The Biosynthesis of C_5—C_{20} Terpenoid Compounds

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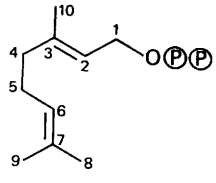
Reviewing the literature published during 1987
(Continuing the coverage of literature in *Natural Product Reports*, 1988, Vol. 5, p. 247)

1 Introduction

The format of this year's review follows that of last year. The major feature of publications this year is the continuing impressive output of Croteau and his group on the monoterpene cyclases. Here, stereospecific synthesis of isotopically labelled precursors and analogues combined with good analytical techniques, applied to biosynthesis in cell-free and partially purified enzyme systems, has been most rewarding. The rapidly advancing work on HMG-CoA synthase and reductase is now concentrated on the regulatory elements of the genes coding for these enzymes.

A joint commission of IUPAC and IUB have made recommendations on the nomenclature of prenols.[1,2] However, consultations of these documents on the numbering of geranyl diphosphate, a compound for which several systems seem to be in current use, leaves this reviewer even more confused. The numbering system most used is shown in (1). However, workers in the iridoid area use the system shown in (2) (*e.g.* see reference 45). The IUPAC/IUB recommendation is shown in (3). In this review the system in (1) is used. In reporting of papers on the iridoid monoterpenes, these have been renumbered accordingly. In all cases in this review the structures are numbered where confusion may arise.



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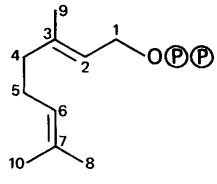

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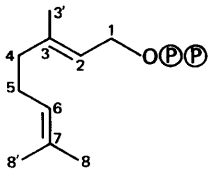


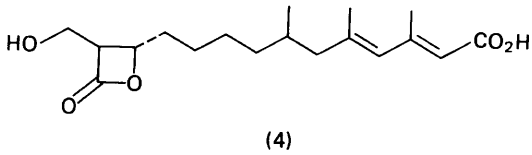
(3)

2 Mevalonic Acid

A new synthesis of (3-*R*,*S*) mevalonolactone which was adapted to the synthesis of [5,5-$^2H_2$]mevalonolactone has been reported.[3] Stereospecific labelling at C-5 has been achieved by Zamir *et al.*[4] by a route incorporating an enzyme reduction of an aldehyde with horse liver alcohol dehydrogenase to produce a stereospecific —CH^{2}HOH group which becomes C-5 of mevalonate. In this way large quantities of (3-*R*,*S*), (5*R*)- and (5*S*)-[$^2H_1$]mevalonolactones were produced.

Anastasis *et al.* have now reported in full[5] their investigations into the role of leucine in mevalonate biosynthesis. The catabolic pathway of leucine and the pathway of mevalonate biosynthesis intersect at (3*S*)-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) (Scheme 1). Thus there is a possibility that five of the six carbon atoms of leucine could be incorporated intact into mevalonate. There have been past reports of the incorporation of leucine into terpenoids. This investigation uses ^{13}C-n.m.r. techniques to look at the labelling pattern in the sesquiterpenoid paniculide A produced by tissue cultures of *Andrographis paniculata*. This system was shown to incorporate [U-^{14}C]leucine into paniculide A with high specific incorporations (15–20%). Feeds of [2-^{13}C]-, [3-^{13}C]-, and (4*R*)-[5-^{13}C]- and (4*S*)-[5-^{13}C]-leucines and interpretation of the ^{13}C n.m.r. of isolated paniculide A revealed the fate of each carbon atom of leucine. It was shown that HMG-CoA arising from leucine breakdown is not incorporated intact into mevalonate. However, the labelling patterns observed could be accounted for if acetyl CoA and acetoacetate produced by breakdown of this catabolic HMG-CoA then entered the terpenoid pathway and were incorporated into mevalonate *via* anabolic HMG-CoA.

The condensation of acetyl CoA and acetoacetate to form HMG-CoA is catalysed by the enzyme, HMG-CoA synthase (E.C. 4.1.3.5) (Scheme 1). A potent inhibitor of this enzyme has been isolated from micro-organisms independently by two groups. This β-lactone (4) was in fact originally isolated[6] in 1971 from a *Cephalosporium* sp. as the antibiotic 1233A. In these new publications[7–9] screens for HMG-CoA synthase inhibition led to the isolation of this compound from *Scopulariopsis* sp. and from *Fusarium* sp. Structure–activity studies showed that the β-lactone ring is essential for activity while the carboxy terminus is not. The inhibition was shown to be reversible and it was suggested[9] that the mode of action was by competitive inhibition with acetyl CoA and possibly also with acetoacetate. Progress in the molecular biology of HMG-CoA synthase was reported last year. In further publications Gil *et al.*[10] have examined the 5′-untranslated region of the mRNA for Chinese hamster HMG-CoA synthase. This region contains an exon of 59 nucleotides located 10 nucleotides



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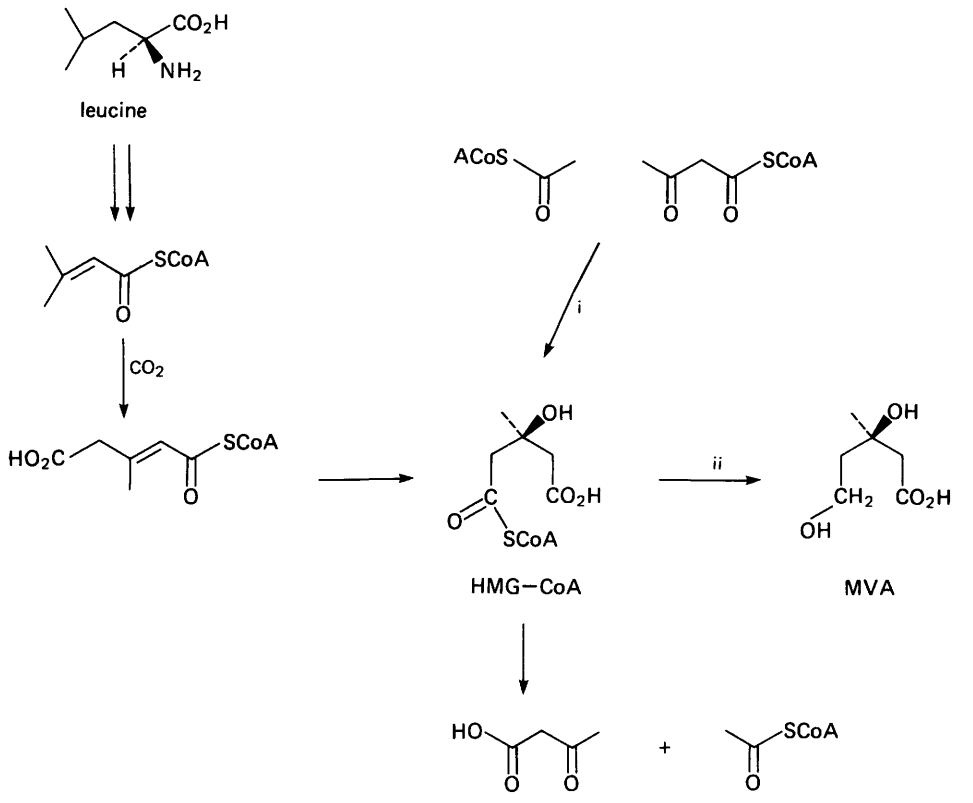


Enzymes: i, hydroxymethylglutaryl-CoA synthase; ii, hydroxymethylglutaryl-CoA reductase

Scheme 1

upstream of the translation start site. This exon was found in about 50% of the mRNAs coding for the enzyme, and the ratio of mRNAs with and without this exon was constant in different tissues of hamster. A cDNA for the human enzyme was found to have a 90% homology in the region corresponding to this optional exon. It was suggested that this exon plays a functional role such as susceptibility to feedback regulation or a specific targeting of these mRNAs to cellular sites such as mitochondria.

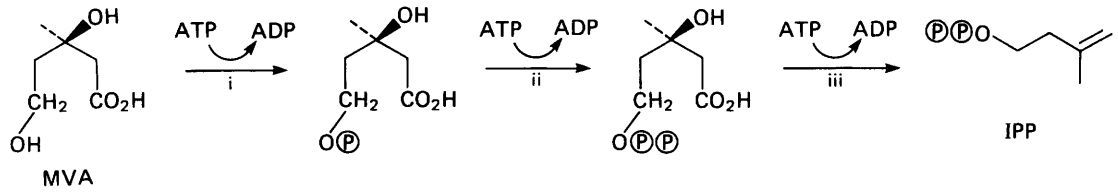
The next enzyme of the mevalonate pathway, HMG-CoA reductase (NADPH) (E.C. 1.1.1.34) (see Scheme 1) has also been extensively studied by molecular biologists. The gene encoding this enzyme was isolated a few years ago and research has moved onto the biochemical mechanism of the regulation of this gene which is thought to involve negative feedback by sterols. Luskey has compared[11] the 5′ end of the human HMG-CoA reductase gene with that of the hamster and has shown that a region of 179 nucleotides is 88% conserved between the species. When fused to *Escherichia coli* chloramphenicol acetyltransferase gene this region of the reductase gene directs the expression of the acetyltransferase in transfected hamster cells, and this expression is regulated by cholesterol. HMG-CoA reductase gene is unusual in that it lacks the classic TATA initiation sequence and CAAT sequence, but it contains five repeats of the sequence GGGCGG or its inverse complement. These GC boses were thought to be important in the regulation of transcription. However, Osborne *et al.*[12] in an examination of the 5′ flanking region of the hamster gene have generated mutations which scrambled various GC boxes. These mutants still transcribed the enzyme in an *in vitro* system based on Hela cell extracts. Thus it appears that these GC boxes have no function in the regulation of this gene. These studies, however, did reveal two regions of the promoter that were crucial for the *in vitro* transcription, and also a region that may be inhibitory.

There are several papers describing attempts to identify the repressor of the HMG-CoA reductase gene. Popjak *et al.*[13] have suppressed sterol biosynthesis with inhibitors such as 2,3-iminosqualene which blocks the cyclization of squalene oxide. This resulted in a rise in reductase levels, indicating that the repressor was indeed a sterol. Boogaard *et al.*[14] using the same approach concluded that there is a non-sterol but mevalonate-derived effector of enzyme activity as well as a sterol repressor. Trzaskos *et al.*[15,16] have isolated 3β-hydroxylanost-8-en-32-aldehyde which they propose is the sterol regulator. In other work, 24,25-epoxycholesterol has been implicated.[17]

As well as repression of new enzyme synthesis, there are other biochemical mechanisms which control HMG-CoA reductase activity. Dotan and Schecter[18] have isolated from rat liver cytosol a 19 kDa protein which activates the enzyme. Inactivation of the enzyme occurs by phosphorylation and this topic has been reviewed by Stonik and Brewer,[19] who also report[20] the purification of a kinase involved in this phosphorylation. Hasumi *et al.* in a screen of microbial metabolites for HMG-CoA reductase inhibitors have reported[21] that phenicin, a known compound from *Penicillium* sp. irreversibly inhibits the enzyme, probably by reaction with sulphydryl groups on the protein molecule. Heathcock *et al.*[22] have summarized the current situation with the powerful inhibitors compactin and mevinolin and describe the synthesis and biological activity of structural analogues.

3 Hemiterpenoids

The formation of isopentenyl diphosphate (IPP) from mevalonate involves the three ATP-requiring enzymes, mevalonate kinase (E.C. 2.7.1.36), phosphomevalonate kinase (E.C. 2.7.4.2), and mevalonate-5-diphosphate decarboxylase (E.C. 4.1.1.33) as depicted in Scheme 2. It has been shown,[23] that etioplasts and etiochloroplasts isolated from white mustard (*Sinapis alba* L.), lack these three enzymes although they were capable of converting IPP into prenyl-lipids. It was considered that, within the plant cell, IPP formation occurs only in the cytoplasm and endoplasmic reticulum where these enzyme activities were easily detected. Chiew *et al.*[24] have described an isolation procedure, from pig liver tissue, which accomplished



Enzymes: i, mevalonate kinase; ii, phosphomevalonate kinase; iii, mevalonate-5-diphosphate decarboxylase

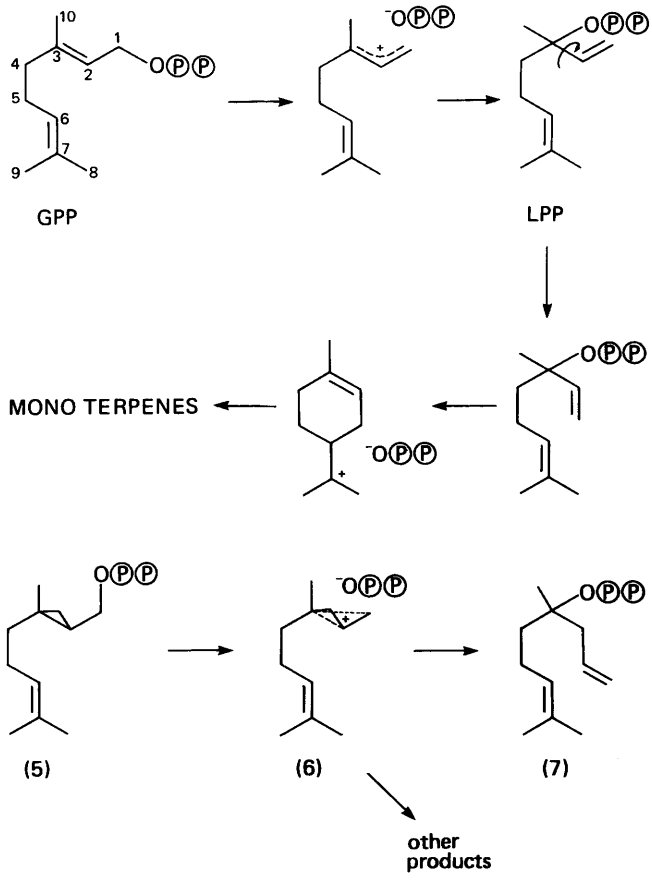
Scheme 2

separation of these three enzyme activities in one operation. The purification of mevalonate-5-diphosphate decarboxylase was continued to apparent homogeneity (MW = 88000) using five chromatographic steps including affinity chromatography on ATP-agarose. [1-^{14}C]-Labelled mevalonate-5-diphosphate was prepared enzymatically from [1-^{14}C]mevalonate using the two isolated kinases and utilized in an assay for the decarboxylase by $^{14}CO_2$ release. The availability of pure decarboxylase and this radioactive assay enabled the authors to reassess the metal and pH requirements and nucleotide specificity of this enzyme as well as examine the effects of phosphorothioate analogues of ATP when used as co-substrates. Reardon and Abeles[25] in a study of inhibition of cholesterol biosynthesis in rat liver homogenates concluded that 3′-fluoro-, 3′,3′-difluoro-3′,3′,3′-trifluoro- and 4,4-difluoromevalonates act by inhibition of mevalonate-5-diphosphate decarboxylase. Using partially purified decarboxylase, it was shown that mono- and di-phosphorylated derivatives of these fluoromevalonates are more effective inhibitors. This confirms earlier literature observations that mevalonate kinase and phosphomevalonate kinase can accept 3′-fluoromevalonate and that the diphosphorylated compound is the active inhibitor of the decarboxylase, although the mechanism of this inhibition is unclear.

Isopentenyl diphosphate isomerase (E.C. 5.3.3.2), which converts IPP into dimethylallyl diphosphate (DMAPP), has been purified to apparent homogeneity for the first time from chromoplasts of *Capsicum annuum* fruits.[26] The purification was achieved by affinity chromatography on Sepharose-coupled aminophenylethyl diphosphate. This procedure also yielded pure geranylgeranyl disphosphate synthase. This part of the paper is reviewed in the diterpenoid section. IPP-Isomerase was shown to be monomeric with a molecular weight of 33500 Daltons and to have a K_m for IPP of 6 μmol dm^{-3} with a pH optimum of 7—8.

Suga *et al.* have described[27] chemical synthesis of stereospecifically labelled (*R*)- and (*S*)-[2-^{2}H]isopentenyl diphosphates. McClard *et al.* have prepared[28] phosphonylphosphinyl analogues of IPP and DMAPP. In these compounds the P—O—P—O— bridges have been changed to P—CH_2—P—CH_2— thereby preventing hydrolysis. Similar derivatives of geranyl diphosphate where the P—O—P—O group has been replaced by P—CH_2—P—O— or by P—CF_2—P—O— have been prepared by this group[29] and by Gotoh *et al.*[30] who also have synthesized the corresponding analogue of DMAPP. The compounds with P—CH_2—P—O groups are recognized by prenyltransferases but are not good substrates. However, the P—CF_2—P—O analogue is a good substrate for farnesyl diphosphate synthetase and also for monoterpene cyclases[29] (see later), presumably because the electronegativity of the CF_2 unit is similar to that of the normal oxygen bridge.

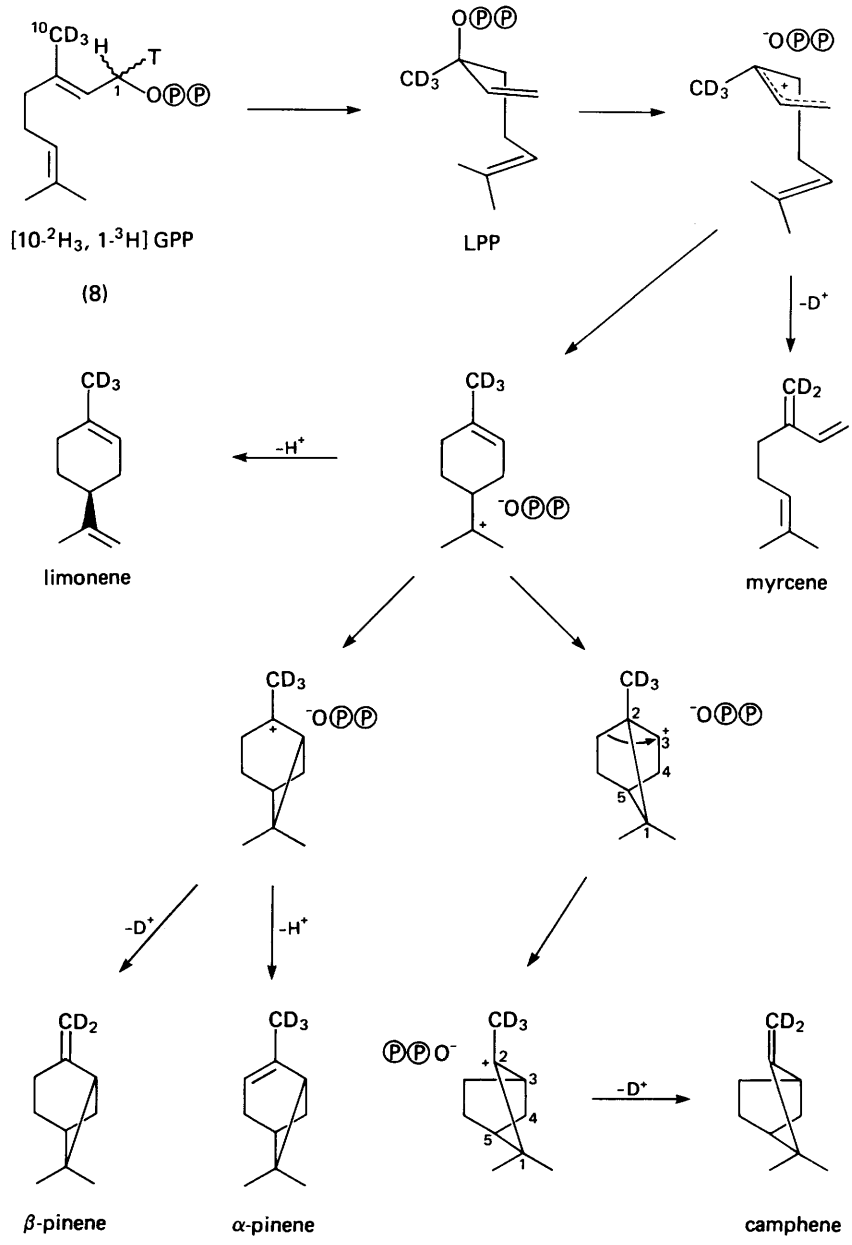
Comparison of the hybridization of cDNA libraries to mRNA of rats fed chlolestrol or the inhibitor mevinolin has been used to identify cDNAs coding for HMG-CoA reductase. This technique was also used[31] to identify and clone a cDNA coding for a prenyltransferase. *In vitro* transcription and translation of this cDNA gave a 39 kDa protein whose amino acid sequence showed good active-site homology with other known prenyltransferases.



Scheme 3

4 Monoterpenoids

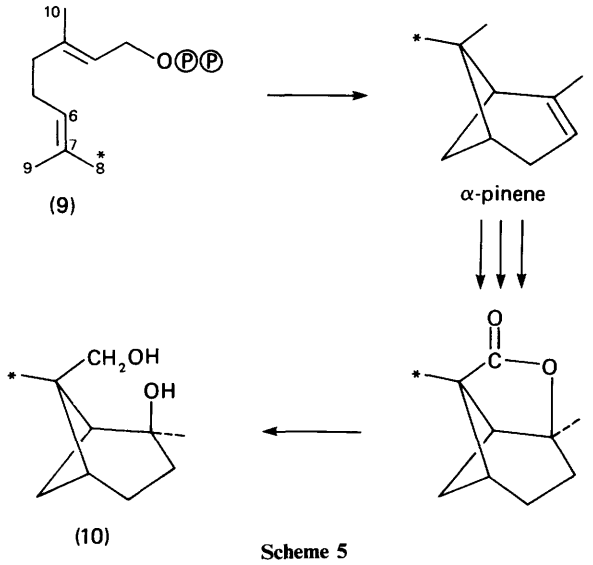
The biosynthesis and metabolism of monoterpenoids has been expertly reviewed by Croteau.[32] His group has published several more papers on the mechanism of monoterpene cyclases which catalyse the conversion of geranyl diphosphate (GPP) into the mono- and bi-cyclic compounds. It is now generally agreed that these cyclases operate by an isomerization–cyclization mechanism. In this mechanism geranyl diphosphate is first converted into the tertiary allylic diphosphate, linalyl diphosphate (LPP), which after a rotation around the C-2—C-3 bond cyclizes to the α-terpinyl cation as shown in Scheme 3. In order to uncouple the isomerization–cyclization reactions Wheeler and Croteau[33] have prepared the substrate analogue 2,3-cyclopropylgeranyl diphosphate (5). Incubation of this compound with a crude cyclase preparation from *Salvia officinalis* gave, among other (enzyme catalysed) solvolytic products, the isomerized tertiary diphosphate (7) which cannot cyclize. This identification of (7) as a product (albeit indirectly by phosphatase treatment of water-soluble products) demonstrated, for the first time, the initial isomerization step of these cyclases. The other products could all be rationalized as arising from water capture or proton loss from the cyclopropylcarbinyl cation intermediate (6), and parallels were



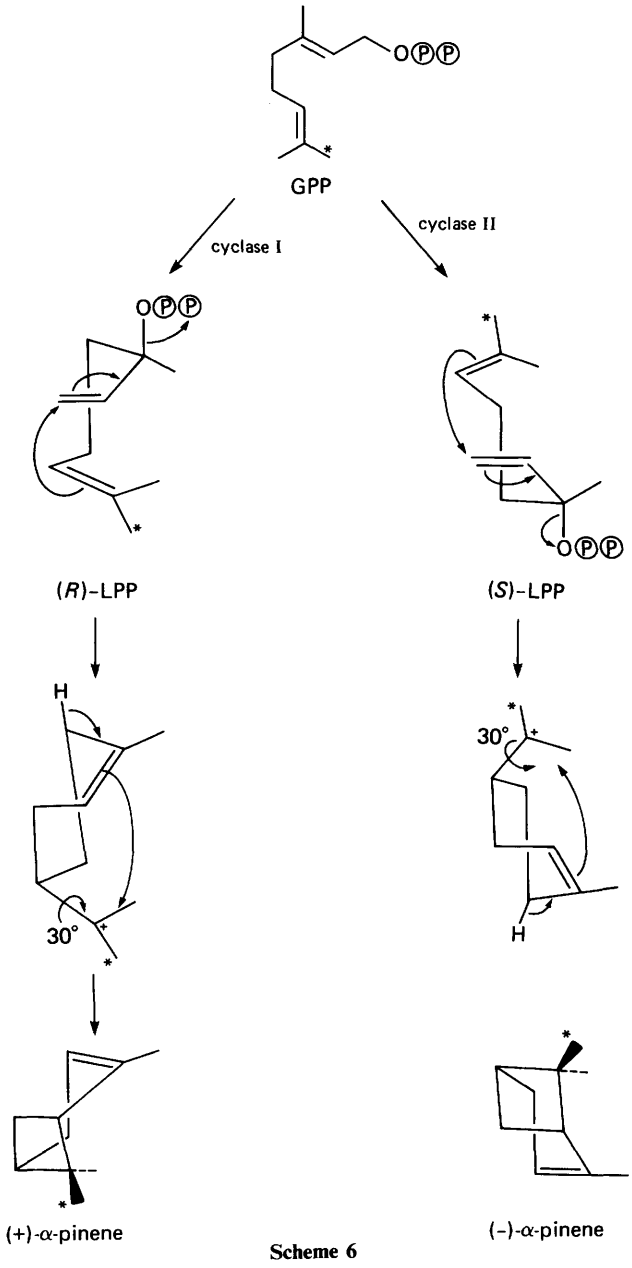
Scheme 4

drawn with other terpene biosynthetic reactions such as presqualene diphosphate→sterols and the formation of irregular monoterpenes from chrysanthemyl diphosphate where this type of intermediate has been implicated. In another study with substrate analogues these authors have determined[34] the functional group requirements for recognition and ionization by these cyclases. A series of analogues of GPP, modified in chain length and in geometry and electronic character of the C-2—C-3 and C-6—C-7 double bonds were examined both as inhibitors of GPP conversion and as substrates for separated (+)-α-pinene and (+)-bornyl diphosphate cyclases. It was concluded that the major recognition site of the substrate is the diphosphate group. The C-2—C-3 olefinic function is a requirement for ionization of the C-1 diphosphate, and is recognized on a geometric basis. The C-6—C-7 olefin is primarily recognized electronically, and plays a stereoelectronic role, in optimizing orbital alignment of the C-2—C-3 π-system with the C-1—OP bond and also excludes water from the active site, preventing capture of the intermediate cations to form alcohols. Stremler and Poulter[29] have demonstrated that a GPP analogue where the P—O—P bridge has been replaced by P—CF_2—P is acceptable as a substrate by cyclases from lemon peel. This compound is stable to the non-specific phosphatases which hydrolyse GPP to geraniol in these systems and thus is a useful mechanistic probe for these cyclases.

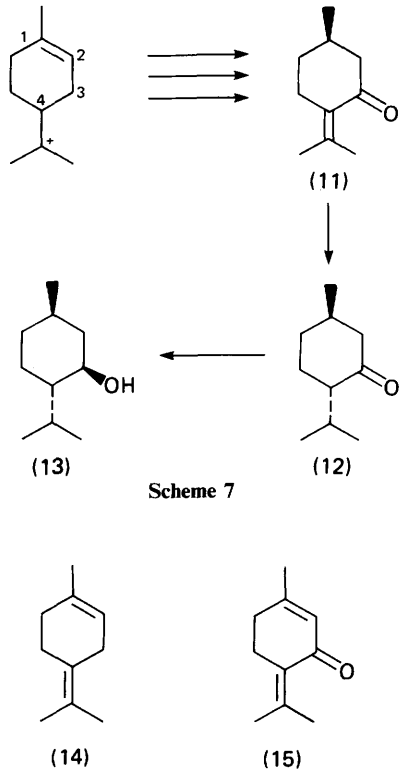
In a mechanistic study of the cyclization step of these enzymes Croteau *et al.*[35] have prepared [10-$^2H_3$, 1-^{3}H]GPP (8) in order to determine whether (−)-α-pinene and (−)-β-pinene are formed by the same enzyme. It has already been shown by this group that two separable enzymes existed, in *S. officinalis*, for the conversion of GPP into (+)-α-pinene or (−)-α-pinene. GPP:pinene cyclase I (MW 96 kDa) converts GPP into (+)-α-pinene accompanied by (+)-camphene, (+)-limonene and the acyclic compound myrcene, while GPP:pinene cyclase II (MW 55 kDa) transforms GPP into (−)-α-pinene, (−)β-pinene as well as (−)-camphene, (−)-limonene and myrcene. The mechanism shown in Scheme 4 has been proposed to account for these products. It can be seen from this scheme that two branch points would be sensitive to primary isotope effects if a 10-C^2H_3 group were present. Incubations of [10-$^2H_3$, 1-^{3}H]GPP and [1-^{3}H]GPP with separated cyclase I and II enzymes gave the following results. (i) There was an overall decrease of the



Scheme 5



Scheme 6



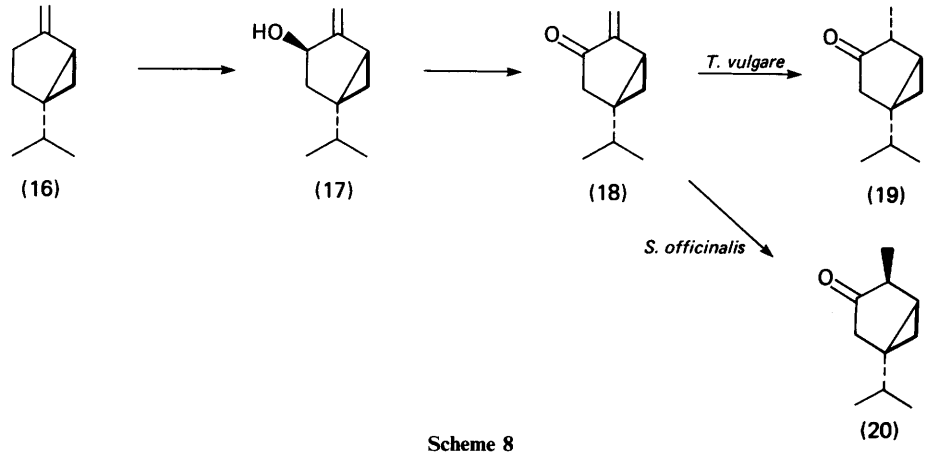
Scheme 7

production of total olefins from the 10-C^2H_3 substrate. This was interpreted as a secondary deuterium isotope effect on the initial ionization of GPP which is considered to be the rate determining step. (ii) The proportion of myrcene produced by both enzymes was decreased for the 10-C^2H_3 GPP as predicted. (iii) The ratio of α-pinene:β-pinene produced by cyclase II was 2.92:1 for the 10-C^2H_3 GPP compared to 1.23:1 for the non-deuteriated substrate. These data give rise to a value of 2.4 for k_H/k_D for the loss of geranyl-C-10 proton in the formation of β-pinene. Thus it was concluded that the proposed mechanism of formation of α-pinene and β-pinene shown in Scheme 4 was correct and that these isomers are formed by the same enzyme from a common intermediate.

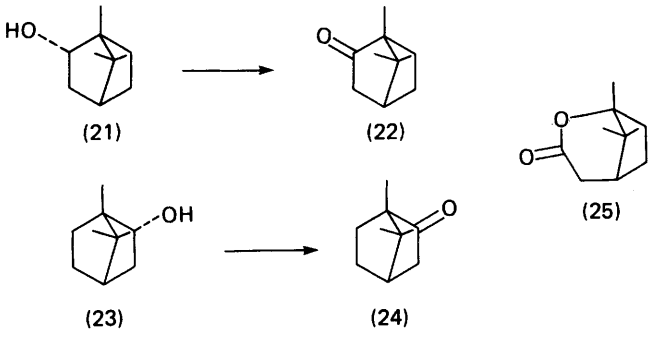
An examination of the stereochemistry of the *gem*-dimethyl groups in (+) and (−)-α-pinene produced by these cyclases has been described by Coates *et al.*[36] (6*E*)-[8-^{3}H]GPP (9) was synthesized and incubated with separated GPP:pinene cyclases I and II. The [^{3}H]-α-pinene produced was isolated and subjected to a chemical degradation such that the *endo*-methyl group of the *gem*-dimethyl group was oxidized as shown in Scheme 5. There was complete retention of ^{3}H in the diol (10) obtained from both (+)-α-pinene produced by cyclase I and (−)-α-pinene produced by cyclase II. Thus the 8-methyl of GPP becomes the *exo*-methyl group in both enantiomers of α-pinene. This can be explained by enantiomeric least motion (30° rotation) cyclizations of the boat-like α-terpinyl cations formed by the *anti-endo* cyclizations of (*R*)- and (*S*)-linalyl diphosphates as shown in Scheme 6.

Biomimetic experiments on the solvolysis of LPP have shown that it forms mono- and bis-metallic complexes with Mg^{2+} and Mn^{2+} and that the rate of hydrolysis of LPP is increased by formation of the bis metallic complex.[37] This rate correlates well with the rate of the enzymic synthesis of cyclic terpenes by crude enzymes of *Citris limonum* and it was inferred that such complexes are involved in the enzymic reaction.

Akhila has carried out[38] some experiments with shoots of *Mentha arvensis* in a study of the biosynthesis of 3-oxygenated monocyclic monoterpenes such as pulegone (11) menthone (12) and menthol (13) (see Scheme 7). However, the author has based his experiments and discussion on pathways involving terpinolene (14) and piperitenone (15) which are now known



Scheme 8



not to be intermediates in menthone biosynthesis. The main pathway involves the 3-hydroxylation of limonene (reviewed in reference 32). The experiments described[38] appear to show that only one of the two hydrogen atoms of C-3 of the terpinyl cation is lost on transformation to menthone (12). Mechanisms involving hydroxylation at C-3 of the α-terpinyl cation with concomitant 1,2-hydride shifts were invoked to explain these observations. However, it is apparent that the majority of products from the GPP cyclases are olefins and that the oxygen is introduced at later stages by conventional oxygenases. Indeed Karp *et al.*[39] have demonstrated the presence of a cytochrome P_{450} dependent oxygenase which converts (+)-sabinene (16) into (+)-*cis*-sabinol (17) (Scheme 8) in a microsomal preparation from *S. officinalis*. This enzyme activity, which catalyses an important step in the biosynthesis of the thujones (19) and (20) was shown to be highly specific for (+)-sabinene and was also demonstrated to be present in *Artemesia absinthium* and *Tanacetum vulgare*. The dehydrogenase responsible for the next step of this pathway, the conversion of (+)-*cis*-sabinol to (+)-sabinone (18) was shown[40] to be present in *S. officinalis* (MW 92 kDa) and *T. vulgare* (MW 70 kDa). In both species this enzyme activity co-eluted on gel filtration with similar enzyme activities which converted (+)-borneol (21) into (+)-camphor (22) (*S. officinalis*) and (−)-borneol (23) to (−)-camphor (24) (*T. vulgare*). However, in both cases these activities were shown to be separable by electrophoresis and are thus different enzymes. The metabolism of (+)-camphor (22) in *S. officinalis* leaves proceeds by oxidation to the lactone (25) followed by hydrolysis and glucosidation.[41]

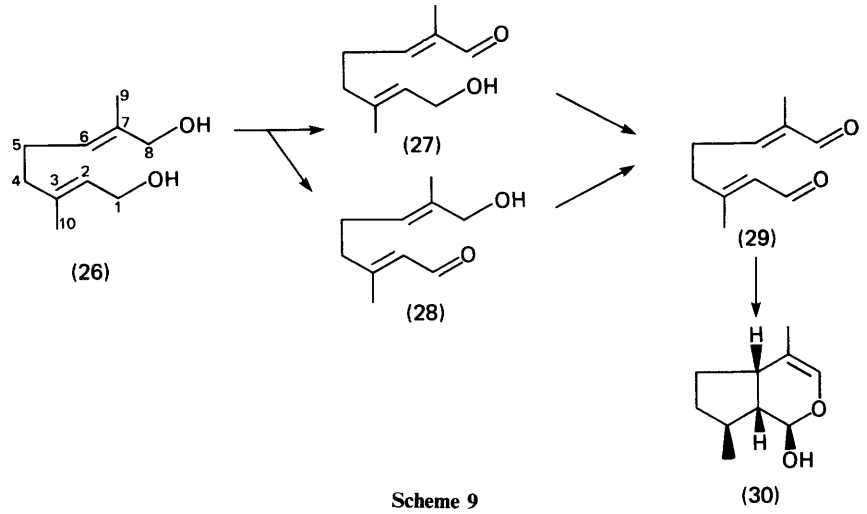
In applications of their work on the enzymes of monoterpene biosynthesis Croteau and his colleagues have examined[42] the effect of application of cytokinins on essential oil content of *Mentha* species. It was shown that the essential oil levels were increased two fold and that this increase was due to an increase in the levels or activities of the biosynthetic enzymes. In a similar study[43] they have investigated the effect of fungal infection or carbohydrate elicitors on terpenoid production by *Pinus contorta* saplings. These trees respond to attack by the production of a resin containing mono- and di-terpenoids. It was shown that this increase in terpenoid production on response to infection was due to an increase in the levels or activities of the relevant terpene cyclases. Pine trees also exude these resins on response to attack by pine beetles. Pierce *et al.*[44] have studied the metabolism of monoterpenoids in the mountain pine beetle, *Dendroctonus ponderosae* fed on *Pinus ponderosa* and *P. contorta*. Three types of oxidative pathways, which the beetles use to de-toxify the volatile monoterpenes, were delineated.

Uesato *et al.*[45] have continued their study of iridoid biosynthesis in *Rauwolfia serpentina*. A cell-free system obtained from suspension cultures converts 8-hydroxygeraniol (26) into iridodial (30) (Scheme 9). They have now succeeded in isolating the mono-aldehydes (27) and (28) and the dialdehyde (29) and have demonstrated their conversion into iridodial (30). The isomeric monoaldehydes (27) and (28) were formed from (26) in a ratio of 7:9 and were both converted into the dialdehyde (29) indicating that the dehydrogenases catalysing these steps are somewhat non-specific. The cyclase which converts (29) into (30) was partially purified (440 fold) and shown to be NADPH dependent and have a molecular weight of 188 kDa with a subunit mass 28.7 kDa.

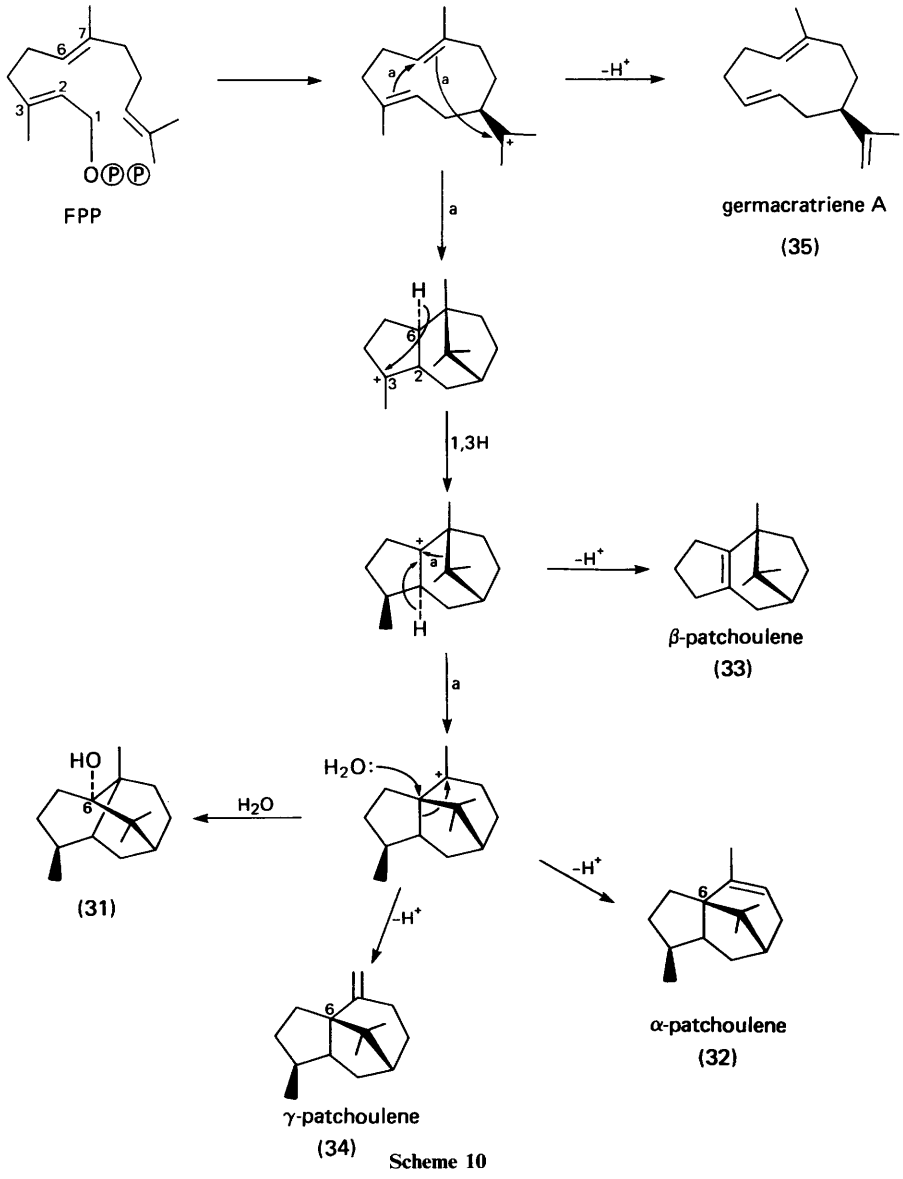
The commercial production of monoterpenes by tissue cultures has never been realised because most cell lines do not accumulate levels of terpenoids as high as those in parent plants. In plants these compounds are located in isolated structures whereas in cell suspension cultures such structures do not exist and thus the cells are susceptible to toxic effects of their own terpenoid products. This problem has been analysed by Brown *et al.*[46] using cultures of *Pelargonium fragrans*, who have shown that monoterpene production proceeds up to 0.3 gm/l, a level at which it was demonstrated to be toxic to fine suspension cultures. Other papers on plant tissue culture of monoterpenes deal with biotransformation of monoterpenoid aldehydes to primary alcohols by *Lavandula angustifolia* cultures[47] and the hydrolysis of (±)-menthyl acetate by cultures of the orchids *Epidendrum ochraceum*, *Cymbidium* and *Dendrobium phalaenopsis*.[48] Microbial transformations described this year are of citronellol and geraniol by *Botrytis cinera*,[49,50] of (−)- and (−)-*cis*-carveol by *Streptomyces*,[51] of α-cedrane and cedrol by *Beauvaria sulpurescens*[52] and the allyl rearrangement and resolution of various acetates of cyclic terpene alcohols by *Pseudomonas* sp.[53]

5 Sesquiterpenoids

Suga *et al.*[54] in a mechanistic investigation of farnesyl diphosphate synthase from *Pisum sativum* have examined the stereochemistry of the condensation of DMAPP with (*E*)- or (*Z*)-[4-^{2}H]IPP (see reference 27 for synthesis). It was found that this condensation proceeds *via* addition to the *si–si* face of IPP in accord with previous studies on pig liver and pumpkin enzyme. However, when the alkylating agent, iodoacetamide was added to this system the reaction proceeded by an unusual *re–re* addition.

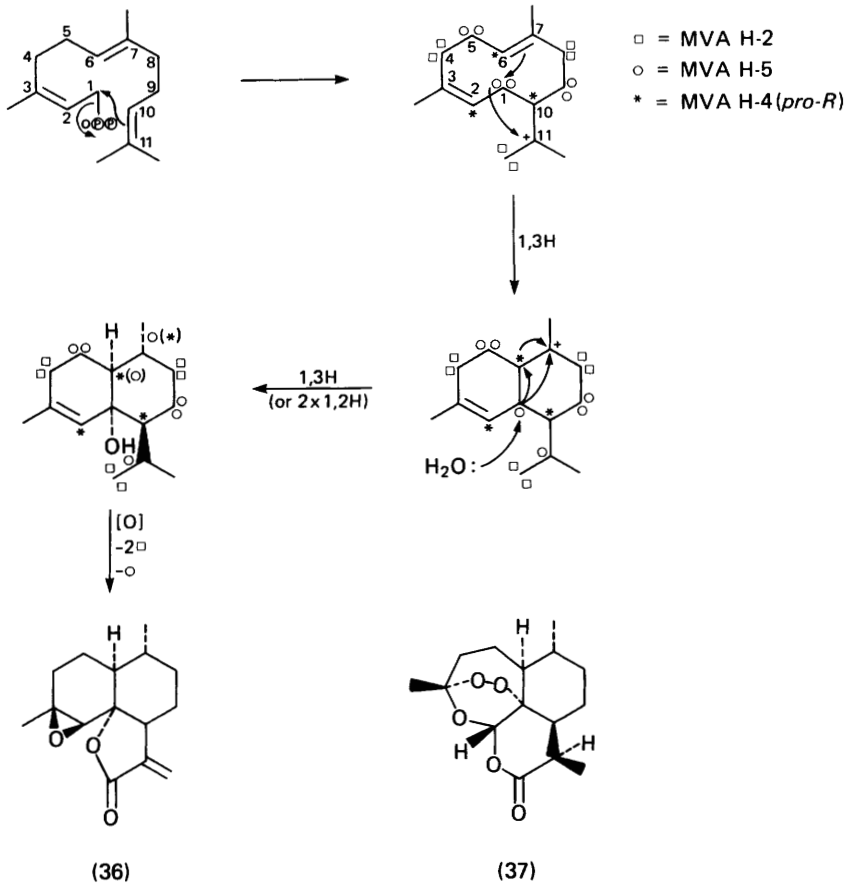


Scheme 9

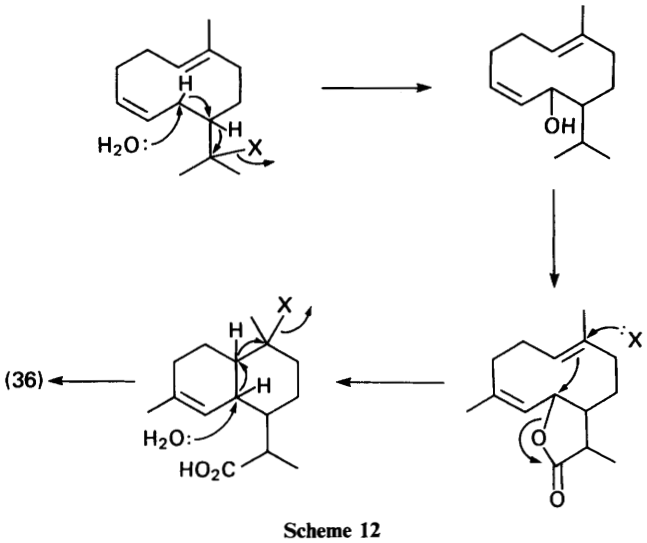


Scheme 10

In contrast to the monoterpene cyclases, the enzymes catalysing the cyclization of farnesyl diphosphate to yield the plethora of sesquiterpene skeleta are poorly understood. For monoterpenes the immediate products of the cyclase enzymes are olefins. These are then transformed to oxygenated terpenes by separate oxidative enzymes. However this is obviously not the case for many sesquiterpenes where hydride shifts and capture of carbocations by water must take place to account for the structures formed. Croteau *et al.*[55] have examined the biosynthesis of patchoulol (31) in a cell-free system of *Pogostemon cablin*. It had been suggested earlier that (31) and olefins such as α-patchoulene (32), β-patchoulene (33), and γ-



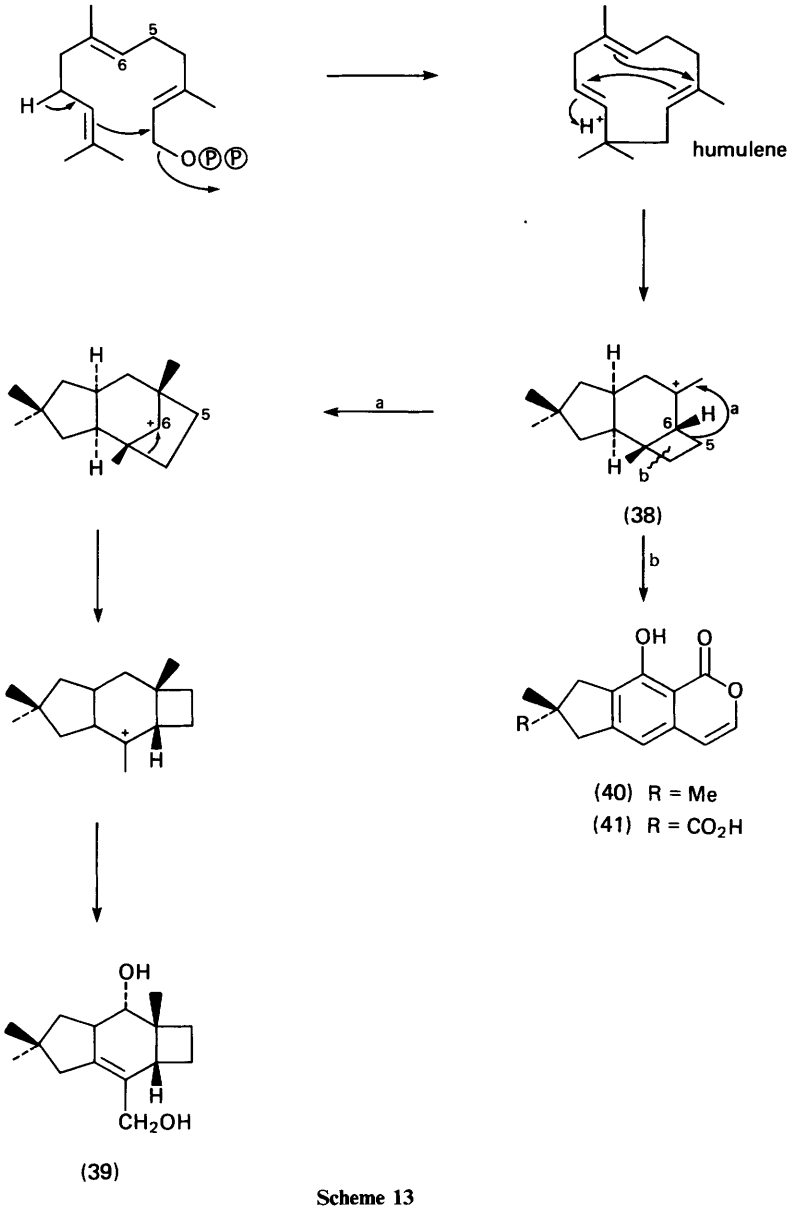
Scheme 11



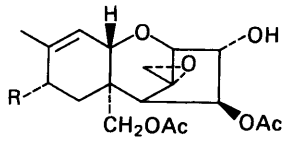
Scheme 12

patchoulene (34), were formed from germacratriene A (35) by protonation and Wagner–Meerwein shifts. Following feeds of [12,13-^{14}C, 1-^{3}H]- and [12,13-^{14}C, 6-^{3}H]-farnesyl diphosphate, an analysis of ^{3}H:^{14}C ratios in patchoulol and certain chemical degradation products led to this type of mechanism being rejected. To account for the labelling pattern observed a mechanism involving a 1,3-shift of H-6 of FPP to H-3 was proposed (see Scheme 10). A short paper by Akhila *et al.*[56] on the same subject is difficult to compare directly as these authors number farnesyl diphosphate in the reverse direction. However, their results with [2-^{14}C, 4*R*-^{3}H$_1$]mevalonate feeds also show no loss of tritium from H-6 of FPP (numbering in Scheme 10) in the biosynthesis of patchoulenes. They proposed two 1,2-hydrogen shifts (H-6→H-2) and (H-2→H-3) to account for this. Of course the 1,3-hydrogen shift proposed above[55] also accounts for this result. Akhila *et al.*[57] have also carried out mevalonate feeds to stems of *Artemisia annua* in a study of the biosynthesis of arteannuin B (36) and artemisinin (qinghaosu) (37). Their results with doubly labelled[^{14}C,^{3}H]mevalonates indicated that two hydrogens from C-2 of mevalonate, one 4-*pro-R* hydrogen and one C-5 hydrogen were lost on the formation of arteannuin B (36) from farnesyl diphosphate. A reasonable mechanism for the formation of (36) is shown in Scheme 11. The shift of H-1 to H-11 is known to occur in cadalane biosynthesis. This mechanism, however, does not account for the loss of a mevalonoid 4-*pro-R* hydrogen atom. In order to explain their results the authors proposed[34] a rather convoluted mechanism involving formation, opening, and re-formation of the lactone function *via* various 1,2-hydrogen shift and X-group mechanisms, as outlined in Scheme 12. A cell-free system from *A. annua* has now been prepared[58] and hopefully use of this with more advanced intermediates such as doubly-labelled FPP will be more helpful in unravelling the biosynthesis of this interesting antimalarial compound.

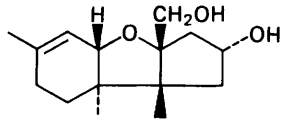
The alternate cyclization of FPP to the humulene system leads to a range of tricyclic sesquiterpenes. Abell and Leech[59] have examined the labelling pattern produced in 7,12-dihydroxysterpurene (39) produced by the fungus *Stereum purpureum* fed with ^{13}C-labelled acetate. Their results indicated that C-5—C-6 of FPP is cleaved during the formation of (39) and the mechanism shown in Scheme 13 involving two 1,2-alkyl shifts from the tricyclic carbocation (38) was proposed. The intermediate (38) is also involved in the biosynthesis of other classes of sesquiterpenes. Donnelly *et al.*[60] have studied the incorporation of ^{13}C-labelled acetate and mevalonate into the



Scheme 13



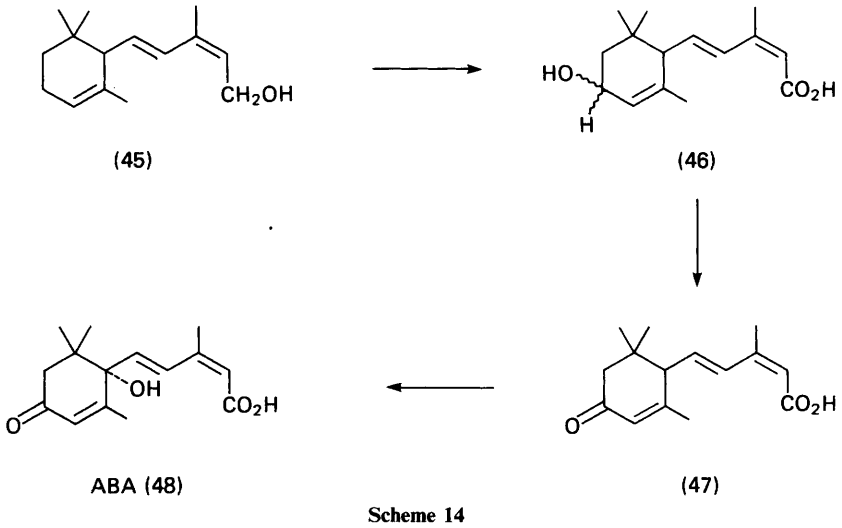
(42) R = $OCOCH_2CHMe_2$
(43) R = H



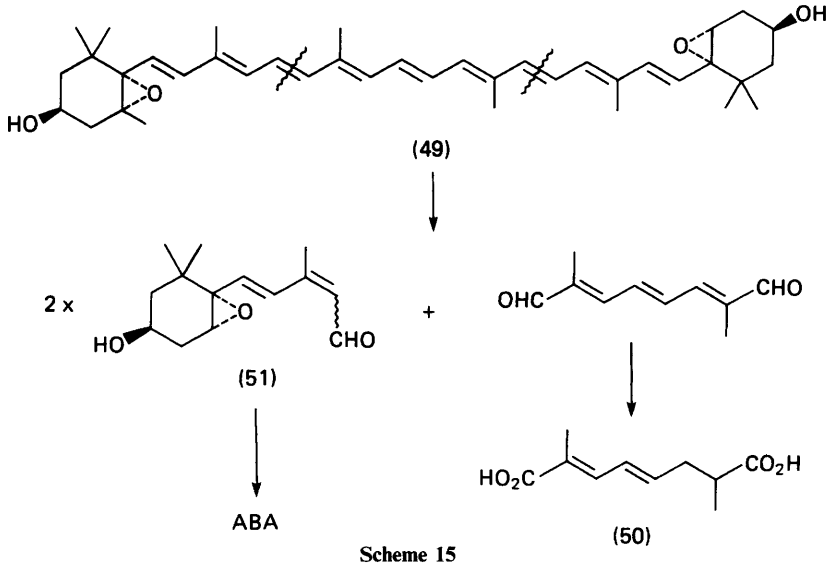
(44)

unusual isocoumarins fomajorin D (40) and fomajorin S (41) in the fungus *Fomes annosus*. It was proposed that (38) (Scheme 13) was transformed into (40) and (41) *via* a cleavage of the cyclobutyl ring although no mechanism was given.

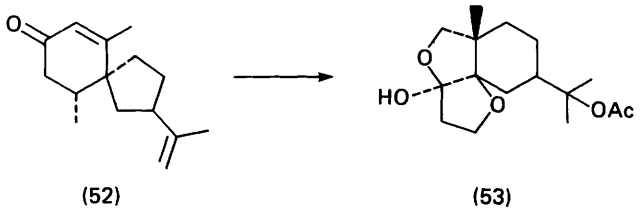
In the trichothecene area, results utilizing the monooxygenase inhibitor ancymidol to block fungal biosynthesis, with the accumulation of the intermediate trichodiene, have now been reported in full.[61] Beremand has reported[62] the mutation of *Fusarium sporotrichioides* by u.v. treatment. Several mutants blocked for the biosynthesis of T-2 toxin were identified using an immunoassay with a monoclonal antibody which recognized the isovalerate moiety of T-2 toxin (42). Two mutants which were blocked for C-8 hydroxylation, and thus accumulated diacetoxyscirpenol (43), were identified along with one which appeared to be blocked very early in the pathway. Zamir *et al.*[63,64] have fed ^{13}C-labelled mevalonates to *Fusarium culmorum* with a view to identifying intermediates between trichodiene and the trichothecanes 3-acetyldeoxynivalenol and sambucinol. This work has led to the characterization of a new 'dead-end' metabolite, apotrichodiol (44).



Scheme 14



Scheme 15

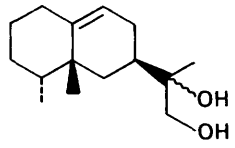


The biosynthesis of abscisic acid (ABA) (48) in the fungus *Cercospora rosicola* has been studied[65] by the feeding of putative precursors. α-Ionylidene ethanol (45) was efficiently incorporated into 1′-deoxy-ABA (47) and ABA. Both epimers of 4-hydroxy-α-ionylidene acetic acid (46) were also shown to be intermediates. The pathway shown in Scheme 14 can therefore be constructed for the fungal system. The 1,4-*trans*-diol (46) has also been identified[66] in pea and avocado where it was shown to be a precursor of ABA. However, in higher plants ABA may be formed by breakdown of a C_{40} precursor e.g. violaxanthin (49). Linforth *et al.*[67] have identified in the ABA-deficient *flacca* mutant of tomato a large amount of 2,7-dimethylocta-2,4-dienedioic acid (50). This compound was not present in isogenic non-mutant plants and was postulated to be formed during ABA biosynthesis as shown in Scheme 15.

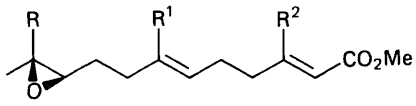
Sindhu and Walton have shown[68] that xanthoxin (51) is converted into ABA by a cell-free system from *Phaseolus vulgaris*. In leaves of the same species results obtained[69] with $^{14}CO_2$ and $^{18}O_2$ incorporation into violaxanthin and ABA were interpreted as confirmation of a C_{40} pathway. Creelman *et al.*[70] have examined $^{18}O_2$ incorporation into ABA in stressed and turgid leaves of *Xanthium strumarium*. They concluded that in stressed leaves ABA is formed from xanthophylls. In turgid leaves, however, they suggested the biosynthetic pathway may be different. Cowan and Railton[71] have presented results on a cell-free system from embryos of *Hordeum vulgare* which appears to incorporate MVA and IPP into abscisic acid. Loveys and Robinson[72] have demonstrated the presence of ABA in protoplasts prepared from *H. vulgare* leaves.

Work on the metabolism of ABA is complicated by the fact that only the racemate is available in labelled form. There are two reports this year of the separation of (+) and (−)ABA on chiral HPLC columns.[73,74] Thus the natural (+)-(*S*)-enantiomer can now be obtained from synthetic mixtures for use in metabolic work. Metabolic work on ABA reported this year includes studies in *H. vulgare*,[75] *Robinia pseudacacia* seeds,[76] and the fungus *Ceratocystis coerulescens*.[77]

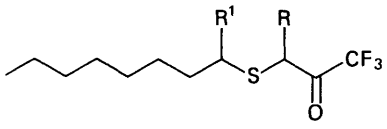
In the phytoalexin area, Murai *et al.*[78] have shown that the vetispirane solavetivone (52), labelled at C-8 with ^2H, is a precursor of the unusual compound phytuberin (53). In studies



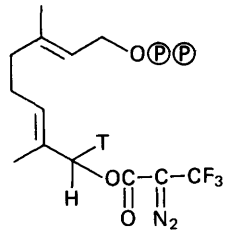
(54)



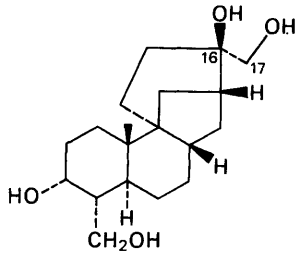
(55) JH I R = R^1 = Et, R^2 = Me
(56) JH II R = Et, R^1 = R^2 = Me
(57) JH III R = R^1 = R^2 = Me



(58) R = Me, R^1 = H
(59) R = H, R^1 = Me



(60)



(61)

of the control of phytoalexin synthesis in potato, Stermer and Bostock[79] have examined the role of HMG-CoA reductase while Zook *et al.*[80] have investigated Ca^{2+} mobilization as a regulatory mechanism. Callus cultures of tobacco treated with cellulase produce the phytoalexin debneyol (54). Brooks *et al.*[81] have analysed (54) produced from [1,2-$^{13}C_2$]acetate and concluded that the angular methyl group arises by migration from C-10 of a eudesmane intermediate. In this system also, Chappell and Noble[82] have shown that HMG-CoA reductase activity is associated with phytoalexin production and that elicitor-induced capsidiol synthesis could be blocked by the HMG-CoA reductase inhibitor, mevinolin.

Efforts in the insect juvenile hormone area can be roughly divided into three areas: biosynthesis, regulation of hormone levels by juvenile hormone esterase, and studies of mode action (*i.e.* hormone binding proteins). A complete issue of *Insect Biochemistry*[83] consists of a collection of papers on all aspects of work in this area. The biosynthesis of JH III (57) is from mevalonate with the methyl ester carbon arising from methionine. However, the ethyl branched homologues JH II (56) and JH I (55) arise by the participation of 3′-homomevalonate formed by the substitution of one propionyl-CoA for one acetyl-CoA. Brindle *et al.*[84] have considered the source of this propionyl-CoA. Measurement of ^{14}C incorporation from likely precursors of propionate into JH II revealed that isoleucine or valine were good sources of this C_3 segment while glucose, succinate, threonine, and β-alanine were not incorporated. Leucine, isoleucine, and glucose were sources of acetate for JH III and for the acetate-derived portion of JH II. Feyereisen and Farnsworth[85] have also examined the metabolic source of carbon for JH III biosynthesis. Inhibitors of JH biosynthesis have been reviewed.[86] Phorbol esters have been shown to inhibit JH III biosynthesis,[87] implicating protein kinase C as a regulatory factor in JH III levels.

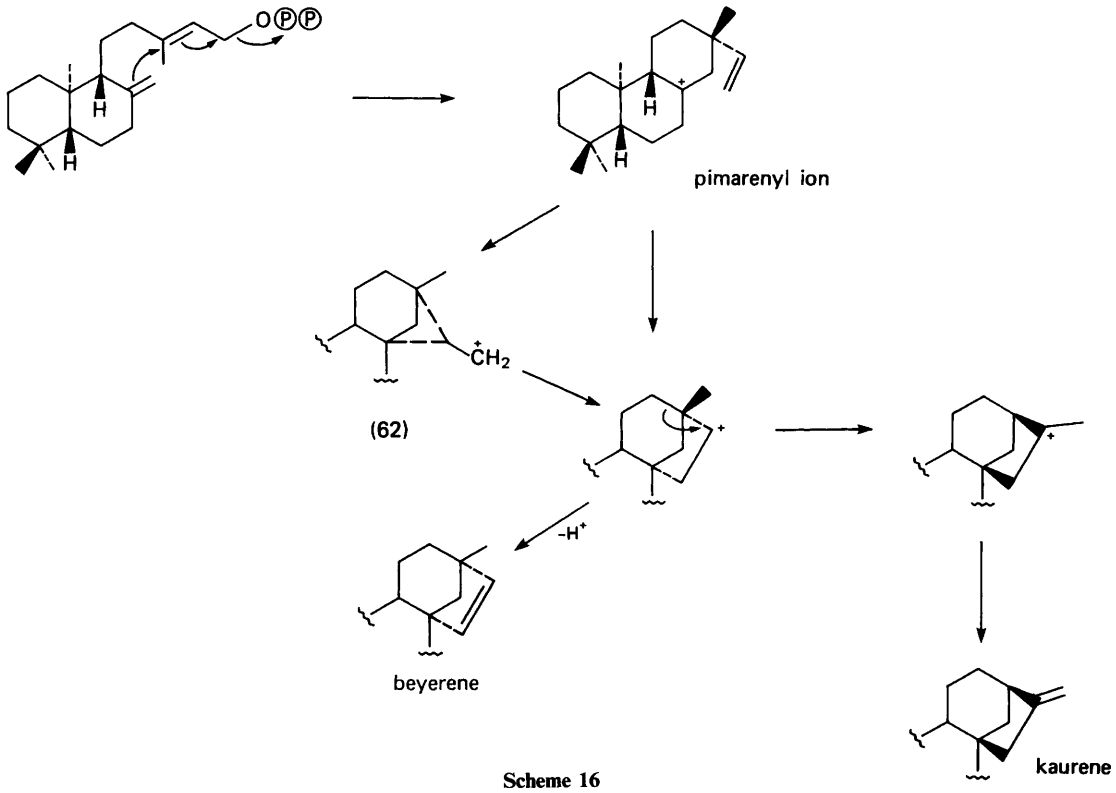
Juvenile hormone esterase is inhibited by trifluoromethyl ketones. Linderman *et al.*[88] have carried out a structure activity study on this type of compound. This led to the design and synthesis of compounds (58) and (59) which are much more active than previously known trifluoroketones. Hanzlik and Hammock[89] have used these types of inhibitor (3-alkylthio-1,1,1-trifluoropropan-2-ones), as affinity ligands for the purification of JH esterase from *Trichoplusia ni*. This technique gave a 1000-fold purification in one step. The product was one band of MW 64 kDa on SDS-PAGE, but isoelectric focusing showed multiple bands, which were all catalytic in JH hydrolysis. The proteins were non-specific in that they would hydrolyse non-JH esters. Two papers by Jones *et al.*[90,91] describe a classical but rapid isolation of the same enzymes, by precipitation with poly(ethylene glycol), gel filtration, and chromatofocusing. As above, different electromorphs were observed and immunological characterization of enzyme in different Lepidoptera was carried out.[92] Papers on the mode of action of JH, other than those in reference 83, include photoaffinity labelling[93] and isolation of a binding protein.[94]

Microbial transformations of sesquiterpenes reported this year are of quadrone by *Cunninghamella echinulata*[95] and of deoxyvulgarin by *Rhizopus nigrians* and *Aspergillus ochraceous*.[96] The biosynthesis of the meroterpenoids, australides, in *A. ustus* has been investigated by de Jesus *et al.*[97] using ^{13}C- and ^{2}H-labelled acetate and ^{13}C-mevalonate feeds. Analysis of the products by n.m.r. techniques led to proposals for the cyclization and oxidative elaboration of the farnesyl moiety of these compounds of mixed terpenoid–polyketide origin.

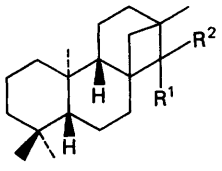
6 Diterpenoids

A procedure for the purification of geranylgeranyl diphosphate (GGPP) synthase from chromoplasts of *Capsicum annum* using affinity chromatography on Sepharose-linked aminophenylethyl diphosphate, a known inhibitor of phytoene biosynthesis, has been developed.[26] This method also yielded pure isopentenyl diphosphate isomerase (see Hemiterpenoid section). GGPP Synthase was found to be a dimer of subunit mass 37 kDa and, unlike enzyme from *Ricinus communis*, catalyses the prenyl transfer reaction of IPP (K_m 3 μM) onto DMAPP (K_m 0.95 μM), GPP (K_m 1 μM), or FPP (K_m 1.2 μM). All of these prenyl transfer activities were inhibited when the enzyme was irradiated with the photolabile substrate analogue (2-diazo-3-trifluoropropionyloxy)geranyl diphosphate (60).

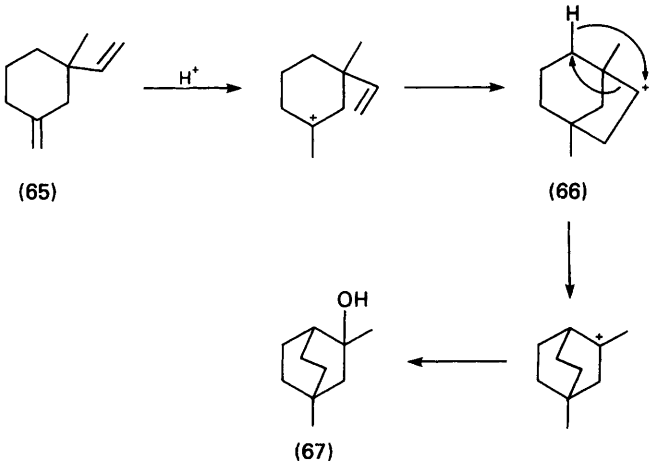
Ackland *et al.*[98] have reported further results in their study of aphidicolin (61) biosynthesis in *Cephalosporium aphidicola*.



Scheme 16



(63) $R^1 = H$, $R^2 = CH_2OH$
(64) $R^1 = CH_2OH$, $R^2 = H$

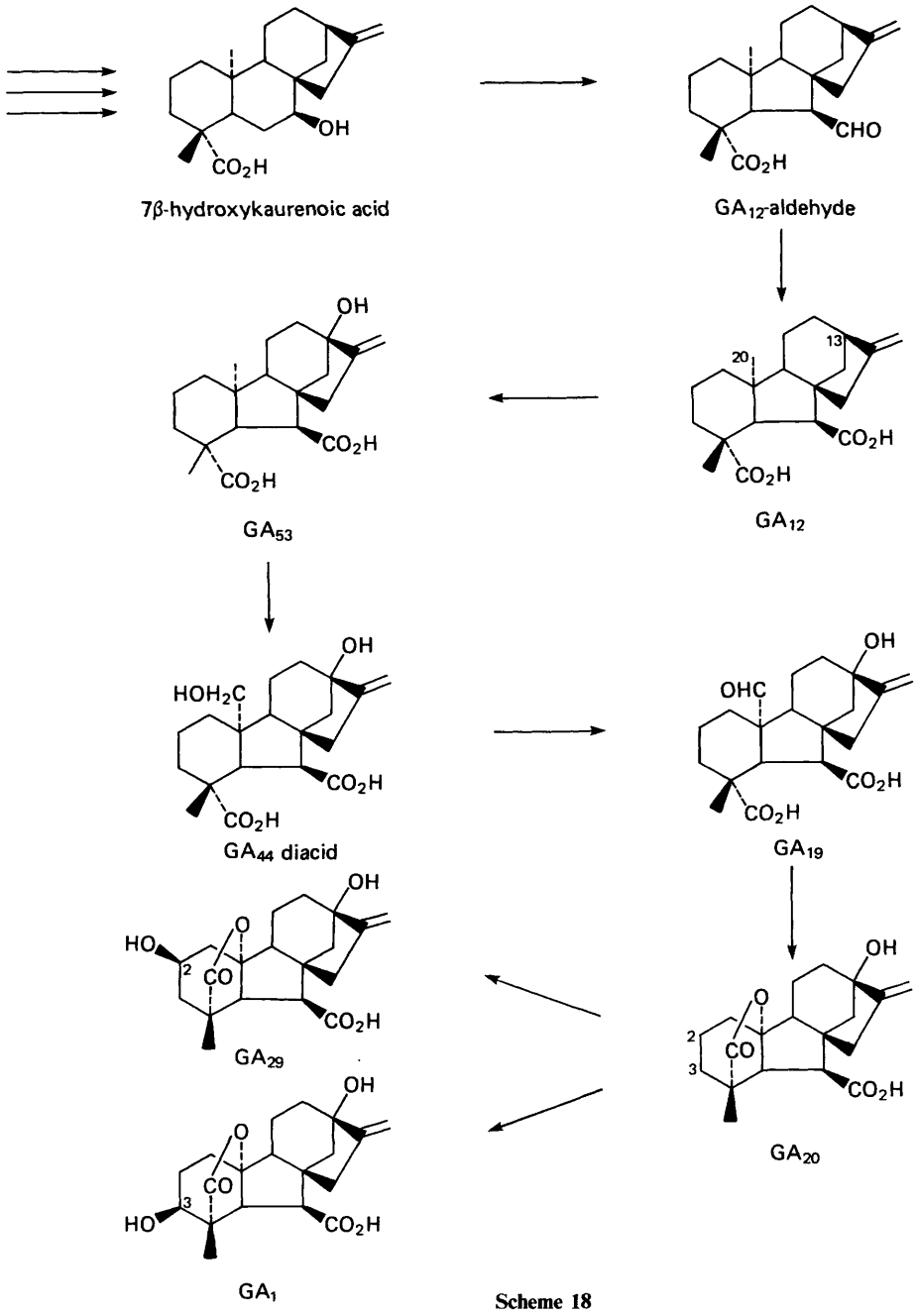


Scheme 17

Their results indicate that the 16β-hydroxy group originates from water, although it is not clear whether this is by addition to the carbocation formed in the cyclization of ring D or by hydration of the exocyclic olefin. The 17-hydroxy group arises by enzymic hydroxylation. A minor route to the 16,17-diol function was also found *via* epoxidation of the 16,17 double-bond and hydrolysis.

The usual proposed mechanism of the formation of ring D in tetracyclic diterpenes such as beyerene and kaurene from the precursor copalyl diphosphate (CPP) is shown in Scheme 16. The cyclization of the pimarenyl ion to the beyerenyl ion is a violation of Baldwin's rules for ring closure and, at this time, had never been achieved in model studies in the laboratory. The alternative exocyclic ring closure to the cyclobutylcarbinyl ion (62) has been considered by Coates and Kang.[99] They have synthesized the epimeric cyclobutycarbinols (63) and (64) and incubated the corresponding diphosphates with a kaurene synthase preparation from *Marah macrocarpus*. However, these were not incorporated into kaurene by this system. Therefore they are not free intermediates in the cyclization of CPP. In contrast, solvolysis of the tosylates gave beyerene, isokaurene, and kaurene as well as some pimaradienes, as predicted in Scheme 16. In further studies[100] on this biosynthetic cyclization, the disallowed 5-*endo*-trig cyclization of pimarenyl ion to beyeranyl ion has now been modelled by the superacid catalysed transformation of 3-methyl-3-vinylmethylenecyclohexane (65) to the bicyclic alcohol (67) (Scheme 17). This occurs *via* the beyerenyl-type ion (66) which then undergoes a 1,3-hydride shift and Wagner Meerwein rearrangement to give (67) which is analogous to the proposed mechanism of the biosynthesis of atiserene.

The early 13-hydroxylation pathway of gibberellin (GA) biosynthesis is shown in Scheme 18. In the long day rosette plant, spinach (*Spinacis oleracia*), the flux of gibberellins through this pathway to the more active C_{19} compounds is controlled by day length. Gilmour *et al.*[101] have begun to purify the oxidative enzymes involved in this pathway in spinach. One of these, GA_{44} oxidase (MW 38.1 kDa), was purified one hundred fold by HPLC techniques. Molecular weight information was obtained for three other enzymes of this part of the pathway–GA_{12}-13-hydroxylase (28.4 kDa), GA_{53}-oxidase (42.5 kDa), and GA_{19}-oxidase (39.5 kDa)–although these activities could not be purified. In further investigations of the genetic block in gibberellin biosynthesis in mutants of pea, Ingram and Reid have shown[102] that the *Na* gene may control the ring contraction of 7β-hydroxykaurenoic acid to GA_{12}



Scheme 18

aldehyde. Similarly the *1h* and *1s* mutations were indicated to be before *ent*-kaurene. In a study of GA biosynthesis in relation to fruit growth of pea, Garcia-Martinez *et al.*[103] have shown that GAs produced in the seed regulate pod growth early in fruit development, whereas later in seed enlargement GAs are not necessary for normal seed development. The *procera* mutant of tomato looks like a normal plant to which exogenous GAs have been applied. This giantism has been studied by Jones[104] who showed it was not due to the overproduction of GA_{20} or GA_1 or to an over-response to exogenous GA. Further reports of radioimmunoassay (RIA) for certain GAs have appeared[105,106] and a comparison of RIA with g.c.–m.s. measurement of GA_9 levels in *Picea abies* has been carried out.[107] Fraga *et al.* have continued their studies on the biotransformation of analogues by *Gibberella fujikuroi*. *Ent*-kaur-15-ene was converted[108] into GA analogues, including GA_3-15-ene, while 7-hydroxy-atis-15-ene-19-oic acid only gave[109] C_{20}-GA analogues in accordance with previous studies with this tetracyclic carbon skeleton.

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