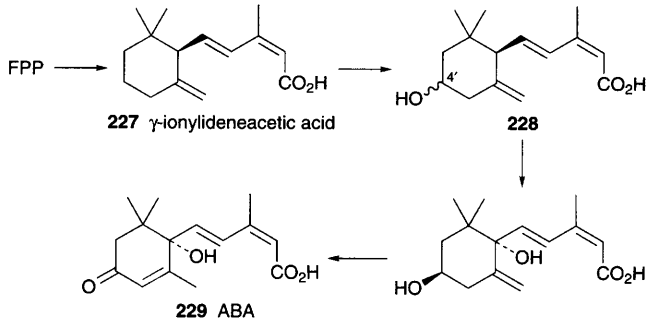
Scheme 45



Scheme 48



Scheme 49

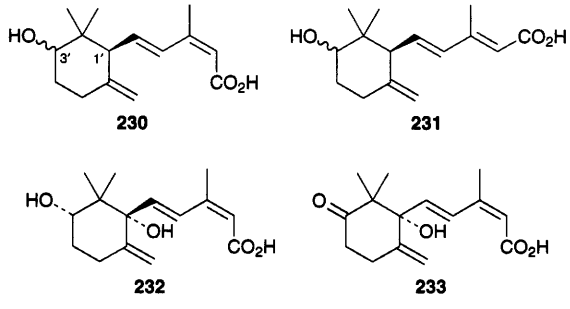


Scheme 50

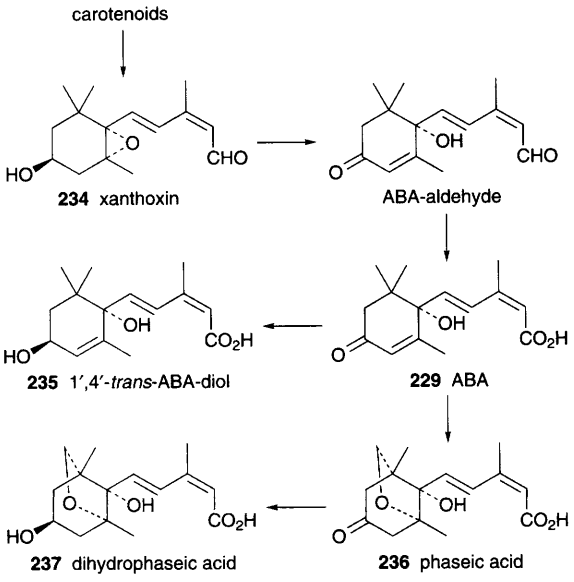
(96%) towards (3*S*)-nerolidol demonstrated by *Phaseolus lunatus* is not found in other plants, and varies quite widely.[180] Thus, only a moderate degree of enantioselectivity (*S*:*R*= 66:34) was found in *Gossypium herbaceum* and *Fragaria magna*, and other figures recorded were *Spathiphyllum wallissi* (94:6), *Gerbera jamesonii* (82:18), *Lycopersicon esculentum* (77:23) and *Humulus lupulus* (77:23), with 76:24 being observed for another cultivar of *P. lunatus*. The formation of these volatile homoterpenes is usually triggered by damage caused by insect infestation. It has also been shown that treatment of healthy undamaged plants of Lima bean with β-glucosidase leads to formation of volatiles, predominantly **224** and the diterpene-derived **225**, identical to those triggered by insect damage.[181]

The irregular C_{12}-terpenoid dehydrogeosmin **226**, the dominant component of the flower scent of the cactus *Rebutia marsoneri* is also a degraded sesquiterpene, and its formation involves loss of the C_3 side-chain from a eudesmane-type intermediate (Scheme 49).[182] Farnesol has been shown to be incorporated into dehydrogeosmin, and studies with a number of ^{2}H-labelled farnesols provided evidence for the 1,2-hydride shift in Scheme 49.

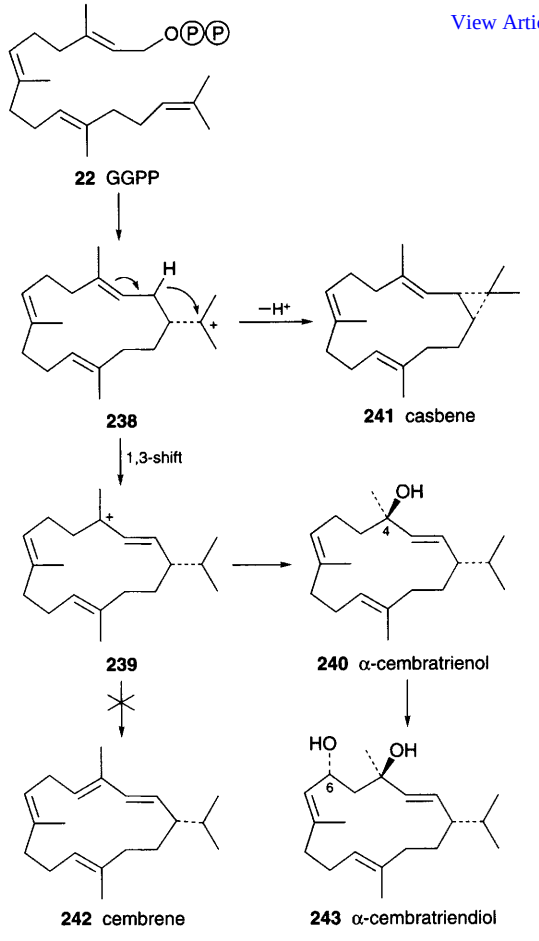
The plant growth regulator abscisic acid (ABA) **229** is produced from FPP by two main routes, which involve either direct cyclization of the C_{15} precursor or alternatively initial formation of a C_{40} carotenoid followed by oxidative metabolism. The former direct pathway is utilized by several species of fungi, whilst plants produce ABA by the indirect carotenoid route. Even in fungi, routes vary, and in *Cercospora cruenta*, a pathway *via* γ-ionylideneacetic acid **227** is favoured. Earlier feeding experiments with labelled **227** had demonstrated that it was converted into ABA *via* epimeric 4′-hydroxyacids **228** (Scheme 50), whilst two epimeric 3′-hydroxy acids **230** also formed appeared to fall outside the ABA pathway. In recent studies, feeding **227** to *C. cruenta* actually gave four 3′-hydroxy acids, the epimeric pair **230** and smaller amounts of the corresponding *E* analogues **231**.[183] Further feeding experiments with the 3′*S* epimer of **230** showed it to be metabolized by 1′-hydroxylation to **232** and then oxidation to the ketone **233**, as well as partial isomerization to the 3′*S*-epimer of **231**.



In plants, carotenoids with a suitable ring system are cleaved through the polyene chain to xanthoxin **234**, which is then modified to ABA (Scheme 51). A cell-free system from *Citrus sinensis* has been shown to achieve ABA synthesis from β-carotene, thus carrying out the necessary carotenoid ring modifications as well as the later steps.[184] Indeed, MVA was also utilized by the cell-free system giving ABA, phaseic acid **236** and 1′,4′-*trans*-abscisic acid diol **235**.[185] The diol **235** was the major product, and was shown to be a metabolite of ABA, rather than its precursor. Thus, addition of unlabelled ABA as a cold-pool trap increased the incorporation of activity into ABA but reduced the levels in the diol and phaseic acid. The major metabolite of ABA in sunflower (*Helianthus annuus*) embryos was the 4′-*O*-glucoside of dihydrophaseic acid **237**.[186] Smaller amounts of phaseic acid **236** and dihydrophaseic acid were also formed. Phaseic acid is formed as a result of



Scheme 51



Scheme 52

hydroxylation of one of the *gem*-methyls followed by cyclization. In tomato (*Lycopersicon esculente*), 1′,4′-*trans*-abscisic acid diol **235** may also serve as a source of ABA.[187]

A number of reviews covering aspects of sesquiterpene biosynthesis have been published. These include discussions on the chemistry and genetics of trichothecene biosynthesis,[188] the biochemistry and regulation of trichothecene biosynthesis,[189] macrocylic trichothecenes,[190] the use of deuterium labelled precursors in the biosynthesis of sesquiterpenes in *Perilla* and *Heteroscyphus*,[191] abscisic acid biosynthesis and metabolism,[192, 193] and aromadendrene sesquiterpenoids.[194]

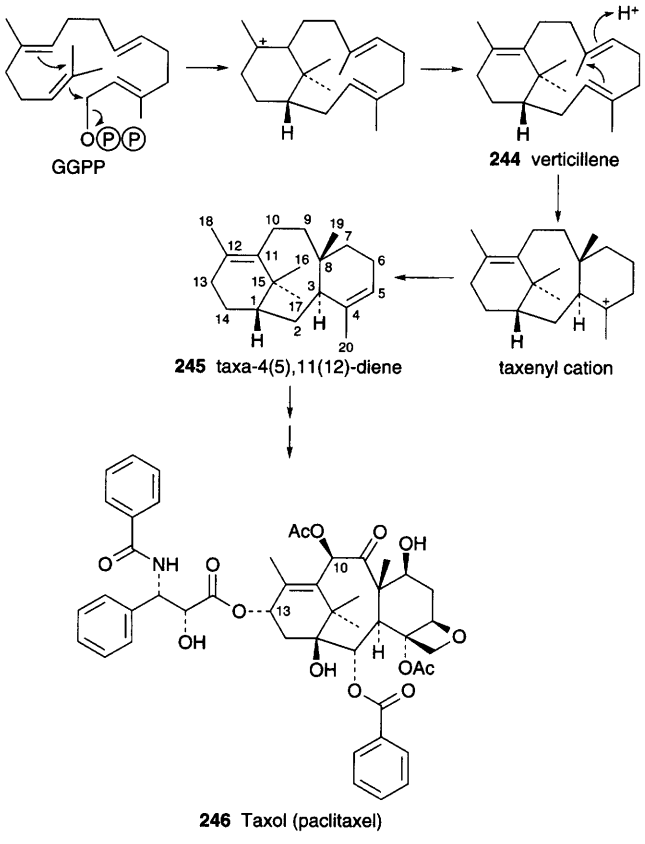
8 Diterpenoids

The cembranoid diterpenes are the principal components of the gummy surface exudates of tobacco (*Nicotiana tabacum*). Cell-free extracts from the trichomes of tobacco catalysed the conversion of geranylgeranyl diphosphate (GGPP) **22** into α-2,7,11-cembratrien-4-ol **240** and its 4β-epimer.[195] The enzyme activity resembled other plant terpene cyclases in its solubility, M_r, pH optimum and requirement for Mg^{2+}. That the products of cyclization were alcohols, rather than the alkene cembrene **242**, necessitates a revised pathway for the biosynthesis of the cembratriendiols, *e.g.* **243** (Scheme 52), in which the initial cyclized cation **238** undergoes charge relocalization to **239** (probably *via* a 1,3-hydride shift) which is then quenched with water. Further hydroxylation gives compound **243**. An earlier suggestion included cembrene **242** as an intermediate, and derived this by ring opening of casbene **241**. Casbene is formed from GGPP by the action of casbene synthase from the castor oil plant, *Ricinus communis*. A near full-length casbene synthase cDNA clone has been isolated from elicited castor seedlings.[196] The deduced amino acid sequence of the encoded protein had 42% identity with 5-*epi*-aristolochene synthase from tobacco, and 31% identity with limonene synthase from spearmint, indicating common features in the biosynthesis of mono-, sesqui- and di-terpenes.

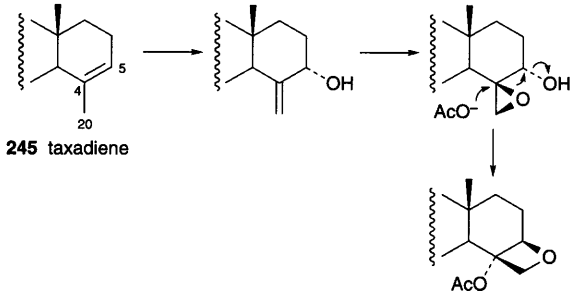
The diterpenoid portion of the important anticancer agent Taxol (paclitaxel) **246** has its origins in GGPP, and a soluble taxadiene synthase preparation from the stem bark and cambium of Pacific yew (*Taxus brevifolia*) has been isolated.[197] Activity levels in yew were low and could not be induced by stem wounding or elicitor treatment in marked contrast to the diterpene cyclase abietadiene synthase from lodgepole pine and grand fir (see below). The partially purified enzyme had similar properties to abietadiene synthase, in respect of kinetic properties, M_r, and a requirement for a divalent metal ion, with Mg^{2+} being the best. It converted GGPP into a taxadiene, but unexpectedly, this was characterized as the 4(5),11(12)-diene **245**, rather than the 4(20),11(12)-diene which had been predicted on the basis of many natural taxoids with double bonds in those positions.[198] This structure has been confirmed by synthesis.[199] The formation of **245** is rationally formulated as shown in Scheme 53, *via* the bicyclic verticillene **244**. Labelled taxadiene **245** was then shown to be a precursor of the complex taxoids by feeding experiments in *Taxus brevifolia* leaf discs, where it was incorporated into several products including Taxol **246** and its 10,13-diol analogue 10-deacetylbaccatin III. The common occurrence of taxoids with 4(20)-ene-5-oxy functionality, and the lack of oxygenated derivatives with a 4(5)-double bond suggests hydroxylation at C-5 of taxadiene with allylic rearrangement may be the next step in the sequence. Then, formation of the oxetane ring is formulated as *via* the epoxide with subsequent ring expansion (Scheme 54).

A novel fungus *Taxomyces adreanae* has been isolated from the inner bark of *Taxus brevifolia* and shown to synthesize Taxol and other taxanes *de novo* in culture.[200] Both ^{14}C-acetate and ^{14}C-phenylalanine were incorporated into Taxol. Unfortunately, the yields of Taxol are very small, and this does not yet provide a satisfactory system for Taxol production. Nevertheless, the possibility that there has been genetic transfer between the tree and an associated fungus is most interesting.

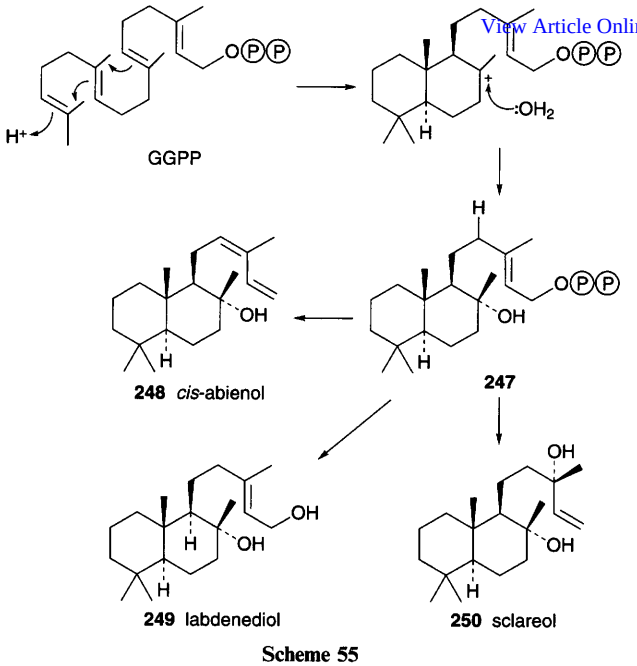
Cell-free extracts from trichome-bearing tissues of tobacco (*Nicotiana tabacum*) convert GGPP into the bicyclic labdane diterpenoid *cis*-abienol **248** (Scheme 55).[201] The enzyme activity was soluble, was stimulated by Mg^{2+} and was unaffected by P-450 inhibitors. Similarly, extracts from *Nicotiana*



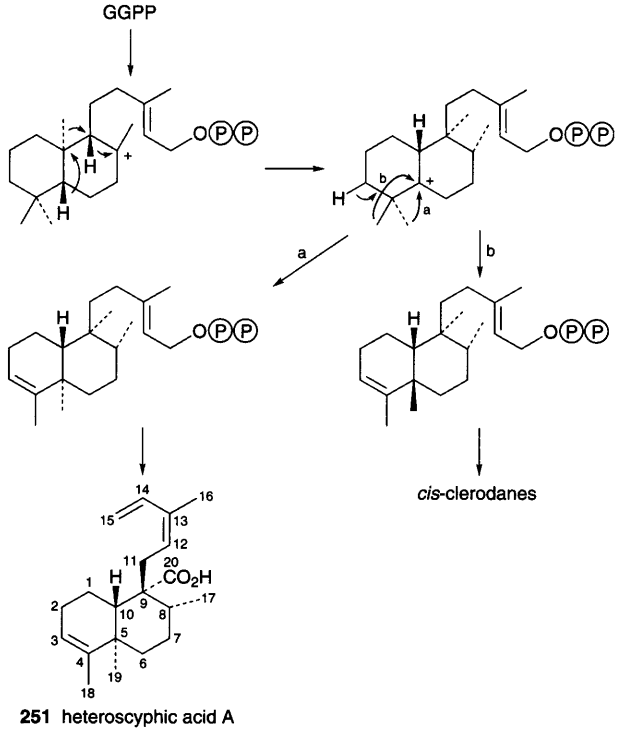
Scheme 53



Scheme 54



Scheme 55

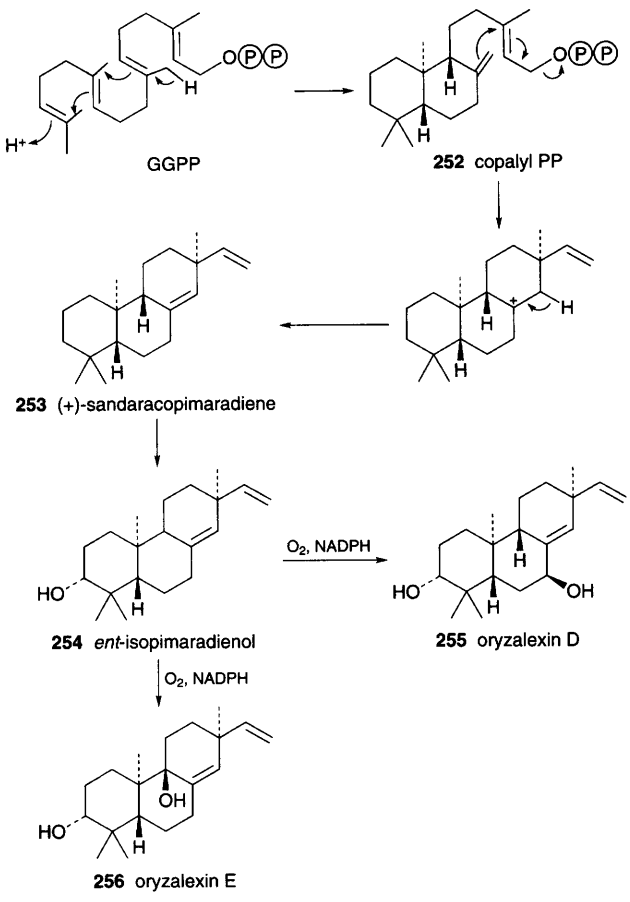


Scheme 56

glutinosa converted GGPP into labdenediol **249** and sclareol **250**,[202] exemplifying variations in the fate of the diphosphate **247** (Scheme 55). The two activities in *N. glutinosa* co-purified throughout the purification process.

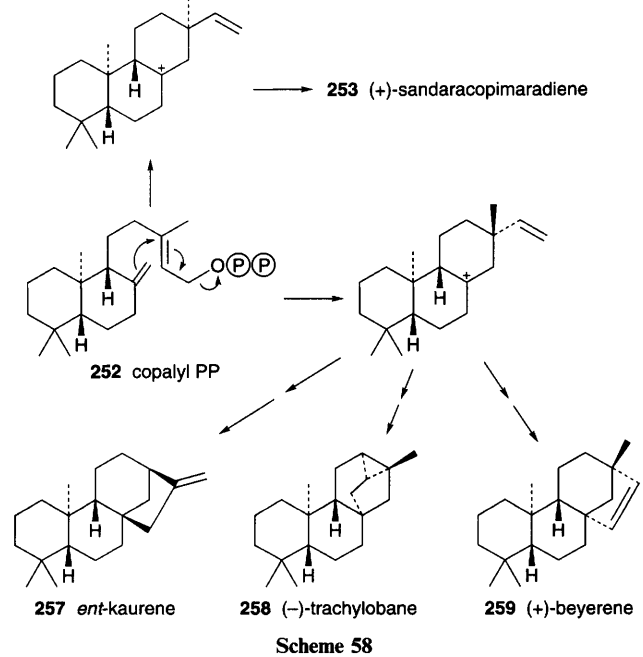
Cultured cells of the liverwort *Heteroscyphus planus*, as well as producing sesquiterpenes of the cadalane type (see above), also synthesize a variety of diterpenes of the clerodane type. The biosynthesis of one of these, heteroscyphic acid A **251** has been investigated by feeding ^{13}C-labelled acetate and mevalonate precursors.[203, 204] It was observed that there was preferential labelling of the C_5 units in the FPP-derived portion of the diterpene, suggesting FPP from exogenous MVA was combining with endogenous IPP, probably in the chloroplasts. In contrast, the sesquiterpene C_5 units were labelled equivalently. A similar non-equivalent labelling from MVA of the phytyl side-chain of chlorophyll a was noted, though if glycine was used as precursor, then equivalent labelling ensued.[205] The labelling patterns in heteroscyphic acid A were consistent with Scheme 56, where folding of the GGPP precursor achieves a different stereochemistry to the labdanes in Scheme 55. The labelling confirmed the involvement of a 1,2-methyl migration as responsible for the introduction of the carboxy group C-20, and also a 1,2-methyl migration of the 19-methyl from C-4 to C-5 in producing the *trans*-clerodane skeleton. The *cis*-clerodanes would be obtained by migration of the alternative C-4 methyl.

The rice phytoalexins, the oryzalexins, are produced by a similar cyclization of GGPP as seen for the clerodanes above, but proton loss generates copalyl diphosphate (PP) **252** (Scheme 57). Further cyclization initiated by loss of the diphosphate allows formation of (+)-sandaracopimaradiene (*ent*-isopimaradiene) **253** This is the likely substrate for oxgenation reactions, beginning with the 3-hydroxy group. The conversion of the *ent*-isopimara-8(14),15-dien-3β-ol **254**, which is formed in UV-irradiated *Oryza sativa* leaves, into



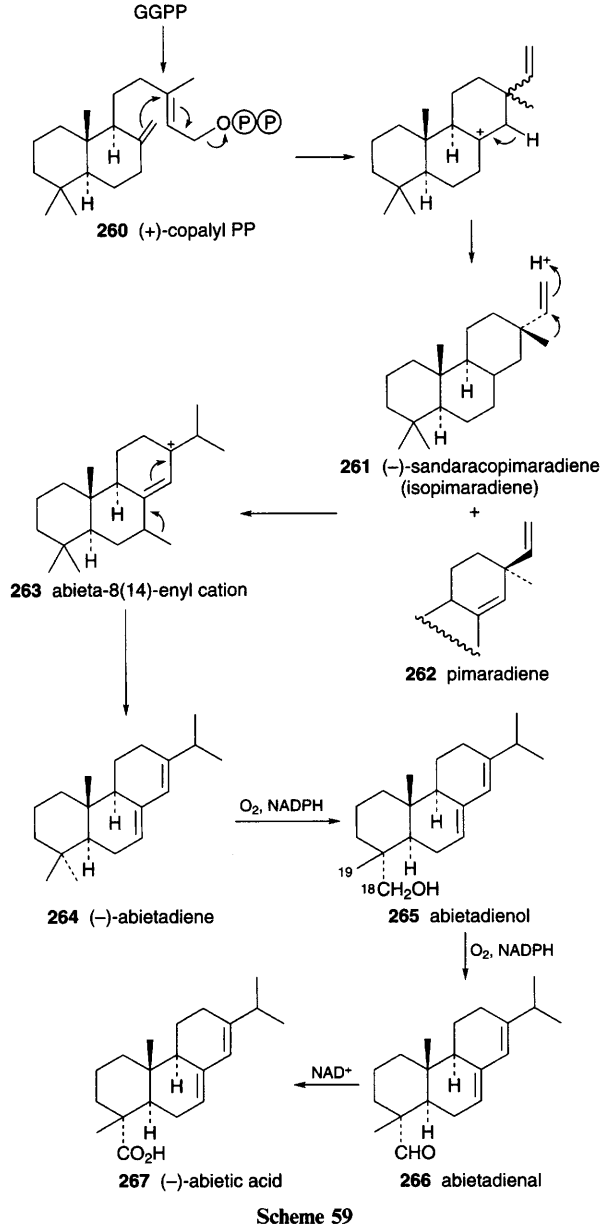
Scheme 57

oryzalexin D **255** and oryzalexin E **256** is catalysed by a microsomal fraction from the leaves.[206] The conversion was dependent on O_2 and NADPH, and appears to be an inducible P-450 hydroxylase system. Diterpene cyclase activities in castor oil plant (*Ricinus communis*) are responsible for alternative cyclizations of copalyl PP **252** (Scheme 58). The four activities, generating (−)-*ent*-kaurene **257**, (+)-beyerene **259**, (−)-trachylobane **258**, and (+)-sandaracopimaradiene **253** from copalyl PP, reported some years ago in a cell-free extract, have recently been resolved and partially purified.[207]



Scheme 58

(−)-Abietic acid **267** is a major component of the rosin fraction of the oleoresin synthesized by grand fir (*Abies grandis*), lodgepole pine (*Pinus contorta*), and many other conifers as a defensive secretion against insect and pathogen attack. High levels of resin acid biosynthesis are induced in grand fir by stem wounding, whereas lodgepole pine produces these materials constitutively. Abietic acid is derived from GGPP *via* (−)-abieta-7(8),13(14)-diene **264**, with subsequent oxidation of the C-18 methyl. Soluble extracts of lodgepole pine stem and mechanically wounded grand fir stem catalysed the Mg^{2+}-dependent cyclization of GGPP to (−)-abietadiene **264**, along with small amounts of pimaradiene **262** and its epimer (−)-sandaracopimaradiene (isopimaradiene) **261**, which are likely to be intermediates in the sequence (Scheme 59).[208] The product mixture from grand fir contained about



Scheme 59

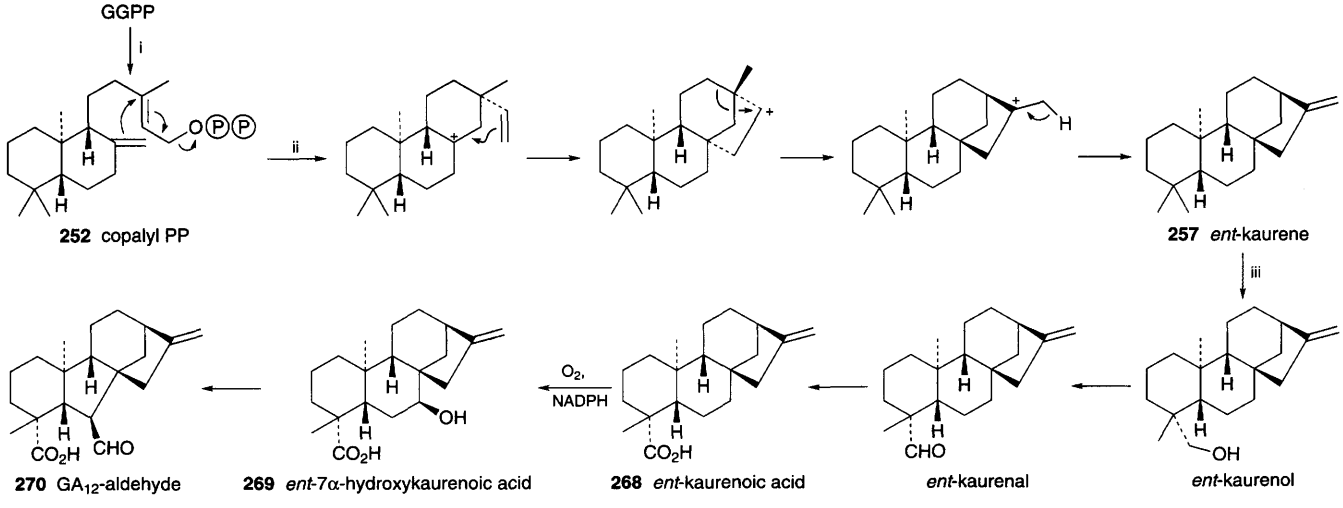
90% abietadiene, whilst that from lodgepole pine contained more byproducts, and yielded about 67% abietadiene. The grand fir enzyme was partially purified, and had similar

properties to other diterpene cyclases. In the metal ion requirement, Mg^{2+} was very much preferred to Mn^{2+}, which contrasts to the monoterpene cyclases from these plants where Mn^{2+} is more effective than Mg^{2+}. There was no evidence for any separation of enzyme activities catalysing parts of the reaction sequence, *e.g.* to (+)-copalyl PP **260** or pimaradiene **262**, although these are logically implicated in the pathway (Scheme 59), the early parts of which mirror those of the oryzalexin pathway described above. Pimaradiene (or sandaracopimaradiene) undergoes a 1,2-methyl migration to generate the abietadiene skeleton *via* the abieta-8(14)-enyl cation **263**. Oxidation of abietadiene to abietic acid **267** is achieved by the successive action of three enzymes, which have been demonstrated in cell-free extracts from both plants.[209] The first two transformations, abietadiene to abietadienol **265** and abietadienol to abietadienal **266**, are catalysed by distinct P-450 systems, requiring O_2 and NADPH cofactors. Oxidation of the aldehyde **266** to the acid **267** is then accomplished by the action of an NAD^+-dependent aldehyde dehydrogenase. The oleoresin secretion from grand fir consists of diterpene resin acids (rosin) and volatile monoterpenes (turpentine) which appear to act as a solvent for the diterpenes.[210] This allows deposition of an antifungal rosin barrier to seal the injury. Monoterpene and diterpene cyclases, the crucial enzymes required, are coordinately induced in wounded stems, reaching levels up to 100 times higher than in the uninjured stems. Interestingly, the last enzyme in the abietic acid pathway, the aldehyde dehydrogenase activity which is not inducible by wounding, is constitutively expressed at a high level, and therefore is not efficiently coupled to the earlier steps.

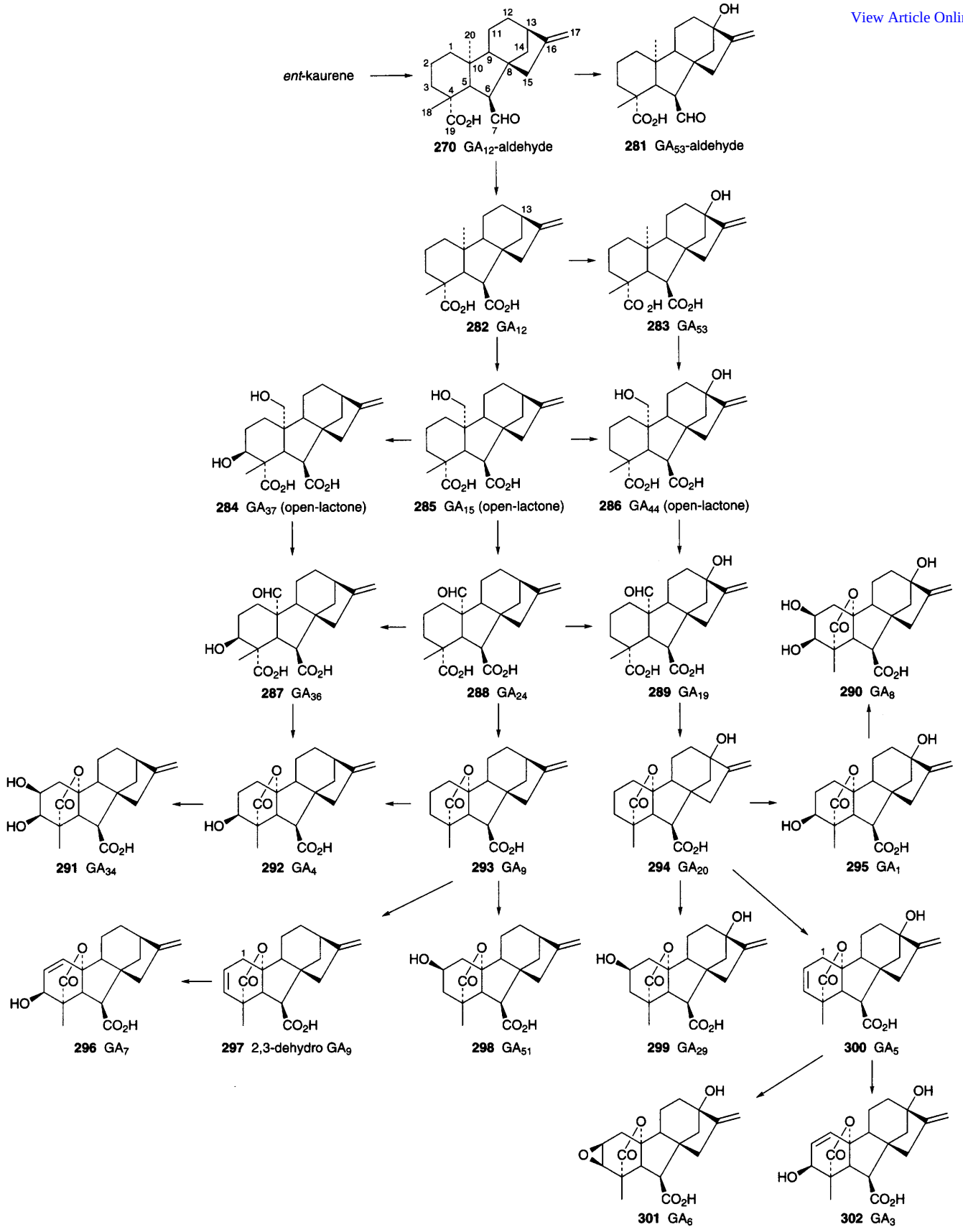
Studies on the biosynthesis and metabolism of the gibberellins (GAs) always account for a considerable proportion of the terpenoid research literature. These diterpenoids, with over 100 different structures now known, play a significant role as plant growth hormones, and have their origins in *ent*-kaurene **257**. *ent*-Kaurene is produced from GGPP by the action of two enzymes, firstly *ent*-kaurene synthetase A, which cyclizes the substrate to copalyl PP **252**, and then *ent*-kaurene synthetase B, which accomplishes the further cyclization and subsequent rearrangement (Scheme 60). *ent*-Kaurene biosynthesis from GGPP has been observed using cell-free extracts from wheat (*Triticum aestivum*) seedlings, pea (*Pisum sativum*) shoots and pumpkin (*Cucurbita maxima*) endosperm, and in each case, the activity was located in the plastids.[211] A gene encoding for *ent*-kaurene synthetase A has been isolated from *Arabidopsis thaliana* and shown to express an active protein in *E. coli*.[212] This gene would complement a dwarf gibberellin-responsive *Arabidopsis* mutant known to be blocked prior to *ent*-kaurene. *Escherichia coli* cells carrying both the *ent*-kaurene synthetase A gene and a gene from *Erwinia urldovora* which encoded GGPP synthase accumulated copalyl PP. The purification of *ent*-kaurene synthetase B from immature seeds of pumpkin has been reported.[213] This activity was fully separated from *ent*-kaurene synthetase A, and found to require a divalent metal ion such as Mg^{2+}, Mn^{2+} or Co^{2+}. The hydroxylase enzyme catalysing the conversion of *ent*-kaurenoic acid **268** into *ent*-7-hydroxykaurenoic acid **269** in the fungus *Gibberella fujikuroi* has also been characterized and solubilized.[214] This is a P-450-dependent enzyme, requiring O_2 and NADPH cofactors, though FAD and monovalent metal ions stimulated the conversion. When *ent*-kaurene **257** and *ent*-kaurenoic acid **268** were fed to shoots of maize (*Zea mays*), GA_{12}-aldehyde **270** was elaborated, but in addition, several minor metabolites were detected.[215] These have been identified as hydroxylated products **271** and **273** from *ent*-kaurene, and **272** and **274** from *ent*-kaurenoic acid. The formation of **271** and **272** gives further examples of hydroxylation at an allylic position with rearrangement of the double bond as seen with the biosynthesis of GA_3 **302** from GA_5 **300** (Scheme 61).

The intensely sweet glucosides **276–280** of the diterpenoid steviol **275** found in the plant *Stevia rebaudiana* are known to be formed from *ent*-kaurene **257**. Three UDPGlc-dependent glucosyltransferase activities from the leaves have been fractionated and characterized.[216] One activity would accept steviol **275** and steviol monoside **276** as substrates, but no other steviol glycosides. A second activity accepted only steviol, whilst the third had rather broad substrate specificity, accepting steviol, steviol monoside, steviol bioside **278** and stevioside **279**. All the glucosyltransferases showed significant activity towards the flavonols quercetin and kaempferol. The conversion of stevioside **279** into rebaudioside A **280** by a partially purified enzyme from fresh leaves of *S. rebaudiana* has been reported, and this enzyme will function when immobilized to DEAE-agarose gel.[217]

Scheme 61 gives the usual interrelationships of the most commonly encountered plant gibberellins as established from various experimental data. The stepwise conversion of GA_{12}-aldehyde **270** may proceed along the early 13-hydroxylation pathway *via* GA_{53} **283** and GA_{44} **286**, or by the early non-hydroxylation pathway which involves GA_{12} **282** and GA_{15} **285**. However, many variations to this generalization are encountered. In potato (*Solanum tuberosum*) shoots, the early 13-hydroxylation pathway was confirmed by the conversion of labelled GA_{12}-aldehyde **270** and GA_{12} **282** into GA_{53} **283**, GA_{44} **286**, GA_{19} **289**, GA_{20} **294**, GA_{29} **299**, GA_1 **295** and GA_8 **290**.[218] GA_{53} **283** appears to be formed predominantly *via* GA_{12} **282** rather than by GA_{53}-aldehyde **281**, although the latter was produced by feeding GA_{12}-aldehyde **270**. The

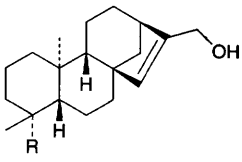


Scheme 60 Enzymes: i, *ent*-kaurene synthetase A; ii, *ent*-kaurene synthetase B; iii, *ent*-kaurene oxidase

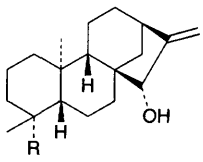


Scheme 61

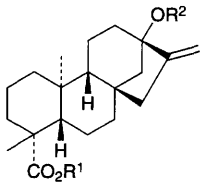
detection of endogenous GA_{51} **298** suggested the non-hydroxylation pathway was also active in this plant. In sorghum (*Sorghum bicolor*) seedlings, GA_{20} **294** was metabolized to GA_1 **295**, GA_8 **290** and GA_{29} **299**, and possible glucosyl conjugates.[219] A gene isolated from maize (*Zea mays*) encoding a protein which appears to have significant sequence homology to P-450 enzymes is suggested to be involved in the early 13-hydroxylation step, probably GA_{12} **282**→GA_{53} **283**.[220]



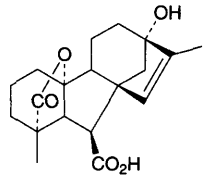
271 R = Me
272 R = CO_2H



273 R = Me
274 R = CO_2H



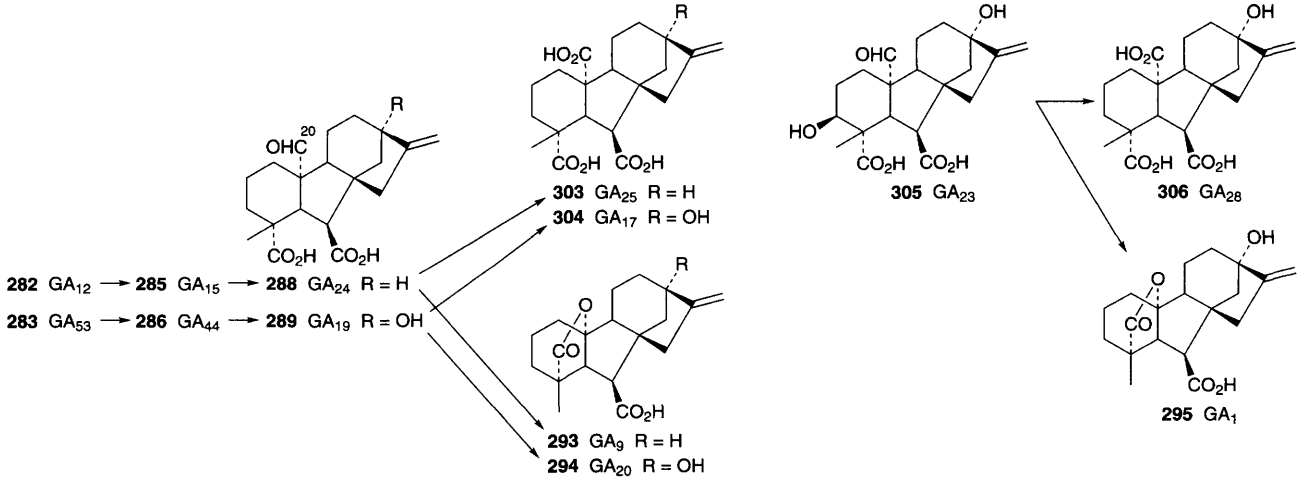
275 steviol $R^1 = R^2 = H$
276 s teviol monoside $R^1 = H$; R^2 = Glc
277 rubusoside $R^1 = R^2$ = Glc
278 steviolbioside $R^1 = H$; R^2 = Glc(2→1)Glc
279 stevioside R^1 = Glc; R^2 = Glc(2→1)Glc
280 rebaudioside A R^1 = Glc; R^2 = Glc(2→1)[Glc(3→1)]Glc



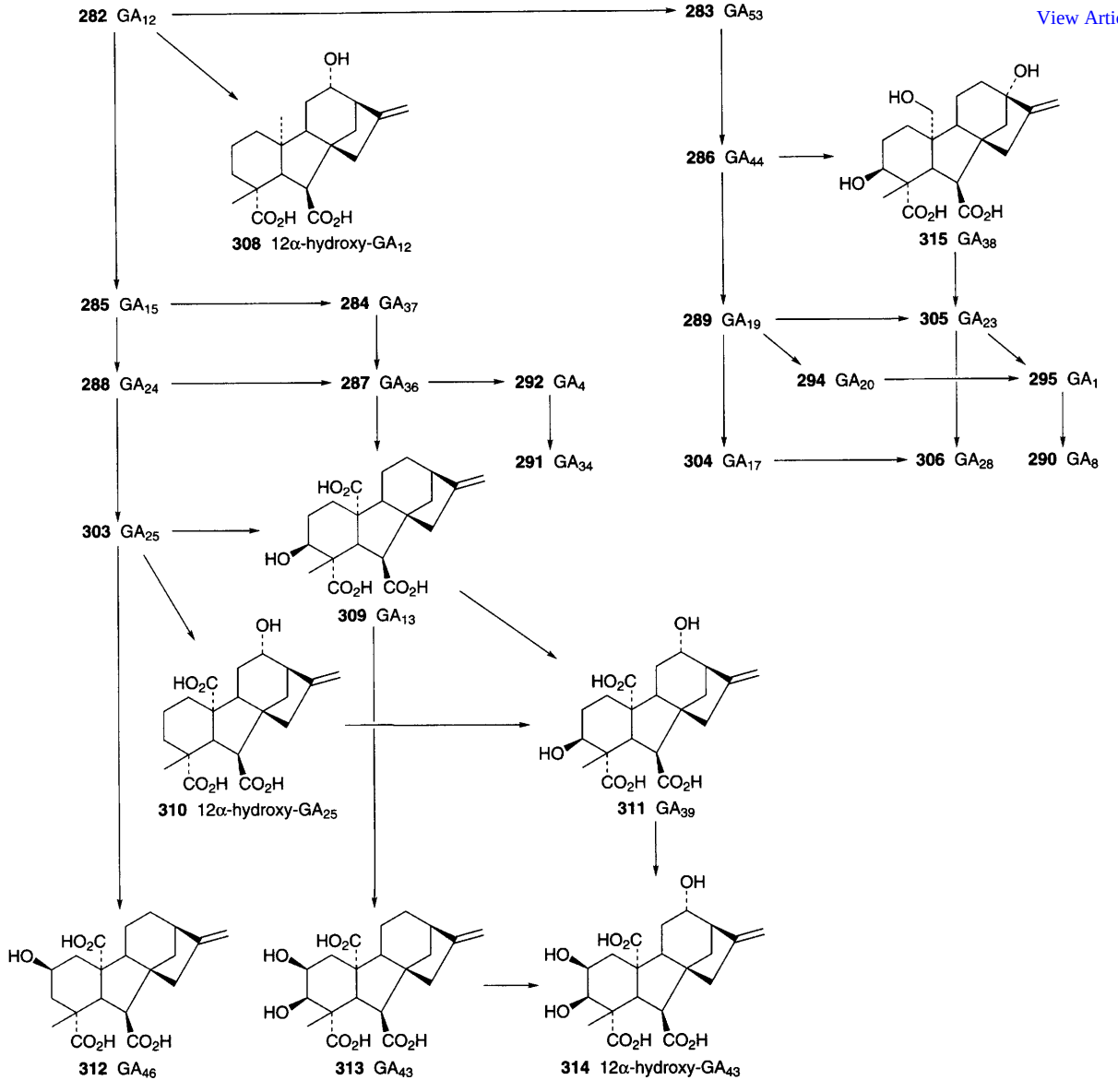
307 GA_{20}-15-ene

Metabolism of GA_{20} **294** to GA_1 **295** has been demonstrated in both tall and dwarf forms of rice (*Oryza sativa*) and *Arabidopsis thaliana*, though this was blocked in some mutants.[221] Tall mutants also produced labelled GA_{29} **299** from the feedings. Three 2-oxoglutarate-dependent dioxygenases have been detected in *Cucurbita maxima*, separated and partially purified.[222] These were a 7-oxidase catalysing oxidation of the aldehyde group in GA_{12}-aldehyde **270** to give GA_{12} **282**, a 3β-hydroxylase converting GA_{15} **285** into GA_{37} **284**, and a 20-oxidase which oxidized the 20-methyl in GA_{12} to give GA_{15} **285** and GA_{24} **288**. A crude enzyme preparation containing all three activities plus a 2β-hydroxylase was able to transform GA_{12}-aldehyde in the presence of $^{18}O_2$ into [$^{18}O_2$]GA_4 **292** and [$^{18}O_5$]GA_{43} **313**, thus demonstrating that all oxygen atoms introduced after GA_{12}-aldehyde were derived from molecular oxygen. This 20-oxidase has been purified further, and a partial amino acid sequence has been established.[223] It was found to catalyse the transformation of GA_{12} **282** into GA_{15} **285**, GA_{24} **288** and also GA_{25} **303**, showing that it could oxidize C-20 sequentially to alcohol, aldehyde, and then acid (Scheme 62). GA_{53} **283** was similarly oxidised to the alcohol GA_{44} **286**, the aldehyde GA_{19} **289** and then to the acid GA_{17} **304**, with small amounts of what was probably the C_{19}-gibberellin GA_{20} **294**. A cDNA clone encoding the 20-oxidase was isolated and expressed in *E. coli*.[224] The recombinant protein indeed catalysed the three step conversions of GA_{12} to GA_{25}, and GA_{53} to GA_{17}, but was also found to convert GA_{24} **288** and GA_{19} **289** into the C_{19}-gibberellins GA_9 **293** and GA_{20} **294** respectively. GA_1 **295** was the product from incubation with the aldehyde GA_{23} **305**. A multifunctional gibberellin 20-oxidase has also been found in *Arabidopsis thaliana*.[225] The gene was isolated by using the pumpkin cDNA as a probe, and it was then expressed as a fusion protein in *E. coli*. The *Arabidopsis* protein had 55% amino acid identity with the pumpkin enzyme. This protein would convert GA_{53} **283** into GA_{44} **286** with small amounts of GA_{19} **289**, and GA_{19} into GA_{20} **294**, thus accounting for oxidation and loss of the C-20 methyl. In a parallel study, three gibberellin 20-oxidase cDNA clones were isolated from *Arabidopsis* and expressed in *E. coli* as fusion proteins.[226] Each of these oxidized GA_{12} at C-20, giving predominantly GA_9 *via* GA_{15} and GA_{24}, with the acid GA_{25} **303** being a minor product. GA_{53} was similarly oxidized, but less effectively than GA_{12}. It is inferred that the acids GA_{25} **303** and GA_{17} **304** are not intermediates in the formation of GA_9 and GA_{20}, but represent side-reactions (Scheme 62). A gibberellin 3β-hydroxylase from bean (*Phaseolus vulgaris*) converting GA_{20} **294** into GA_1 **295** and GA_5 **300**, as well as the epoxidation of GA_5 to GA_6 **301** has been partially purified.[227] A C-20 hydroxylase converting GA_{12} **282** into GA_{15} **285** was also reported.[228]

Variants on the pathways of Scheme 61 have been reported in several plants. In *Brassica rapa* GA_9 **293** was converted into GA_{20} **294** by a late 13-hydroxylation, and GA_4 **292** similarly into GA_1 **295**.[229] GA_1 can apparently be produced by at least two metabolic pathways in this plant. The same conversions can occur in cultured cells of *Raphanus sativus*, and a major pathway to GA_1 is *via* GA_9 and GA_4.[230] Although GA_{20} **294** may also act as a minor source of GA_1, the main metabolite of this gibberellin was found to be GA_{20}-15-ene **307**. The 13-hydroxylation of GA_4 giving GA_1 has also been noted in several genotypes of *Zea mays*, *Oryza sativa* and *Arabidopsis thaliana*.[231] Transformations in cell-free extracts of *Cucurbita*



Scheme 62

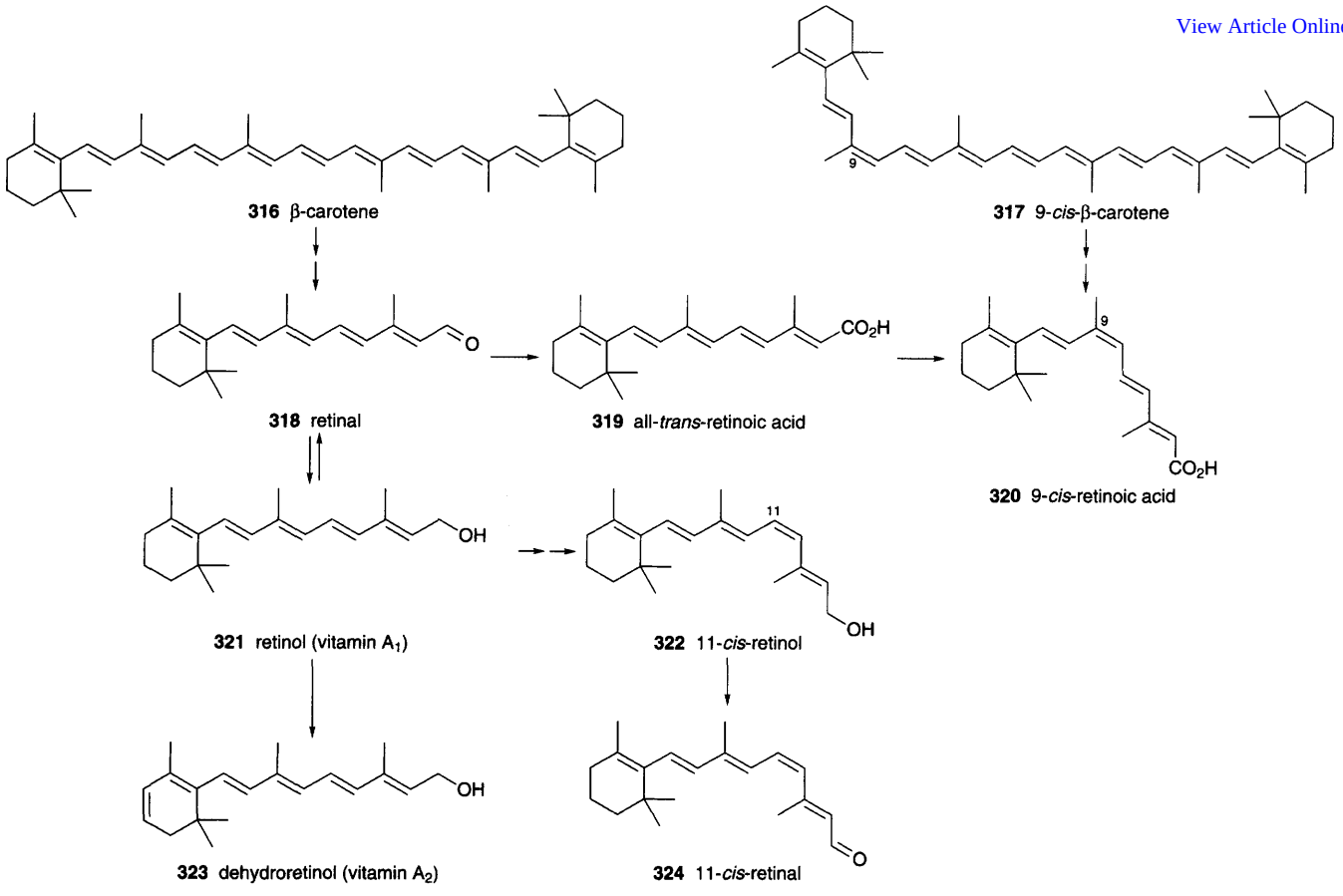


Scheme 63

maxima which may be superimposed on those of Scheme 61 are outlined in Scheme 63.[232,233] Metabolism of GA_{12} **282** in an endosperm system yielded GA_{13} **309**, GA_{43} **313** and 12α-hydroxy-GA_{43} **314** as major products, with GA_4 **292**, GA_{37} **291**, GA_{39} **311** and GA_{46} **312** as minor products. Intermediates GA_{15} **285**, GA_{24} **288** and GA_{25} **303** accumulated at low protein concentrations. GA_{53} **283** is formed from GA_{12} by a microsomal oxidase, and converted predominantly into GA_1 **295**, GA_{23} **305**, GA_{28} **306** and GA_{44} **288**. GA_{19} **289**, GA_{20} **294** and GA_{38} **315** are minor products. In further studies using an embryo preparation, 12α-hydroxy-GA_{25} **310** and 12α-hydroxy-GA_{12} **308** were also detected in addition to those mentioned in the endosperm work. At low 2-oxoglutarate and ascorbate concentrations, 3β-hydroxy products predominated, whilst at high concentrations of cofactors, 12α-hydroxy GAs were formed in increasing amounts. Only C_{19} GAs were 2β-hydroxylated, a characteristic of embryo systems. Glucosides and glucosyl esters are major products formed when gibberellins are fed to *Dalbergia dolichopetala*.[234] GA_{20} **294** and GA_5 **300** give predominantly their glucosyl esters, whilst GA_1 **295** and GA_4 **292** are converted into their 3-*O*-glucosides. Where modifications in the gibberellin portion occurred, these were in accord with Scheme 61, and the product was sometimes bound as a glucoside.

The major gibberellin in *Phaeosphaeria* sp. L497 has been identified as GA_1 **295**, and it is formed in a pathway from GA_{12}-aldehyde **270** *via* GA_9 **293** and GA_4 **292**, or *via* GA_9 and GA_{20} **294**.[235,236] The pathway was confirmed by feeding experiments, and is analogous to that seen in some plants (see above), but has not been noted previously in fungi.

Retinoids possess a diterpenoid skeleton but are actually derived by oxidative cleavage of a C_{40} carotenoid. Cleavage of β-carotene **316** may occur at the central double bond, theoretically giving two molecules of retinal **318**, or by an excentric process, liberating just one molecule of retinal. Retinal is then reduced to retinol (vitamin A_1) **321** *via* an alcohol dehydrogenase activity, an enzyme which is also required in the production of retinoic acid **319** from vitamin A_1 *via* retinal (Scheme 64). Human alcohol dehydrogenase consists of a family of five related enzymes, one of which, denoted class IV, is most active as retinol dehydrogenase. The gene for this enzyme has been cloned and sequenced, and shown to be expressed principally in the human stomach, but not in the liver.[237] The mouse has a simpler alcohol dehydrogenase gene family than humans, with only three types of enzyme, though a highly conserved class IV enzyme analogous to the human retinol dehydrogenase has been detected.[238] Two retinol dehydrogenase activities in microsomes of rat liver have been obtained by gene



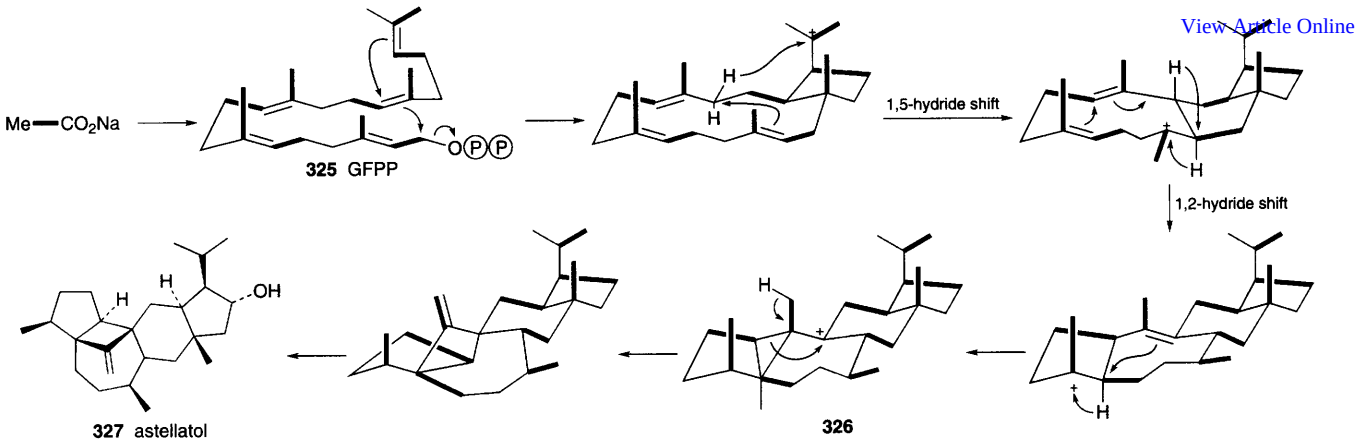
Scheme 64

cloning and expression.[239, 240] The proteins had amino acid sequences some 82% identical, and both had higher activity with $NADP^+$ than NAD^+. Both enzymes recognized retinol in its physiological form bound to cellular retinol-binding protein. Enzyme activity was detected in liver, brain, kidney, lung and testes, though expression was in the liver. The biosynthesis of 11-*cis*-retinal **324**, the chromophore of visual pigments, requires isomerization of all-*trans*-retinol **321** to 11-*cis*-retinol *via* a retinyl ester. The final step is catalysed in bovine retinal pigment epithelium by a membrane-associated 11-*cis*-retinol dehydrogenase, which has been isolated and partially characterized.[241] This is a member of a family of short-chain alcohol dehydrogenases, and is active with NAD^+, but not $NADP^+$. Dehydroretinol (vitamin A_2) **323** constitutes some 20–25% of the total retinoid content in human epidermis, and its formation directly from labelled retinol **321** has been demonstrated in cultures of human skin keratinocytes.[242] No labelled retinal, dehydroretinal, retinoic acid or dehydroretinoic acid could be detected.

An NAD^+-dependent aldehyde dehydrogenase that catalyses oxidation of retinal to retinoic acid **319** has been purified from rat kidney.[243] A cytosolic retinal oxidase activity from rabbit liver has been characterized as a metalloflavoenzyme containing two FADs, eight iron and two molybdenum atoms.[244] This enzyme required molecular oxygen for the conversion of retinal into retinoic acid, but this oxygen was not incorporated into the product, water supplying the new oxygen.

A variety of biological effects appear to be mediated by retinoids, and two families of receptors are recognized, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). 9-*cis*-Retinoic acid **320** is a specific ligand for RXR, whereas both 9-*cis*- and all-*trans*-retinoic acid **319** activate the RAR family. The isomerization of all-*trans*-retinoic acid to 9-*cis*-retinoic acid and also 13-*cis*-retinoic acid has been observed in bovine liver membranes.[245] The isomerization presumably occurs at the acid oxidation level since all-*trans*-retinol and all-*trans*-retinal were not converted into 9-*cis*-retinoids by the microsomes. The reaction is shown to be dependent on the presence of thiol groups in the protein, and since isomerization was inhibited in the presence of α-tocopherol and ascorbic acid, a free radical mechanism is surmized.[246] An enzyme from rat kidney has been described which catalysed the oxidation of both 9-*cis*-retinal and all-*trans*-retinal into the corresponding acids, suggesting that in this system isomerization occurs at the retinol oxidation level, analogous to the 11-*cis* isomerization in the visual pigment pathway.[247] Rat kidney enzyme also accepts both isomers of retinal as substrates with similar efficiency.[248] Rapid isomerization of all-*trans*- and 9-*cis*-retinoic acids has been reported in rat serum.[249] To add yet a further variant, the conversion of 9-*cis*-β-carotene **317** into both 9-*cis*-retinoic acid and all-*trans*-retinoic acid in human intestinal mucosa has been described.[250] The addition of all-*trans*-β-carotene to the system increased the rate of all-*trans*-retinoic acid biosynthesis, but did not affect the rate of 9-*cis*-retinoic acid formation, indicating a direct pathway from 9-*cis*-β-carotene to 9-*cis*-retinoic acid. 9-*cis*-β-Carotene is not usually a constituent of fresh vegetables, but processed vegetables may contain up to 50% of the total β-carotene as the 9-*cis* isomer.

Recent reviews covering aspects of diterpenoid biosynthesis include discussions of taxol biosynthesis,[251–253] Taxol production by fungi associated with Pacific yew,[254] taxane diterpenoids,[255] biosynthesis and metabolism of gibberellins in higher plants,[193,256] and the genetics of gibberellin biosynthesis.[257–259]



Scheme 65

9 Sesterterpenoids

It is an unusual event to record biosynthetic studies on sesterterpenoids although many examples of this least common group of natural terpenoids are now known. The origins of the complex ring systems of astellatol **327** in *Aspergillus variecolor* have been investigated by feeding [$^{13}C_2$]acetate.[260] The labelling pattern supports a pathway *via* cyclization of geranylfarnesyl diphosphate (GFPP) **325** and subsequent rearrangements, but retaining all ten pairs of coupled carbons (Scheme 65). It is suggested that these include an early 1,5-hydride shift (as seen with other sesterterpenoids), then cyclization accompanying two 1,2-hydride shifts. Ring expansion of a cyclopropyl-containing cation **326** is suggested to account for the final ring structure. Note that the published scheme[260] contains a number of errors, and Scheme 65 is a corrected version.

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