The Biosynthesis of Triterpenoids, Steroids, and Carotenoids

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Reviewing the literature published between January 1984 and December 1985
(Continuing the coverage of literature in *Natural Product Reports*, 1985, Vol. 2, p. 525 and 1986, Vol. 3, p. 205)

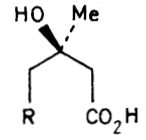
1 Introduction

In earlier volumes of this journal, the biosynthesis of triterpenoids and steroids[1] was treated separately from the biosynthesis of carotenoids.[2] These two complementary strands are brought together again in this Report. The papers that are discussed were selected mainly through the perusal of *Chemical Titles*, and of the major chemistry and biochemistry journals, for the appropriate period. As before, it has often been difficult to decide which publications to discuss and which to exclude. This was particularly true of Sections 2, 5.2, and 5.3, for which I have endeavoured to select those papers that present new results, of interest to the biosynthetic chemist, from among the overwhelming number of related publications that are mainly of medical or biological interest. Studies on the isolation, purification, properties, and inhibition of the enzymes of biosynthesis are treated in an illustrative rather than a comprehensive fashion.

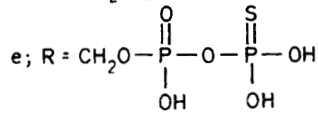
Two volumes of *Methods in Enzymology* are devoted in large part to the enzymes of steroid and carotenoid biosynthesis.[3] Some individual contributions to these works, and other reviews, will be cited at appropriate places in the text.

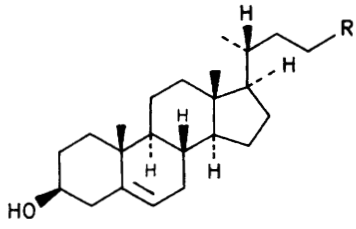
2 Mevalonic Acid

Mevalonic acid (1a) is formed *via* the thiohemiacetal (1b) by the stepwise reduction of (3*S*)-3-hydroxy-3-methylglutarylco-enzyme A (HMG-CoA) (1c) with NADPH, catalysed by the enzyme hydroxymethylglutaryl-CoA reductase (NADPH) [E.C. 1 . 1 . 1 . 34]. The mammalian reductase remains the subject of intensive study, owing to its critical role in the regulation of the biosynthesis of cholesterol (2a). The complete amino-acid sequence of the reductase of hamster has been deduced from sequence studies on the cloned cDNA.[4] This reductase is a glycoprotein, of mol. wt. 97092, containing 887 amino-acid residues, the *N*-terminal domain of which remains

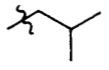


(1) a; R = CH_2OH
b; R = CH(OH)S-CoA
c; R = C(O)S-CoA
d; R = CH_2OⓅ

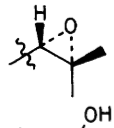




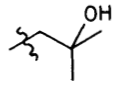
(2) a; R =



b; R =



c; R =



membrane-bound; soluble enzymically active proteins of lower molecular weight are formed by adventitious proteolysis of the native enzyme during its isolation.[5] The native reductase of rat liver is a dimer,[6] the monomer units of which are linked by a carbohydrate residue.[7] A human HMG-CoA reductase has been prepared by using cloned cDNA;[8] this reductase, which contains 888 amino-acid residues, shows close homology with the enzyme of hamster, especially in the catalytic domain and in the membrane-bound region. The latter region is responsible for the sterol-regulated degradation of the enzyme.[8] Antibodies that were raised to the HMG-CoA reductase of rat liver cross-reacted with the reductase of human origin.[9]

It is known that mammalian HMG-CoA reductase (NADPH) is subject to rapid modulation of activity by means of a reversible phosphorylation–dephosphorylation cycle.[1,10] The less active (phosphorylated) form of the enzyme is re-activated by dephosphorylation, catalysed by endogenous non-specific phosphatases.[11] In the reverse direction, the more active form of the reductase is partially inactivated by ATP-dependent phosphorylation, catalysed by the enzyme [hydroxymethyl-glutaryl-CoA reductase (NADPH)] kinase [E.C. 2 . 7 . 1 . 109], which is itself subject to modulation of activity by reversible

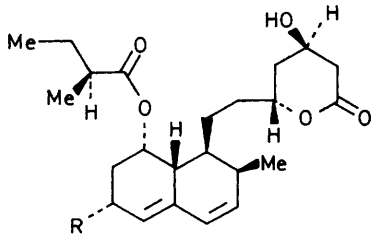
phosphorylation; the phosphorylated reductase kinase is the more active form.[1,10] A reductase kinase of rat liver has been purified to homogeneity.[12]

Further evidence has been adduced for the contention that the short-term modifications of the activity of mammalian HMG-CoA reductase (NADPH) that can be achieved by altering the diet of experimental animals is due to reversible phosphorylation of the enzyme.[13,14] In particular, administration of mevalonolactone to the rat stomach resulted in a 38% reduction in the activity of the reductase of the liver, within 20 minutes, in parallel with an increase in the level of phosphorylation both of the reductase and of the reductase kinase.[14] Furthermore, it has been shown that the fraction of the reductase of rat liver that is in the active (unphosphorylated) form is subject to diurnal variation *in vivo*.[15]

It was suggested earlier that the regulation of the cholesterol-utilizing enzymes cholesterol acyltransferase [E.C. 2 . 2 . 3 . 1 . 26] and cholesterol 7α-mono-oxygenase [E.C. 1 . 14 . 13 . 17] is linked in each case, by means of a phosphorylation–dephosphorylation cycle, to the regulation of HMG-CoA reductase (NADPH) and that the former two enzymes are both more active in the phosphorylated state.[1] Doubt has been cast on the validity of this proposal by the report that conditions which modulated the activity of the reductase by reversible phosphorylation had no effect on the activity of the acyltransferase.[16] Furthermore, a cytosolic protein which stimulated the mono-oxygenase, in the presence of glutathione, had no effect on the reductase; moreover, neither the reductase kinase nor phosphoprotein phosphatases had any effect on the activity of the mono-oxygenase.[17]

The fungal metabolite compactin (3a) (also known as ML-236B) and related compounds are important as potential hypocholesterolemic agents that function by inhibiting HMG-CoA reductase (NADPH).[18] Several mammalian cell lines are resistant to compactin, owing to an extraordinary overproduction of the enzyme. Such cell lines are important sources of the mammalian reductase (*e.g.* ref. 4). Studies have been reported on the mechanisms of over-accumulation of HMG-CoA reductase (NADPH) in compactin-resistant cells[19] and on the mode of interaction of compactin with the HMG-CoA reductase of the yeast *Saccharomyces cerevisiae*.[20] Several structural analogues of compactin that were isolated from *Monascus ruber* have been described.[21] The growth of tissue explants of *Helianthus tuberosus* was inhibited by compactin (3a) or mevinolin (3b); the addition of mevalonic acid resulted in restoration of growth.[22] New synthetic inhibitors of HMG-CoA reductase (NADPH) include derivatives of 5-alkylated 3,5-dihydroxypentanoic acids and the derived δ-lactones,[23] various 3-alkyl-3-hydroxypentanedioic acids,[24] and a non-reducible analogue of the thioacetal (1b).[25]

Hydroxymethylglutaryl-CoA reductase (NADPH) is inhibited if it is incubated with a soluble protein of rat liver, named fermodulin, in the presence of Fe^{2+} ions. The inhibitory protein has been purified and characterized.[26] The effects of thiols[27] and of coenzyme A derivatives[28] on the activity of the rat liver reductase have been described. Neither NAD^+ nor NADH is a substrate for the HMG-CoA reductase (NADPH) of rat liver microsomes, but both were found to be allosteric activators of the enzyme; various analogues of these coenzymes have been tested as potential activators of the reductase.[29] Kinetic studies have been reported on the reductase of radish.[30] The sub-cellular distribution of the HMG-CoA reductase and of mevalonate kinase [E.C. 2 . 7 . 1 . 36] in leaves of *Nepeta cataria* has been investigated.[31]



(3) a; R = H
b; R = Me

3 The Biosynthesis of Squalene from Mevalonic Acid

The enzyme mevalonate kinase[32] [E.C. 2 . 7 . 1 . 36] catalyses the reaction between mevalonic acid and ATP, in the presence of a divalent metal cation (usually Mg^{2+}), to furnish 5-phosphomevalonic acid (1d) and ADP. The manner in which the metal cation is bound to the ionized triphosphate unit of ATP, at the active site of porcine mevalonate kinase, has been determined by kinetic studies in which a series of adenosine 5′-thiotriphosphates were utilized as analogues of the natural substrate and a range of metal ions were used, including Cd^{2+} and Mg^{2+}.[33] Procedures have been described for the purification and assay of porcine phosphomevalonate kinase [E.C. 2 . 7 . 4 . 2].[34] The thiodiphosphate (1e) of mevalonic acid has been prepared with the aid of the latter enzyme.[35]

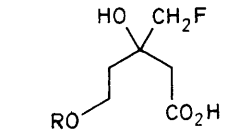
The ATP-requiring enzyme diphosphomevalonate decarboxylase [E.C. 4 . 1 . 1 . 33] has been isolated from the lemon grass *Cymbopogon citratus*. The enzyme has an optimum pH of 6.0 and requires Mg^{2+} (or Mn^{2+}) ions for activity.[36] On the basis of responses to dietary changes, it has been suggested that the decarboxylase may have a role in the regulation of cholesterol biosynthesis in the chick.[37] The fluoromevalonate derivative (4a) inhibits the incorporation of mevalonic acid [but not of isopentenyl diphosphate (5a)] into cholesterol in a rat liver enzyme system.[38] The observed inhibition is a result of the metabolic conversion of the alcohol (4a) into the diphosphate (4b), which is a potent competitive inhibitor of diphosphomevalonate decarboxylase. The isolation of diphosphomevalonate decarboxylase from avian liver has been described.[39]

The equilibration of isopentenyl diphosphate (5a) with dimethylallyl diphosphate (6a) is catalysed by the enzyme isopentenyl-diphosphate Δ-isomerase [E.C. 5 . 3 . 3 . 2].[40] The subsequent condensations of dimethylallyl diphosphate with isopentenyl diphosphate to furnish geranyl diphosphate (7), and of the latter with isopentenyl diphosphate to give 2-*trans*,6-*trans*-farnesyl diphosphate (8), are both catalysed by the enzyme dimethylallyl*trans*transferase [E.C. 2 . 5 . 1 . 1], which is often referred to as farnesyl pyrophosphate synthetase or prenyltransferase. The latter term is sometimes used also to encompass geranylgeranyl-diphosphate synthase activity. The names that were used for these enzymes in the papers that are cited below will be retained in this Report.

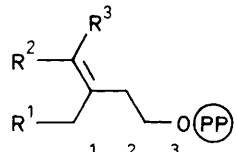
The fluorinated derivatives (5b) and (6b), of isopentenyl diphosphate and dimethylallyl diphosphate respectively, each proved to be potent inhibitors of the isopentenyl-diphosphate Δ-isomerase of *Claviceps* SD58, apparently owing to covalent modification of the enzyme.[41] Furthermore, the amine (9) inhibited the isomerase of the same strain of *Claviceps*[41] and of *Saccharomyces cerevisiae*;[42] presumably the protonated amine acts as an analogue of the carbo-cation that is formally an intermediate in the enzyme-catalysed equilibration of (5a) and (6a).

The three homologues (5c), (5d), and (5e) of isopentenyl diphosphate and the two homologues (6c) and (6d) of dimethylallyl diphosphate were each converted into the same equilibrium mixture, in which the homoallylic diphosphate (5d) predominated, when incubated with the isomerase of pig liver.[43] The reactions that took place in deuteriated water were also studied. The results were interpreted in terms of a model for the topology of the active site and the postulated involvement of two catalytic groups, for proton abstraction and proton donation respectively.

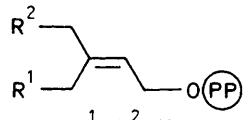
Isopentenyl pyrophosphate isomerase, farnesyl pyrophos-



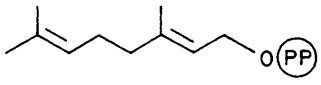
(4) a; R = H
b; R = O(PP)



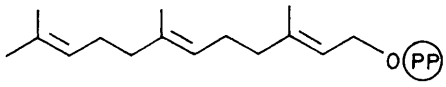
(5) a; $R^1 = R^2 = R^3 = H$
b; $R^1 = F$, $R^2 = R^3 = H$
c; $R^1 = Me$, $R^2 = R^3 = H$
d; $R^1 = R^3 = H$, $R^2 = Me$
e; $R^1 = R^2 = H$, $R^3 = Me$



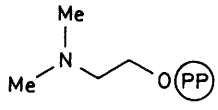
(6) a; $R^1 = R^2 = H$
b; $R^1 = F$, $R^2 = H$
c; $R^1 = Me$, $R^2 = H$
d; $R^1 = H$, $R^2 = Me$



(7)



(8)



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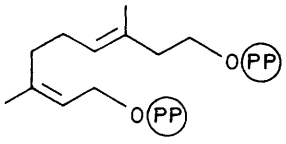
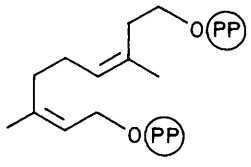


(10)



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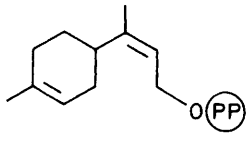

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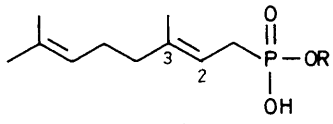
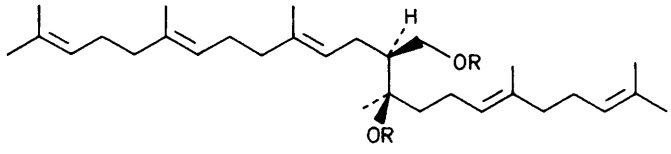
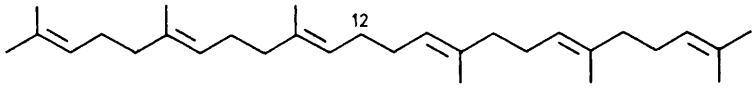


(13) a; R = (P)
b; R = (P); 2,3-dihydro
c; R = H
d; R = H; 2,3-dihydro



(14) a; R = (PP)
b; R = H

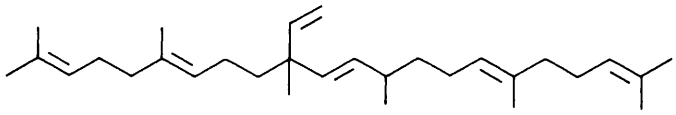


(15)

phate synthetase, and geranylgeranyl pyrophosphate synthetase have been detected in cell-free extracts of the silkworm *Bombyx mori*, and partially purified.[44] Two non-interconvertible forms, I and II, of the farnesyl pyrophosphate synthetase were identified;[44] synthetase II showed preference for the homodimethylallyl diphosphate (6c) and the homoisopentenyl diphosphate (5c) as substrates, and may be involved in the biosynthesis of juvenile hormones.[45] The enzymes isopentenyl pyrophosphate isomerase and prenyltransferase have been isolated from plastids of tomato fruit and purified.[46] Both enzymes required Mg^{2+} or Mn^{2+} ions for activity; the isomerase was totally inactivated by iodoacetamide at a concentration of 1 mmol dm^{-3}.[46] An affinity-chromatography technique has been described for the rapid and efficient one-step purification of farnesyl pyrophosphate synthetase.[47] The latter enzyme is regarded as requiring Mg^{2+} or Mn^{2+} cations for activity; it has now been reported that the enzyme from porcine or avian liver shows higher catalytic activity in the presence of Zn^{2+} ions (0.2 mmol dm^{-3}) instead.[48]

The unnatural substrates (10) and (11) each furnished a mixture of monocyclic diphosphates, in which the *cis*-compound (12) predominated, when incubated with avian farnesyl pyrophosphate synthetase.[49] The phosphonyl phosphates (13a) and (13b) were shown to be powerful inhibitors of pig liver prenyltransferase; not surprisingly, the phosphonates (13c) and (13d) were less good as inhibitors.[50]

It was suggested earlier that the dimer (14a) of 2-*trans*, 6-*trans*-farnesyl diphosphate is an intermediate in the biosynthesis of squalene (15). The racemic diol (14b) has been synthesized, with a view to testing that proposal.[51] Practical accounts have been published concerning the isolation of prenyltransferases from yeast,[52] from liver,[52,53] and from pumpkin.[54] The isolation of squalene synthetase,[55] and the aberrant formation of 12-*cis*-12,13-didehydrosqualene,[56] were



(16)

discussed also. The reactions that are catalysed by prenyltransferase and squalene synthetase have been reviewed.[57]

The unicellular alga *Botryococcus braunii* synthesizes a range of hydrocarbons, including the irregular triterpene (16) and its homologues. In a series of preliminary experiments, sodium [U-^{14}C]acetate and [U-^{14}C]leucine were incorporated into (16) in 11.8% and 5.2% yields respectively, while [2-^{14}C]mevalonate was incorporated to an extent of only 0.2%.[58]

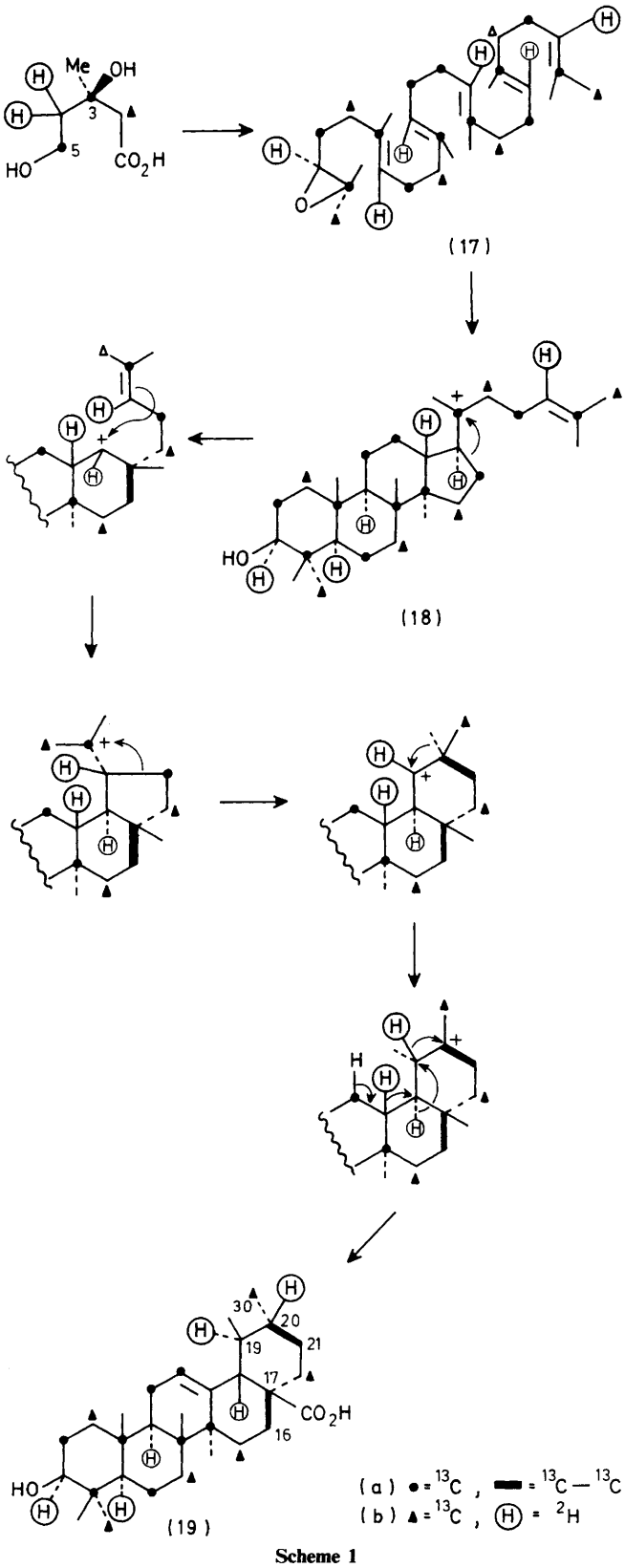
4 The Biosynthesis of Triterpenoids from Squalene

The next step in the formation of sterols is the oxidation of squalene to furnish (3*S*)-squalene 2,3-epoxide (17). The action of the enzyme squalene epoxidase [squalene mono-oxygenase (E.C. 1.14.99.7)] in a washed microsomal fraction of the yeast *Candida albicans* was studied, utilizing the conversion of [4,8,12,13,17,21-^{3}H]squalene into [^{3}H]squalene epoxide as the assay.[59] The epoxidase system required molecular oxygen, NADH or NADPH, and FAD for activity. Unlike the epoxidase of rat liver, the *Candida* enzyme was more active with NADH than with NADPH and its activity was destroyed if it was mixed with Triton X-100. The epoxidase system was stimulated by a soluble fraction from the cytoplasm.[59] The antimycotic agents naftifine and SF 86-327 are potent inhibitors of the squalene epoxidase of fungi; the epoxidase of rat liver is much less sensitive to these compounds.[60] The isolation of squalene epoxidase from rat liver microsomes has been reviewed.[61]

Further studies have been reported on the role of 'supernatant protein factor' (SPF) in the stimulation of mammalian squalene epoxidase; it has now been shown that SPF, in the presence of anionic phospholipids, stimulates uptake of squalene by microsomes as well as stimulating the epoxidase.[62] The efficacy of SPF in stimulating squalene epoxidase is destroyed, in a time- and concentration-dependent manner, if it is incubated with ATP, especially in the presence of Mg^{2+} ions.[63]

Further evidence has been adduced (by ^{13}C n.m.r.) in support of the proposal that the biosynthesis of the pentacyclic triterpene ursolic acid (19) follows the route that is summarized in Scheme 1.[64] Thus [3,5-$^{13}C_2$]mevalonate was incorporated into ursolic acid, by cell cultures of *Perilla frutescens* var. *acuta*, with the labelling pattern shown in (19a). In particular, the observation of coupling between ^{13}C-labelled carbons 16 and 17 and between C-20 and C-21 demonstrated, for each coupled pair of carbon atoms, that the bond between them was formed by a Wagner–Meerwein rearrangement within the *same* mevalonate unit. Furthermore, the β-isotope shift that was observed for C-30 in ursolic acid that had been labelled biosynthetically, following a feeding experiment with [2-^{13}C, 4,4-$^{2}H_2$]mevalonate, confirmed that the postulated 1,2-migration of hydride ion from C-19 to C-20 (ursene numbering), had occurred, as shown in Scheme 1(b).[64] The results provide compelling evidence for the essential features of the biosynthetic scheme that was advanced over 30 years ago by Ruzicka *et al.*[65]

It was established earlier that protonated 2-aza-2,3-dihydrosqualene (20a) and related compounds are potent inhibitors of the enzyme-catalysed cyclization of squalene 2,3-epoxide to



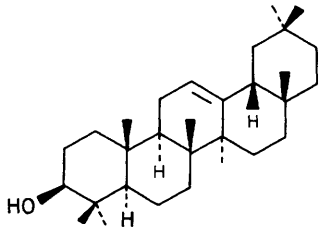
Scheme 1

furnish triterpenoids.[1,66] It has been reported that the corresponding *N*-oxides, especially (20b), are yet more potent as inhibitors of the conversion of squalene epoxide both into lanosterol (21a) [catalysed by lanosterol synthase (E.C. 5 . 4 . 99 . 7)] in rat liver homogenates and into β-amyrin (22) in enzyme extracts of pea.[67] Presumably these inhibitors act by mimicking the formal carbo-cation intermediate that is generated by protonation of squalene epoxide. In contrast, the ammonium ion (23) was a poor inhibitor of the cyclization of squalene epoxide to β-amyrin, in an enzyme system from pea, despite being a structural analogue of the (formal) intermediate cation (18).[68]

The decalins (24a) and (24b), which are inhibitors of lanosterol synthase in hamster cells, act as non-competitive inhibitors of the synthase in rat liver systems and do not inhibit the cyclization of squalene epoxide to triterpenoids in plants. Aza-derivatives, such as (24c), showed no inhibitory activity towards the cyclization of squalene epoxide in any system that was tested.[69] The antimalarial drug chloroquine blocks the biosynthesis of sterols in mammalian cells by inhibiting lanosterol synthase.[70] Other inhibitors of the latter enzyme have been studied.[71]

The bicyclic triterpenoid (25), isolated from gum mastic (the resin of the shrub *Pistacia lentiscus*), may be regarded as the product of the trapping, by water, of a cationic intermediate in the cyclization of squalene epoxide to tetracyclic and pentacyclic triterpenoids.[72] The bicyclic hydrocarbons (26a) and (26b) similarly may be considered to be products of the interruption of the proton-initiated cyclization of squalene to furnish pentacyclic triterpenoid hydrocarbons.[73]

The prokaryotes in general lack the ability to synthesize steroids, at least in quantity, though there are some notable exceptions.[74] On the other hand, triterpenoids of the hopane variety are widespread among the prokaryotes, and may constitute the evolutionary forerunners of steroids.[74] It has been shown that the biosynthetically intriguing C_{35} bacterio-



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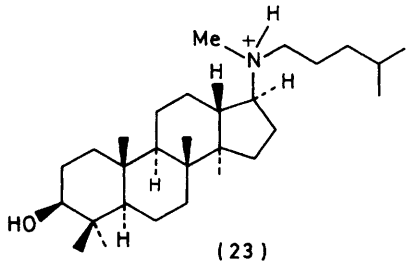

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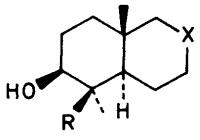
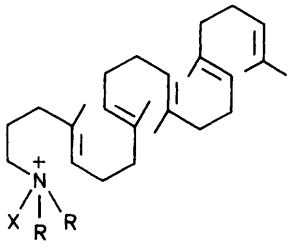
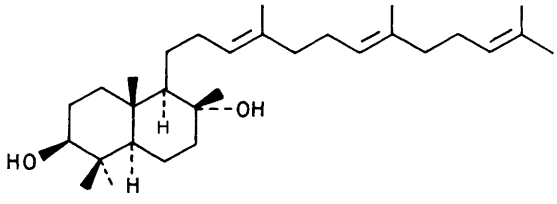


(24) a ; R = Me , X = CH_2
b ; R = H , X = CH_2
c ; R = H , X = $NHCH_2Ph$



(20) a ; X = H , R = Me
b ; X = O^- , R = Et



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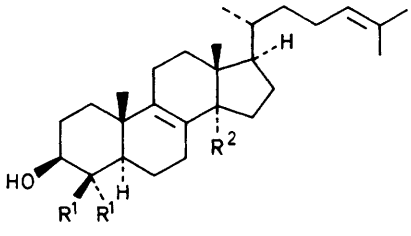
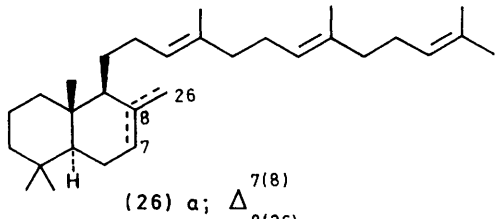


(21) a ; $R^1 = R^2 = Me$
b ; $R^1 = Me$, $R^2 = H$
c ; $R^1 = R^2 = H$



(26) a; $\Delta^{7(8)}$
b; $\Delta^{8(26)}$

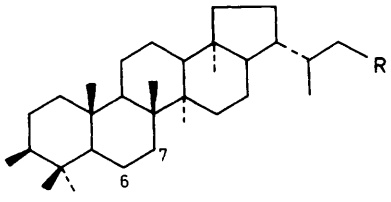
hopanepolyols co-occur with their 3β-methyl-derivatives (27a), (27b), and (27c), and also that 3β-methyldiplopterol (28a) is formed alongside diplopterol (28b), in *Acetobacter pasteurianus* subsp. *pasteurianus*. The 3β-methyl-hopanoids become the major triterpenoids when the latter species is grown in the presence of methionine.[75] Other bacteria have furnished 2β-methyl-hopanoids, such as 2β-methyldiplopterol (28c).[76] The results of biosynthetic feeding experiments, in which either label from a mixture of [*Me*-^{3}H]methionine and [*Me*-^{14}C]-methionine or from [*Me*-$^2H_3$]methionine was incorporated into 2β- and 3β-methyl-hopanoids, were consistent in each case with the transfer of an intact methyl group.[77] In particular, 2β-[$^2H_3$]-methyldiplopterol and 3β-[$^2H_3$]methyldiplopterol were formed when L-(*Me*-$^2H_3$)methionine served as precursor in *Methylobacterium organophilum* or in *Acetobacter pasteurianus* subsp. *pasteurianus* respectively. The mixed bacteriohopanepolyols (27a), (27b), and (27c) from the latter experiment were degraded to the alcohols (27d), (27e), and (27f), respectively, prior to their assay (by mass spectrometry of the derived acetates). In each case the mass spectrum confirmed the presence of $^2H_3$-labelled species, though the precise location of isotopic label was not determined. The attractive possibility that the 3β-methyl-hopanoids could arise from initiation of the cyclization of squalene by means of a (formal) methyl cation is not readily reconciled with the formation of 2β-methyl-hopanoids; it was suggested that both series of triterpenoid are formed by alkylation of a Δ^2-olefin precursor.[77]

5 The Formation of Sterols in Vertebrates

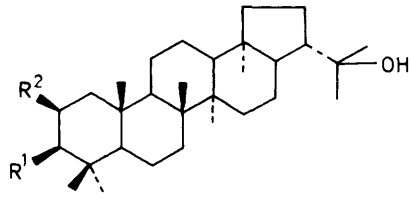
5.1 The Biosynthesis of Cholesterol

Studies have been reported on the conversion of [24,25-^{3}H]-24,25-dihydrolanosterol (29) into 4,4-dimethyl-5α-cholest-8-en-3β-ol (Scheme 2) by rat liver microsomes in which oxidative demethylation at C-4 was inhibited by cyanide ion (1 mmol dm^{-3}).[78] The conditions that were used for the microsomal oxidation of the dihydrolanosterol could be manipulated to permit the accumulation of the oxygenated intermediates (30) and (31) or the conjugated diene (32) or 4,4-dimethyl-5α-cholest-8-en-3β-ol (33). It is known that the oxidation of the 14α-methyl group of 24,25-dihydrolanosterol is performed by a cytochrome-*P*-450-based mixed-function oxidase system. The cytochrome *P*-450 component has now been solubilized and partially purified.[78]

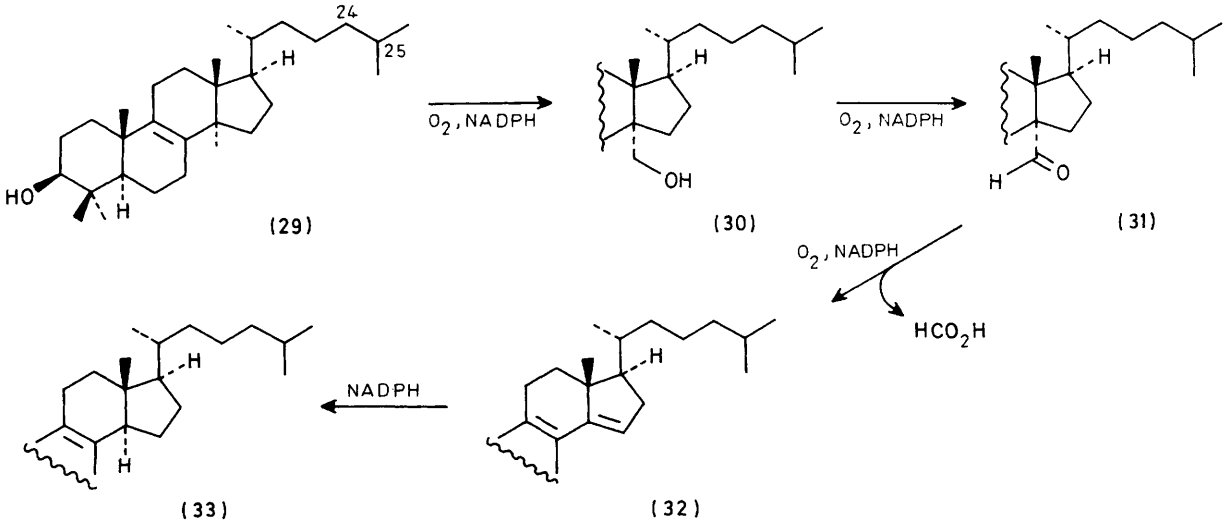
The reductase that catalyses the NADPH-dependent reduction of 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol (32) to the Δ^8-sterol (33) in rat liver microsomes has been studied;[79] anaerobic conditions were chosen to prevent further metabolism of the latter sterol. It was found that the reductase was inhibited by reagents that bind to thiol groups, and by the drug AY-9944, but not by cyanide ion. The enzyme displayed its maximum activity at pH 7.4 and there was no requirement for a metal ion. The membrane-bound enzyme has been partially purified; in particular, the reductase activity has been separated from the other microsomal enzymes of cholesterol biosynthesis.



(27) a; R = $CH_2CH(OH)CH(OH)CH(OH)CH(OH)CH_2OH$
b; 6,7-didehydro; R = $CH_2CH(OH)CH(OH)CH(OH)CH(OH)CH_2OH$
c; R = $CH(OH)CH(OH)CH(OH)CH(OH)CH(OH)CH_2OH$
d; R = CH_2CH_2OH
e; 6,7-didehydro; R = CH_2CH_2OH
f; R = CH_2OH



(28) a; R^1 = Me, R^2 = H
b; R^1 = R^2 = H
c; R^1 = H, R^2 = Me



Scheme 2

The purified reductase showed essentially the same properties as the membrane-bound enzyme.[79]

The penultimate step in the biosynthesis of cholesterol (2a) is the oxidation of 5α-cholest-7-en-3β-ol (34) to give the conjugated diene (35) (Scheme 3). The 'desaturase' enzyme has been solubilized, and purified 70-fold, from rat liver microsomes.[80] The purified enzyme required cytochrome b_5 and cytochrome-b_5 reductase [E.C. 1.6.2.2] for activity; the reconstituted enzyme system converted (34) into (35) in the presence of NADH and oxygen. It was shown also that one mole of NADH was consumed for each mole of diene formed (but for technical reasons this stoicheiometry was determined by using an epimer, α-NADH, of the natural coenzyme); it follows that the enzyme system is a mixed-function oxidase. Particularly noteworthy was the removal of the 4-methylsterol oxidase activity from the desaturase during purification of the latter.[80] Thus the two activities are not associated with the same terminal oxidase, contrary to an earlier suggestion.

It was reported previously that the microsomal dehydrogenation of 5α-cholest-7-en-3β-ol (34) to the diene (35) was promoted by a 'sterol carrier protein' (SCP), and it was suggested that SCP may be identical to 'fatty acid binding protein' (Z-protein). The identity of SCP with Z-protein has now been established.[81,82] It has also been claimed that the apparent binding of sterols to Z-protein is an artefact of the assays used, but that 'sterol carrier protein 2' (SCP-2), which was implicated in the microsomal conversion of lanosterol into cholesterol and in the metabolism of the latter in the adrenal cortex, is distinct from Z-protein.[82] The primary structure of the SCP-2 from bovine liver has been reported.[83] The literature on sterol carrier proteins SCP (*i.e.* Z-protein)[84] and SCP-2[85,86] has been reviewed.

The natural minor steroids (24*S*)-24,25-epoxycholesterol[87,88] (2b) and 25-hydroxycholesterol[88] (2c) both inhibit the biosynthesis of cholesterol in mammalian cell cultures; the main site of action in each case is HMG-CoA reductase (NADPH). A series of derivatives of 22-hydroxycholesterol has been tested, both *in vitro* and *in vivo*, as inhibitors of HMG-CoA reductase (NADPH); some of these compounds showed promise as hypocholesterolemic agents.[89] Other oxygenated sterols inhibited the biosynthesis of cholesterol from 24,25-dihydrolanosterol in an enzyme system from liver;[90] analogues of lanosterol, with lengthened side-chains, were ineffective as inhibitors of the biosynthesis of cholesterol from lanosterol in the same enzyme system.[91]

The biosynthesis of cholesterol from acetyl-coenzyme A[92] and the membrane-bound enzymes which effect the biosynthesis of cholesterol from squalene[93] have been reviewed.

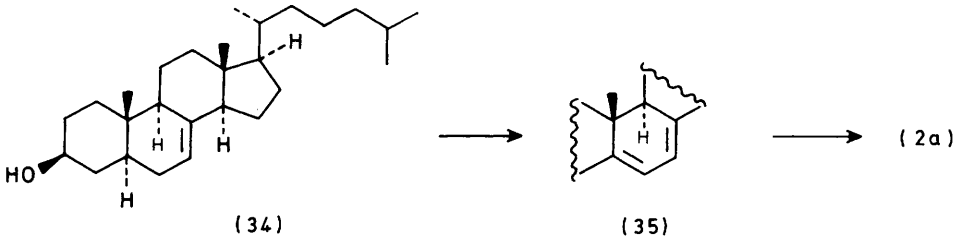
5.2 The Biosynthesis of Steroidal Hormones

The first committed step in the biosynthesis of the steroidal hormones[94] is the oxidative cleavage of cholesterol to furnish pregnenolone (36a) and 4-methylpentanal. This transformation requires oxygen and NADPH, is catalysed by a cytochrome-*P*-450-dependent mixed-function oxidase [cholesterol monooxygenase (side-chain-cleaving) (E.C. 1.14.15.6)], and involves the transient formation of (22*R*)-22-hydroxycholesterol (37a) and (22*R*)-20,22-dihydroxycholesterol (37b). Powerful new evidence for the intermediacy of these two compounds was obtained by studying the kinetics of oxidation of cholesterol by a reconstituted enzyme system that included the side-chain-cleaving cytochrome *P*-450 [cytochrome *P*-450_{scc}] of adrenal mitochondria and the iron–sulphur protein adrenodoxin.[95] In particular, by utilizing anaerobic conditions for reductive steps, it was possible to perform successive single reduction–oxygenation cycles on cholesterol and to observe the sequential appearance of 22-hydroxycholesterol (37a), 20,22-dihydroxycholesterol (37b), and pregnenolone (36a).[95]

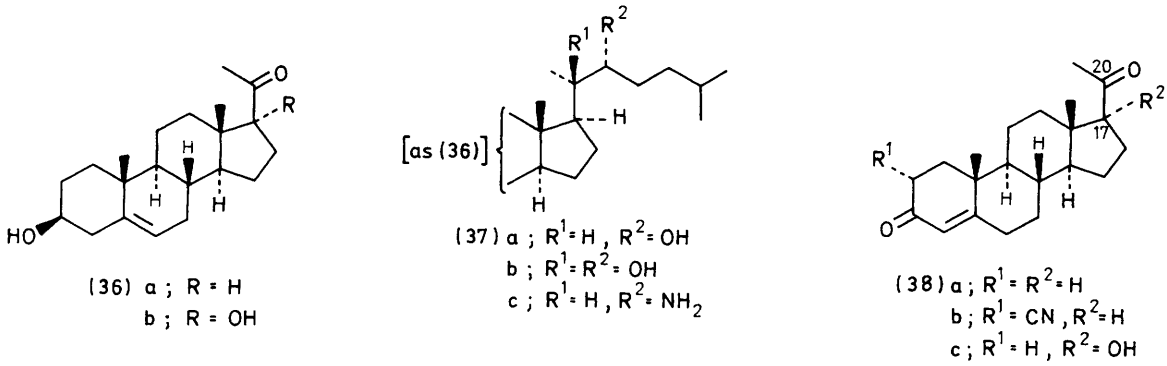
The cytochrome *P*-450_{scc} of bovine adrenal mitochondria consists of a single polypeptide chain, with two independently folded domains; the *N*-terminal domain contains the haem-binding and the adrenodoxin-binding sites.[96] The complete amino-acid sequence of the cytochrome has been reported.[97]

It has been shown that the sterol carrier protein SCP-2 stimulates the biosynthesis of pregnenolone from cholesterol by assisting the transfer of the latter steroid from the outer to the inner membrane of adrenal mitochondria.[98] Several C_{27} derivatives of cholest-4-en-3-one, all of which contained a 22*R*-hydroxyl group, were oxidized to progesterone (38a) by adrenal mitochondria; the parent compound, cholest-4-en-3-one, was not metabolized.[99] It seems likely that these observations merely reflect the lack of specificity of cytochrome *P*-450_{scc} for its normal substrate.[100]

Studies continue on the development of mechanism-based inhibitors of cytochrome *P*-450_{scc}. The silicon-containing



Scheme 3



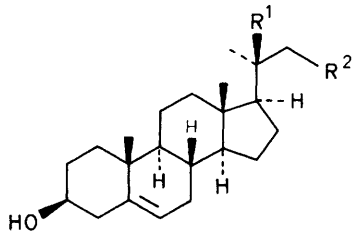
analogue (39a) of cholesterol caused the inactivation of cytochrome $P\text{-}450_{scc}$ in a time-dependent fashion, perhaps owing to its oxidation by the enzyme to form a transient 22-carbo-cation, followed by silylation of the enzyme [see arrows in structure (40)].[101] (22*R*)-22-Aminocholesterol (37c)[102] and the analogues (39b) and (39c), with shorter side-chains,[103] were each potent inhibitors of cytochrome $P\text{-}450_{scc}$. It is believed that, in each case, the inactivation arises from binding of the inhibitor to the cholesterol-binding site on the cytochrome in such a way that the amino-group co-ordinates to the haem iron. Various inhibitors of cytochrome $P\text{-}450_{scc}$ have been tested for their ability to inhibit the production of corticosterone by rat adrenal cells.[104] Model systems have been described for the cleavage of the triol (37b), catalysed by cytochrome $P\text{-}450_{scc}$.[105]

The biosynthesis of progesterone (38a) involves the oxidation of pregnenolone (36a) to give the dione (41a) [catalysed, for example, by the NAD^+-dependent enzyme 3(or 17)β-hydroxysteroid dehydrogenase (E.C. 1.1.1.51)] followed by isomerization of the dione [catalysed by steroid Δ-isomerase (E.C. 5.3.3.1)]. 2α-Cyanoprogesterone (38b) is a potent inhibitor of the biosynthesis of progesterone from [7-^{3}H]-pregnenolone in mammalian cell-free enzyme systems; it was shown that only the dehydrogenase was inhibited in these systems.[106] However, 2α-cyanoprogesterone was shown to be an irreversible inhibitor of the Δ-isomerase in the bacterium *Pseudomonas testosteroni*.[107] An incompletely identified enzyme that catalyses the NAD^+- or $NADP^+$-dependent oxidation of pregnenolone (36a), to furnish pregn-5-ene-3,20-dione (41a), has been isolated from bovine adrenal microsomes and purified. This dehydrogenase also oxidized 17α-hydroxypregnenolone (36b) to the dione (41b).[108]

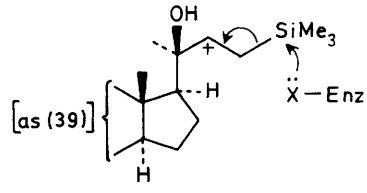
The biosynthesis of androst-4-ene-3,20-dione from progesterone (38a) involves two discrete steps, namely (i) hydroxylation of progesterone to furnish 17α-hydroxyprogesterone (38c) and (ii) cleavage of the 17–20 bond of the latter. Both activities are believed to reside at the same active site of a cytochrome-*P*-450-dependent hydroxylase, which will also convert pregnenolone into dehydroepiandrosterone (42) *via* 17α-hydroxypregnenolone (36b). The components of the hydroxylase have been isolated from mammalian sources and purified.[109–111] The enzymic activity (17α-hydroxylase/17,20-lyase) of the cytochrome *P*-450 component could be reconstituted *in vitro* by addition of NADPH–cytochrome-*P*-450 reductase; the reconstituted enzyme system required NADPH and molecular oxygen for the conversion of progesterone into 5α-androst-4-ene-3,20-dione.[109,110] Other workers have identified a requirement for cytochrome b_5, cytochrome-b_5 reductase [E.C. 1.6.2.2], and NADH for reconstitution of enzymic activity under conditions of low NADPH concentration.[111;*cf.*110] It was confirmed that oxygen-18 is incorporated into the 17-carbonyl group of the product, *i.e.* androstenedione, when progesterone is incubated with the reconstituted testicular enzyme system in an atmosphere of [^{18}O]oxygen.[111] The latter enzyme system also oxidized testosterone to androstenedione; no oxygen-18 was found in the androstenedione that was formed when the enzymic oxidation of testosterone was conducted under an atmosphere of [^{18}O]oxygen.[111]

The Δ^{16}-steroid androsta-4,16-dien-3-one (43) represents an alternative fate for progesterone in porcine testicular microsomes. It has been claimed that the Δ^{16}-steroid-forming activity arises from the 17α-hydroxylase/17,20-lyase activity (discussed above) and is promoted by cytochrome b_5.[112] Androsta-5,16-dien-3β-ol (44) is converted by rat liver microsomes into the α-epoxide (45) and thence into the triol (46).[113]

The biosynthesis of oestrogens from androgens, which involves the placental enzyme system 'aromatase', remains the subject of intensive mechanistic study. Earlier work had established, *inter alia*, that the conversion of androst-4-ene-3,17-dione (47a) or testosterone (47b) into the respective oestrogens involves three discrete oxidative steps, each requiring molecular oxygen and NADPH. It was shown also that the primary alcohol (48a) *or* (48b) and the aldehyde (49a) *or* (49b), respectively, are intermediates, and that C-19 of the androgen precursor is released as formic acid. These and other conclusions from the earlier work are summarized in Scheme 4. These general conclusions have now been extended to encompass the biosynthesis of oestriol (50c) from 16α-hydroxytestosterone (47c) by microsomes from human placentae.[114] In particular it was shown that the triol (48c) and the aldehyde (49c) are intermediates and that the conversion of the former compound into the latter proceeds with retention of the 19(*pro*-19*S*)-proton. Furthermore, it was shown that the 19-oxygen atom of the aldehyde (49c) is retained in the formic acid that is released, while the second oxygen of the latter is supplied by the third



(39) a; R^1 = OH, R^2 = CH_2SiMe_3
b; R^1 = H, R^2 = CH_2NH_2
c; R^1 = H, R^2 = NH_2



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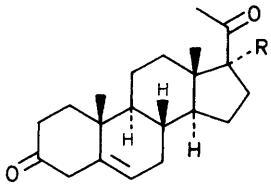
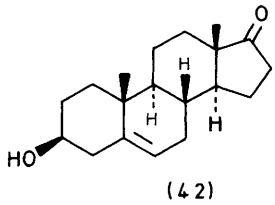


(41) a; R = H
b; R = OH



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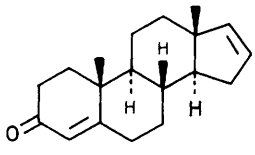

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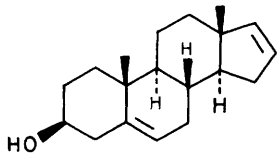

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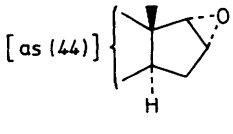
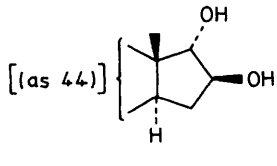


(45)



(46)

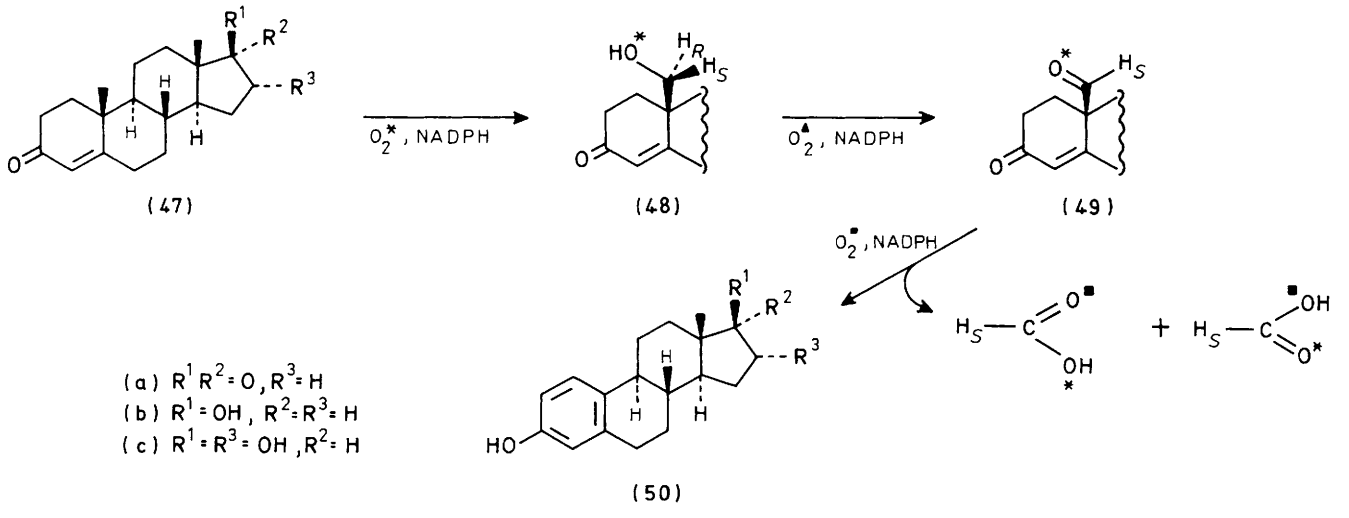
equivalent of molecular oxygen.[114] A similar enzyme system converted [$^{18}O_2$]androst-4-ene-3,17-dione (47a) and [3-^{18}O]-testosterone (47b) into oestrone (50a) and oestradiol (50b) respectively, with retention in each case of over 90% of the oxygen-18 label that was present in the precursor.[115] This work, which confirms an earlier report by another group,[1] demonstrates that no Schiff base is formed as an intermediate at any stage in the aromatization reaction.

Until recently, the available evidence suggested that the 2β-hydroxy-aldehyde (51) is an intermediate in the aromatase-catalysed conversion of the aldehyde (49a) into oestrone (50a).[1] For example, the hydroxy-aldehyde (51) was trapped during an aromatase-catalysed reaction and was shown to undergo spontaneous aromatization at pH 7.1, to furnish oestrone and formic acid, concomitant with loss of the 1β-proton. Thus the postulate that (51) is an intermediate was consistent with the established loss of both the 1β- and the 2β-proton during the conversion of an androgen into an oestrogen and with the suspicion that the final step in the formation of oestrogens is a spontaneous, non-enzyme-catalysed process. Supporting evidence was furnished by the report that the rate of biosynthesis of oestrogens by placental microsomes was reduced by antibodies that bind to 2β-hydroxy-androgens.[116]

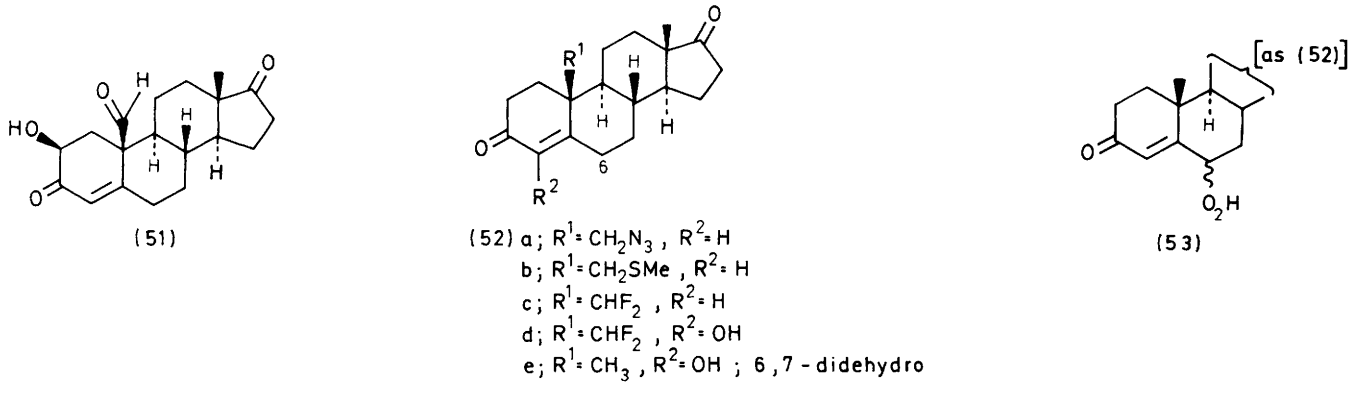
Notwithstanding the previous arguments, it has now been concluded, on the basis of the evidence that is summarized below, that the 2β-hydroxy-aldehyde (51) is *not* an intermediate in the biosynthesis of oestrone.[117] Thus it is known that [^{18}O]formic acid is released when the 19-oxo-androgen (49a) is incubated with placental microsomes under an atmosphere of [^{18}O]oxygen,[1;cf.114] and this observation has been confirmed.[117] If the 2β-hydroxy-compound (51) were an obligatory intermediate in the latter process, its 2β-hydroxyl group should be labelled with oxygen-18; furthermore the same hydroxyl group would be the source of the oxygen-18 label in the formic acid that is ultimately released. However, when the synthetic [19-^{3}H,2-^{18}O]-2β-hydroxy-aldehyde (51) was allowed to aromatize, in the presence or absence of aromatase, the tritium-labelled formic acid that was formed was devoid of oxygen-18. It was concluded that the hydroxy-aldehyde (51) is not an intermediate in the biosynthesis of oestrone from the 19-oxo-androgen.[117] Further consideration must now be given to an alternative suggestion for the mechanism of the final step in the aromatase-catalysed biosynthesis of oestrone.[114]

The syntheses of (19*R*)- and (19*S*)-3β-hydroxy[19-$^2H_1$, 19-$^3H_1$]androst-5-en-17-one (42) have been described briefly.[118] These compounds, each of which contains a 'chiral methyl' group, were used earlier to determine that the initial hydroxylation reaction, catalysed by aromatase, proceeds with retention of configuration.[1] The two components of placental aromatase, *i.e.* the cytochrome *P*-450$_{AROM}$ and the NADPH–cytochrome-*P*-450 reductase, have been purified by h.p.l.c.[119] Monoclonal antibodies have been raised to cytochrome *P*-450$_{AROM}$.[120]

Syntheses of the 19-azidoandrostenedione (52a) and the 19-methylthio-derivative (52b), both of which are potent inhibitors of aromatase, have been described; ultraviolet–visible difference spectroscopy showed that the sulphur atom of the latter inhibitor was co-ordinated to the haem iron when the steroid was bound to aromatase.[121] Other sulphur-containing inhibitors of the same type have been described.[122] Several different types of inhibitor of aromatase have been prepared, including the 19,19-difluoro-steroids (52c) and (52d),[123] the potent inhibitor 4-hydroxyandrosta-4,6-diene-3,17-dione (52e),[124] and the 6α- and the 6β-hydroperoxides (53) of androst-4-ene-3,17-dione;[125]



Scheme 4



the latter compounds may function as inhibitors of aromatase by causing oxidation of cysteine residues. Irreversible inhibitors of aromatase have been reviewed.[126]

Further study has been reported on the cytochrome-*P*-450-dependent adrenal hydroxylase that is responsible for hydroxylation at C-11 in the biosynthesis of the corticosteroids. The active 11β-hydroxylase was reconstituted from purified cytochrome *P*-450$_{11\beta}$ by adding adrenodoxin, adrenodoxin reductase [ferredoxin–NADP$^+$ reductase (E.C. 1 . 18 . 1 . 2)], and Tween 20.[127] Synthetic 18,19-dihydroxy-11-deoxycorticosterone (54a) was shown to be identical to the minor product of the hydroxylation of 18-hydroxy-11-deoxycorticosterone (54b) by a reconstituted 11β-hydroxylase.[128] In the presence of oxygen and NADPH, the same enzyme system converted [1,2,6,7-^{3}H]corticosterone (54c) into aldosterone (55) *via* the 18-hydroxy-steroid (54d).[129]

The role of cytochrome-*P*-450-dependent oxidations in the biosynthesis of the steroidal hormones has been reviewed.[130]

5.3 The Biosynthesis of Bile Acids and the Metabolism of Vitamin D

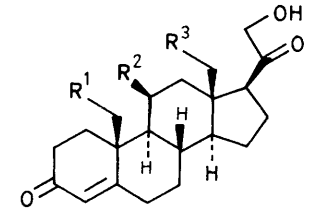
The hydroxylation of cholesterol to furnish 7α-hydroxycholesterol (56), catalysed by the key enzyme cholesterol 7α-mono-oxygenase [E.C. 1 . 14 . 13 . 17], is normally regarded as the rate-limiting step in the biosynthesis of the bile acids. The hydroxylase is stimulated by a cytosolic protein, of mol. wt. 25000, in the presence of reduced glutathione; the stimulatory protein is not affected by ATP or by Mg^{2+} ions.[131;*cf.*17] The biosynthesis of 7α-hydroxycholesterol by liver microsomes is also stimulated by the cytosolic protein SCP-2.[132]

A soluble enzyme that catalyses the NADPH-dependent reduction of 7α-hydroxycholest-4-en-3-one (57) to the 5β-steroid (58) has been isolated from rat liver and purified 230-fold.[133] A cytochrome *P*-450 that is active in the 26-hydroxylation of C_{27} steroids has been isolated from rabbit liver mitochondria.[134] The 26-hydroxylase activity of the purified cytochrome was restored by the addition of ferredoxin and a ferredoxin reductase. The best substrate was the triol (59a), but the diol (59b) was hydroxylated also.

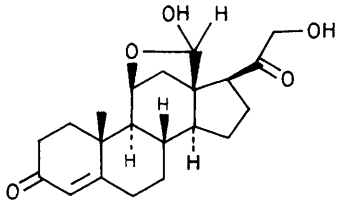
The terminal steps in the biosynthesis of cholic acid have been investigated; the (25*R*)- and the (25*S*)-[7β-^{3}H$_1$]-diastereoisomers (60) were both converted, predominantly, into the same two metabolites, namely the (24*E*)-cholest-24-enoic acid (61) and (24*R*,25*S*)-3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid (62), when incubated with a rat liver homogenate.[135]

The biosynthesis of bile acids has been reviewed.[136] A practical account has been published on the isolation of cytochrome-*P*-450-dependent hydroxylases that are involved in the biosynthesis of bile acids.[137]

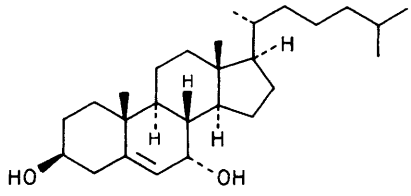
The first step in the metabolism of vitamin D$_3$ [calciol] (63a) is its oxidation to the 25-hydroxy-derivative calcidiol (63b), which is catalysed by a cytochrome-*P*-450-dependent mixed-function oxidase. The requisite cytochrome *P*-450 has been isolated from rat liver microsomes and purified; 25-hydroxylase activity could be reconstituted by supplying NADPH–cytochrome-*P*-450 reductase and NADPH in the presence of oxygen.[138] It is known that the biosynthesis of the dihydroxylactone (64a) from the diol (63b) involves the intermediacy of the triol (63c) and the tetraol (63d). The lactol (65) has now been isolated from chick kidneys and shown (without the benefit of isotopic label) to be a precursor of the lactone (64a) in kidney homogenates.[139] (23*S*)-1α,23,25-Trihydroxyvitamin D$_3$ [(1*S*,23*S*)-1,23,25-trihydroxycalciol] (63e) has been isolated from bovine kidney homogenates that were incubated with calcitriol (63f) and shown to be a precursor of the trihydroxylactone (64b).[140]



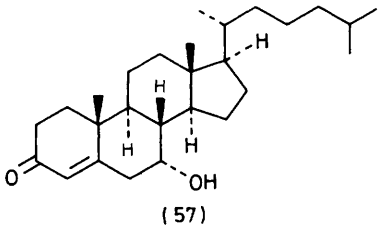
(54) a; $R^1 = R^3 = OH$, $R^2 = H$
b; $R^1 = R^2 = H$, $R^3 = OH$
c; $R^1 = R^3 = H$, $R^2 = OH$
d; $R^1 = H$, $R^2 = R^3 = OH$



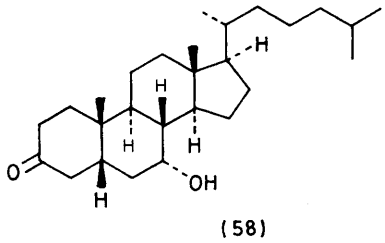
(55)



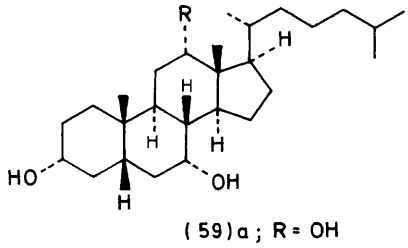
(56)



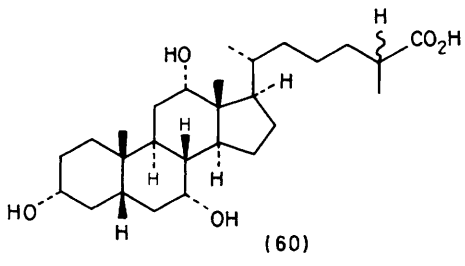
(57)



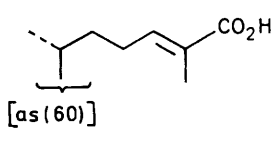
(58)



(59) a; R = OH
b; R = H

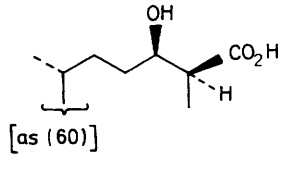


(60)



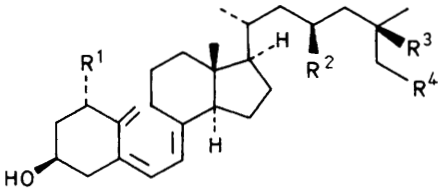
[as (60)]

(61)

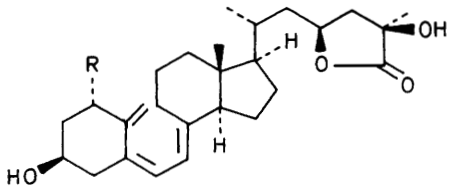


[as (60)]

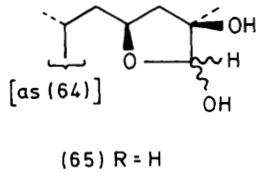
(62)



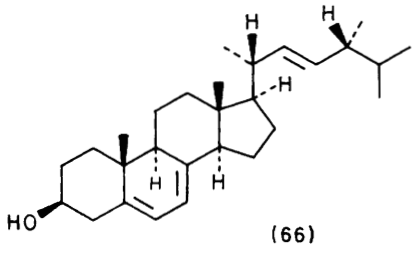
(63) a; $R^1 = R^2 = R^3 = R^4 = H$
b; $R^1 = R^2 = R^4 = H$, $R^3 = OH$
c; $R^1 = R^4 = H$, $R^2 = R^3 = OH$
d; $R^1 = H$, $R^2 = R^3 = R^4 = OH$
e; $R^1 = R^2 = R^3 = OH$, $R^4 = H$
f; $R^1 = R^3 = OH$, $R^2 = R^4 = H$



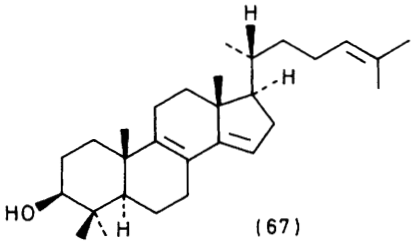
(64) a; R = H
b; R = OH



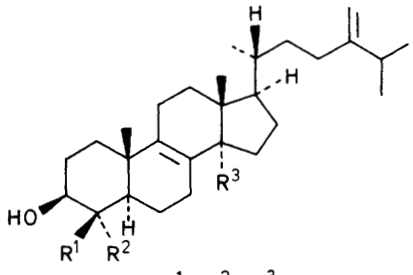
(65) R = H



(66)



(67)



(68) a; $R^1 = R^2 = R^3 = H$
b; $R^1 = R^2 = R^3 = Me$
c; $R^1 = H$, $R^2 = R^3 = Me$
d; $R^1 = R^2 = H$, $R^3 = Me$

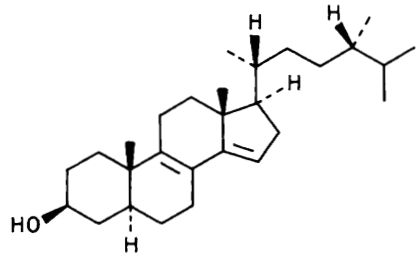
6 Triterpenoids and Steroids in Higher Plants, Algae, and Fungi

6.1 The Biosynthesis of Sterols in Fungi

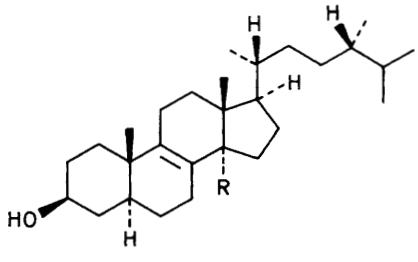
The biosynthesis of ergosterol (66) from [U-^{14}C]acetate or from [2-^{14}C]mevalonate was inhibited, in *Candida albicans*, by the antimycotic drug naftifine[141] and in *Candida albicans*, *Candida parapsilosis*, *Torulopsis glabrata*, and *Trichophyton mentagrophytes* by the related antimycotic agent SF88-327.[142] The main site of action for both fungicides was squalene epoxidase [squalene mono-oxygenase (E.C. 1 . 14 . 99 . 7)], as judged by the accumulation of squalene in treated fungal cultures.[141,142] However, at very high concentrations, naftifine appeared also to inhibit the transmethylation step in the biosynthesis of ergosterol by *Candida albicans*.[143]

The first step in the biosynthesis of ergosterol from lanosterol (21a), in the yeast *Saccharomyces cerevisiae*, is the oxidative removal of the 14α-methyl group by a microsomal cytochrome-*P*-450-based mono-oxygenase system.[1] Details have now been published of the isolation, purification, and properties of the cytochrome *P*-450 component (cytochrome *P*-450$_{14DM}$).[144] The cytochrome *P*-450$_{14DM}$ required NADPH–cytochrome-*P*-450 reductase for reconstitution of its mono-oxygenase activity; incubation of lanosterol (21a) with the reconstituted mono-oxygenase, in the presence of oxygen and NADPH, gave a metabolite that was identified as the triene (67).[145] Yeast microsomes converted lanosterol into 4,4-dimethylzymosterol (21b), presumably *via* the triene (67), when incubated with cyanide ion (to block demethylation at C-4) and NADPH. This conversion was prevented by antibodies that had been raised to cytochrome *P*-450$_{14DM}$ and by known inhibitors of cytochromes *P*-450.[145]

Further transformations in the main route for the biosynthesis of sterols in the yeast *Saccharomyces cerevisiae* include stepwise demethylation of 4,4-dimethylzymosterol (21b) to furnish zymosterol (21c), which is converted into fecosterol (68a) and thence into ergosterol. On the other hand, lanosterol is the preferred substrate for the transmethylation



(69)



(70) a; R = H
b; R = Me

reaction in *Ustilago maydis* and in *Penicillium italicum*, and the first 24-substituted sterol intermediate in the biosynthesis of ergosterol by these two species is 24-methylene-24,25-dihydrolanosterol (68b). The effect of the systemic fungicide tridemorph on the biosynthesis of ergosterol has been clarified. In *Saccharomyces cerevisiae*, at a concentration of 10 μmol dm^{-3}, this fungicide markedly reduced the formation of ergosterol and caused the accumulation of the normal intermediates (67) and (68a), together with abnormal 8,14-dienes, such as (32) and (69); in *Ustilago maydis* that had been similarly grown in the presence of tridemorph, the main sterols were (68a), (70a), and

(71).[146] The related fungicides fenpropimorph and fenpropidin gave results with the latter two species that were qualitatively similar to those given by tridemorph, but with the difference that the 8,14-diene (69) accumulated at the expense of its isomer (68a). It was concluded that the three fungicides inhibit, to differing extents, both the reduction of 8,14-dienes and the isomerization of Δ^8-sterols to Δ^7-sterols.[146]

Other workers have observed the accumulation of the 8,14,24(28)-triene (72) upon growth of *Penicillium italicum* in the presence of fenpropimorph; by contrast, the imidazole-based fungicide imazalil blocked the oxidative removal of the 14α-methyl group of precursors of ergosterol in the same species.[147] Fungicidal triazoles of the dichlorobutrazol series similarly blocked 14α-demethylation of sterols in *Ustilago maydis*, leading to the accumulation of obtusifoliol (68c) and the other 14α-methyl-sterols (68d) and (70b).[148] [^{14}C]Lanosterol has been prepared by the growth of yeast cultures, in which 14α-demethylation was blocked by buthiobate (100 μmol dm^{-3}), in the presence of [1-^{14}C]isopentenyl diphosphate, while zymosterol (21c) was isolated from yeast cells that were adapted to aerobic growth in the presence of ethionine, which blocks the transmethylation reaction.[149] The C_{27} triene (73) and the tetraene (74) were detected by gas chromatography–mass spectrometry in yeast cultures that were grown under the latter conditions.[149] Exogenously supplied cholesterol and ergosterol each inhibited the biosynthesis of ergosterol by *Saccharomyces cerevisiae*.[150]

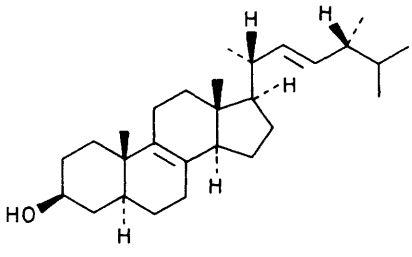
The three isomeric ergostatrienols (75), (76), and (77) have been isolated as minor sterols from *Gibberella fujikuroi*. 5α-[2,4-^{3}H]Ergosta-8,14,22-trien-3β-ol (75) and [^{14}C]ergosta-5,8,22-trien-3β-ol (76) (prepared biosynthetically from [2-^{14}C]-acetate) were incorporated into ergosterol (66), in radiochemical yields of 6.0 and 15.4% respectively, in the same organism; under identical conditions, 5α-[2,4-$^3H_2$]ergosta-6,8(14),22-trien-3β-ol (77) was not incorporated into ergosterol, and must be regarded as an end product of biosynthesis.[151] On the basis of isolation studies, a scheme has been proposed for the biosynthesis of cholesterol, ergosterol, and β-sitosterol (78a) by the ascomycete *Aureobasidium pullulans*.[152]

The biosynthesis of ergosterol,[153] the inhibition by fungicides of the biosynthesis of ergosterol,[154] and the use of yeast mutants in the study of the biosynthesis of sterols[155] have been reviewed.

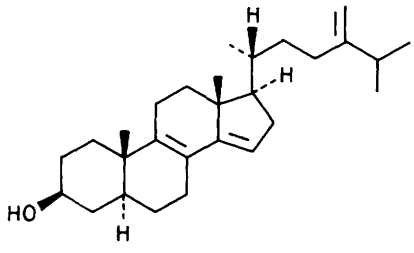
6.2 The Biosynthesis of Phytosterols

Feeding experiments with tissue cultures of *Andrographis paniculata* have shown that [3-^{13}C]leucine is incorporated into the phytosterols β-sitosterol (78a), stigmasterol (79a), and campesterol (78b) only after its complete breakdown to [2-^{13}C]acetate.[156] Similarly, [4-^{14}C, 2-^{3}H]-β,β-dimethylacrylic acid was incorporated into the same sterols only after its extensive degradation, as revealed by a substantially lower ^{3}H:^{14}C atomic ratio in the metabolites.[156]

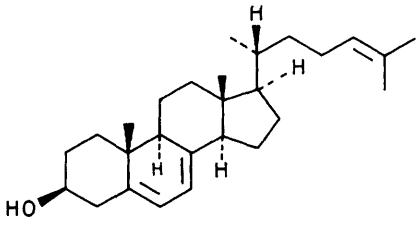
Barley seedlings (*Hordeum vulgare*) that were supplied with [4-^{14}C, 22,23-^{3}H]-β-sitosterol (78a) yielded stigmasterol (79a) with the expected 50% reduction in the ratio of ^{3}H:^{14}C.[157] Conversely, [4-^{14}C]-β-sitosterol was not incorporated into stigmasterol in seedlings of tobacco (*Nicotiana tabacum*), despite an apparent precursor–product relationship for the time-course of labelling of these two sterols when [2-^{14}C]-mevalonate was supplied to the seedlings.[158] It was suggested that the two sterols derive from a common phytosterol precursor in tobacco.



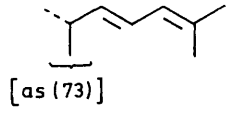
(71)



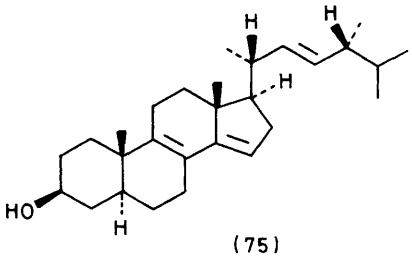
(72)



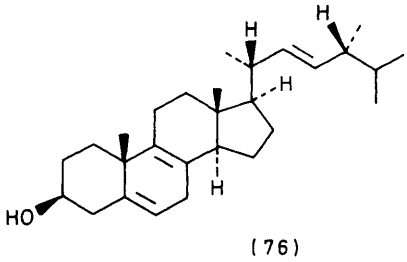
(73)



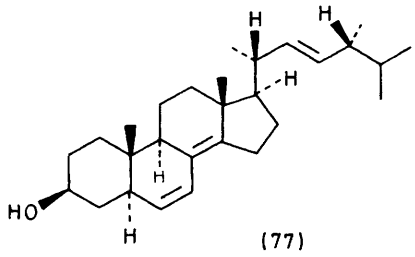
(74)



(75)

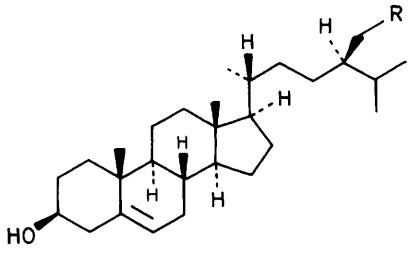


(76)

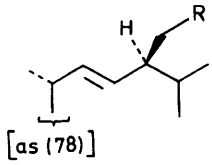


(77)

In cell cultures of bramble (*Rubus fruticosus*) that are grown under normal conditions the Δ^5-sterols β-sitosterol (78a), isofucosterol (80a), and campesterol (78b) constitute about 96% of the phytosterol content, and are formed from cycloartenol (81) *via* the route that is summarized in Scheme 5. Critical steps in this sequence are the alkylation of cycloartenol by *S*-adenosylmethionine (SAM) to furnish 24-methylene-cycloartanol (82), the opening of the cyclopropane ring of cycloeucalenol (83) to give obtusifoliol (68c), and the isomerization of the Δ^8-sterol (84) to the Δ^7-sterol (85). It has been reported that the tertiary amine (24c) is a powerful inhibitor of the isomerization of Δ^8-sterols to their Δ^7-isomers in this system, owing to the structural and electronic resemblance of the protonated amine to the putative carbo-cation intermediate

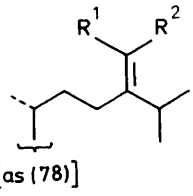


(78) a; R = Me
b; R = H



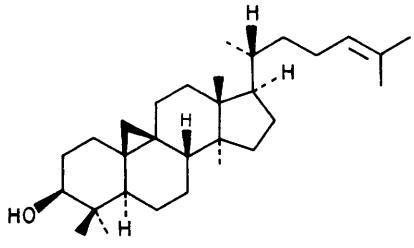
[as (78)]

(79) a; R = Me
b; R = H
c; R = CH_2F

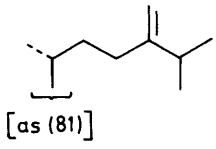


[as (78)]

(80) a; R^1 = H, R^2 = Me
b; R^1 = R^2 = H
c; R^1 = Me, R^2 = H

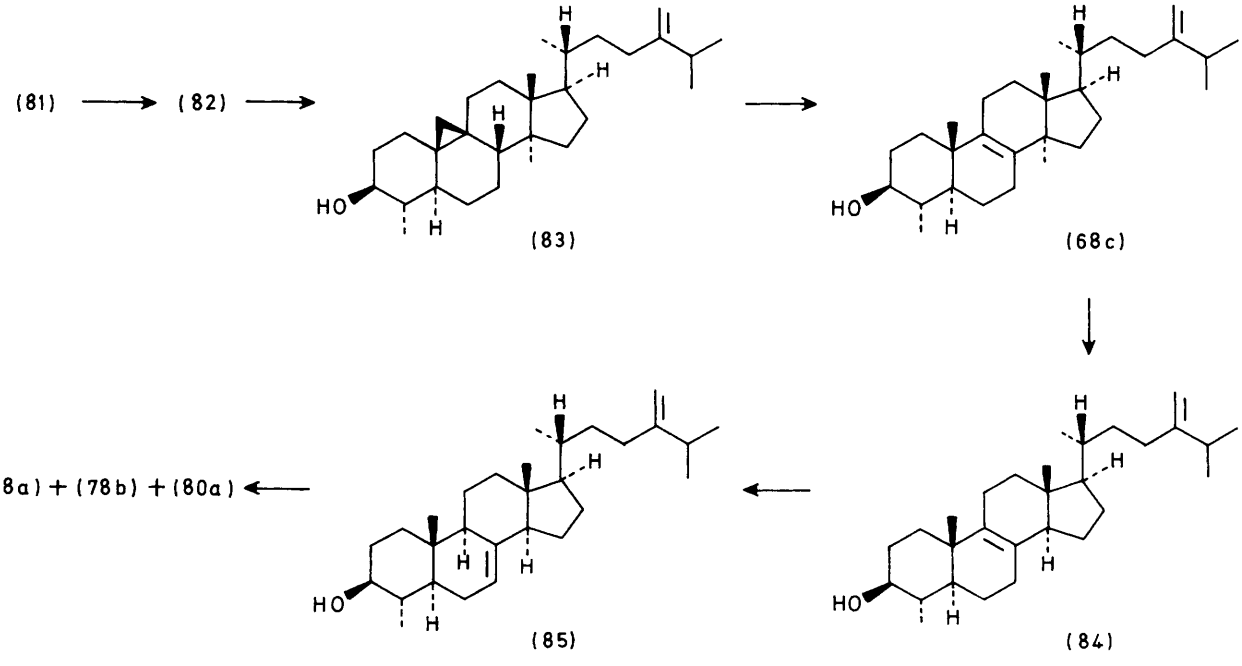


(81)



[as (81)]

(82)



Scheme 5

in the enzyme-catalysed reaction.[159,cf.66] Δ^8-Sterols, such as (86a) and (87), constituted the major phytosterols (87%) when the cell culture was incubated with the inhibitor at a concentration of 1 mg dm^{-3}.[159] At higher concentrations of the inhibitor the isomerization of cycloeucalenol (83) to obtusifoliol (68c) was retarded also, and 9,19-cyclo-steroids such as cycloeucalenol and the 24-substituted pollinastanol derivatives (88), (89a), and (89b) accumulated. The sites of inhibition of sterol biosynthesis by the amine (24c) were confirmed by studies with a cell-free enzyme preparation from maize (*Zea mays*).[159]

The inhibition of biosynthesis of sterols by triarimol, tridemorph, and triparanol in cell cultures of tobacco (*Nicotiana tabacum*), carrot (*Daucus carota*), and soybean (*Glycine max*) has been studied.[160] In the absence of inhibitor, each of these cell cultures furnished the Δ^5-sterols campesterol (78b), β-sitosterol (78a), and stigmasterol (79a) as the only significant sterols. Triarimol inhibited both the 14α-demethylation of obtusifoliol and the second transmethylation reaction, leading to the accumulation of obtusifoliol (68c) and 14α-methyl-5α-ergost-8-en-3β-ol (70b), while tridemorph inhibited both the conversion of cycloeucalenol into obtusifoliol and the second transmethylation reaction. In the cultures of tobacco and carrot cells, treatment with triparanol led principally to the accumulation of 14α-methyl-Δ^8-sterols such as (70b) and (86b), but cycloeucalenol and 24-methylenecycloartanol were detected also.[160]

The 4α-methylsterol (84) has been isolated from horse chestnut (*Aesculus hippocastanum*) and may have a major role as an intermediate in the biosynthesis of phytosterols.[161] The biosynthesis of cholesterol and phytosterols from [2-^{14}C]acetate has been demonstrated in leaves of sorghum (*Sorghum bicolor*).[162] The incorporation of [2-^{14}C]acetate into phytosterols by tissue cultures of *Cucurbita maxima* has been reported.[163] Studies of biosynthetic relevance have been published on the sterol content of seeds of the latter species.[164]

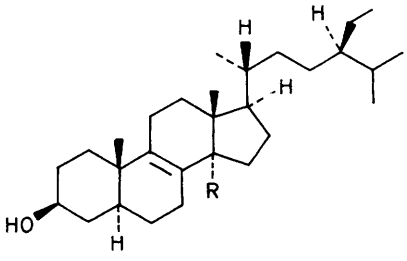
The metabolism of cholesterol in higher plants has been reviewed, and it was suggested that cholesterol may be more widely synthesized in plants than has been recognized hitherto.[165] Reviews have been published on the biosynthesis of phytosterols[166] and on the membrane-bound enzymes[167] and the regulation[168] of phytosterol biosynthesis.

6.3 Alkylation of the Sterol Side-chain

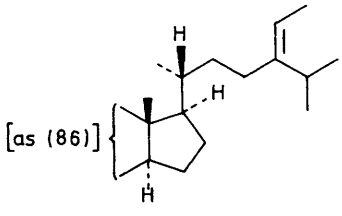
Poriferasterol (90a) that was labelled biosynthetically by supplying [*Me*-$^2H_3$]acetate to the crysophyte alga *Ochromonas malhamensis* acquired deuterium label in an integrated ratio of 2:3 for the 25(*pro*-25*R*)- and the 25(*pro*-25*S*)-methyl group, respectively; therefore the 25(*pro*-25*R*)-methyl of (90a) is derived from C-2 of mevalonate.[169] This result is consistent with the alkylation by *S*-adenosylmethionine of a sterol precursor at the *re* face of the 24–25 double-bond, as shown in Scheme 6. For the purposes of assay, the deuterium-labelled poriferasterol was first degraded to (2*S*, 3*R*)-2-([$^2H_2$]methyl)-[1,1,1,2-$^2H_4$]pentan-3-ol, the ^{2}H n.m.r. spectrum of which, if recorded in the presence of the shift reagent Eu(dpm)$_3$, permitted the absolute configuration of C-2 to be determined.[169]

The biosynthetic origins of the 25(*pro*-25*R*)- and the 25(*pro*-25*S*)-methyl groups of β-sitosterol (78a) and clionasterol (91a) were determined earlier,[170] based on the implied assumption that the biosynthetic results for isofucosterol (80a) in *Pinus pinea*[171] were applicable also to that sterol in *Physalis peruviana*. The conclusions with respect to the phytosterols (78a), (80a), and (91a) in the latter species[170] have now been confirmed by unambiguous assignment of the ^{13}C n.m.r. resonances due to the diastereotopic 25-methyls of clionasteryl acetate.[172] These assignments were performed with the aid of [^{13}C]clionasterol (91a) that was derived chemically from [^{13}C]poriferasterol (90a), which in turn had been labelled biosynthetically by growing *Ochromonas malhamensis* in the presence of sodium [$^{13}C_2$]acetate.

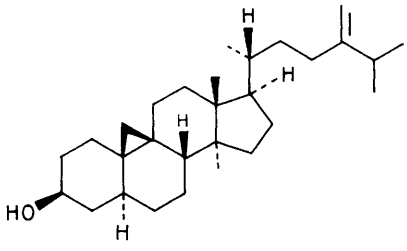
Sodium [$^{13}C_2$]acetate was incorporated into ergosterol (66) in *Saccharomyces cerevisiae*, and the ^{13}C n.m.r. spectrum of the derived 5α-ergost-8(14)-en-3β-ol (92a) showed that the 25(*pro*-25*S*)-methyl of ergosterol is derived from C-2 of mevalonate in the latter species,[173] as had been shown previously for ergosterol in *Claviceps paspali*.[174] 22,23-Dihydrobrassicasterol (91b) and



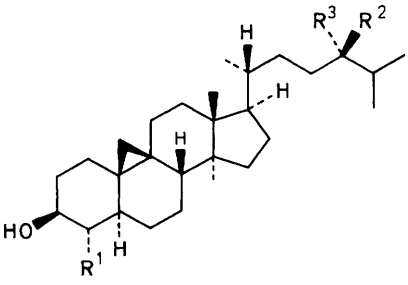
(86) a ; R = H
b ; R = Me



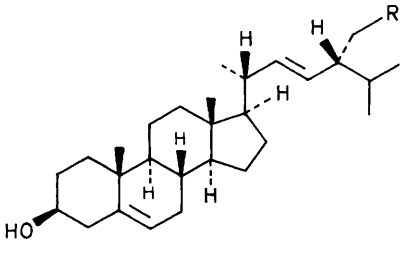
(87)



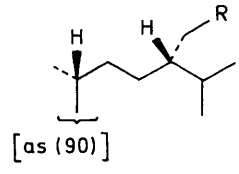
(88)



(89) a; $R^1 = R^2 = H$, $R^3 = Me$
b; $R^1 = R^3 = H$, $R^2 = Me$
c; $R^1 = R^3 = Me$, $R^2 = H$
d; $R^1 = R^2 = Me$, $R^3 = H$



(90) a; R = Me
b; R = H

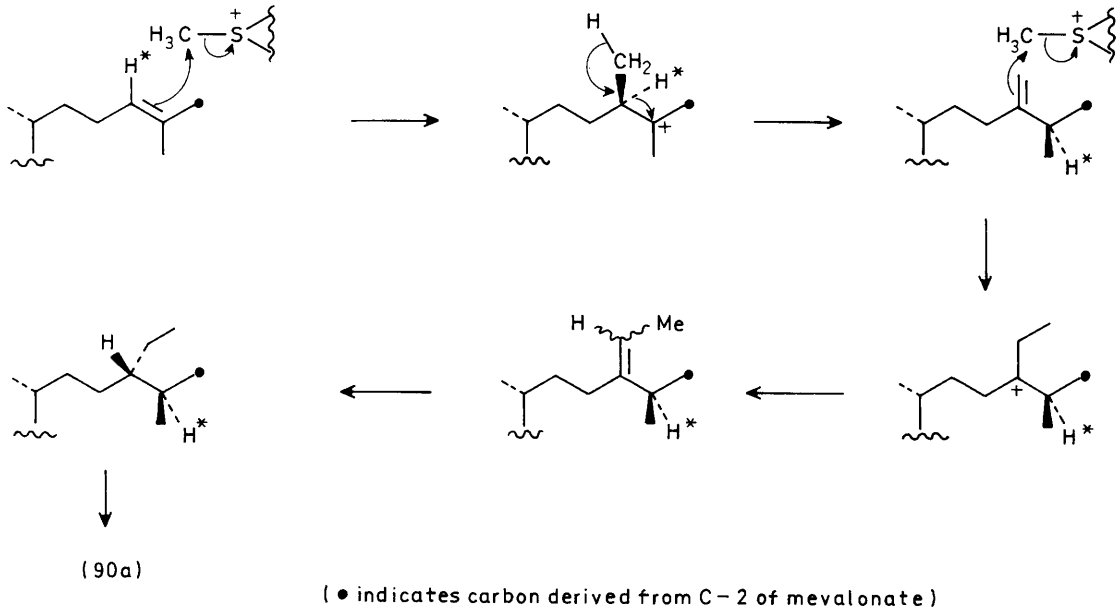


(91) a; R = Me
b; R = H

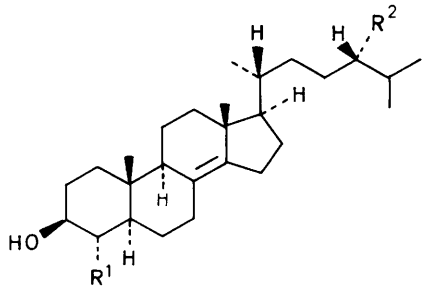
campesterol (78b) were isolated from suspension cultures of *Physalis peruviana* and *Dioscorea tokoro*, each of which had been supplied with [$^{13}C_2$]acetate; the ^{13}C n.m.r. spectra of these labelled steroids, from either source, and of 24-methylenecholesterol (80b) from *P. peruviana*, revealed that C-2 of mevalonate furnishes the 25(*pro*-25*R*)-methyl group of 22,23-dihydrobrassicasterol but the 25(*pro*-25*S*)-methyl of campesterol and of 24-methylenecholesterol.[173] The result for the latter sterol is the reverse of that published previously.[170] In this study the assignments of carbon resonances for the diastereotopic 25-methyls of 22,23-dihydrobrassicasterol were made by comparison with those for the $\Delta^{8(14)}$-sterol (92a), which has the same side-chain. Other assignments were made with the aid of a mixed sample of ^{13}C-labelled 22,23-dihydrobrassicasterol and campesterol which was prepared by hydrogenation of the ^{13}C-labelled 24-methylenecholesterol (80b).[173] Irrespective of the correctness or otherwise of the new assignments for (80b), it is clear from the present results that campesterol, but not 22,23-dihydrobrassicasterol, could be biosynthesized by direct reduction of 24-methylenecholesterol in *P. peruviana*. The authors have interpreted their results in terms of the proposed biosynthetic routes (*a*) and (*b*), in Scheme 7, for the sterols (78b) and (91b) respectively.[173] Some support for this interpretation was afforded by the demonstration that 24-methyldesmosterol (93a) incorporated ^{13}C label stereospecifically from [$^{13}C_2$]acetate in cultures of *P. peruviana* (see Scheme 7).[175] The present status of studies on the biosynthetic origins of the 25-methyls of phytosterols is summarized in Table 1.

Important, though circumstantial, evidence was discussed in the previous Report[1] for the view that 24α-methyl-sterols are formed in *Zea mays* by route (*a*) while 24β-methyl-sterols are biosynthesized *via* route (*b*) or (*c*) of Scheme 7. A study on the sterol composition of shoots of the latter species has confirmed, *inter alia*, the presence, and the biosynthesis from [2-^{14}C]mevalonate and from [*Me*-^{14}C]methionine, of sterols with the side-chains that are shown in Scheme 7.[176] However, evidence against the operation of routes (*b*) or (*c*) was afforded by the investigation of the biosynthesis of sterols in maize seedlings that had been treated with tridemorph.[177] The major sterols that were formed under the latter growth conditions were the mixed diastereoisomeric 24-methylpollinastanols (89a) and (89b), cycloeucalenol (83), the mixed dihydrocycloeucalenols (89c) and (89d), and, significantly, the 24-methyl-Δ^{24}-sterol (94). 24-Methylenecycloartanol, cycloeucalenol, 24-methylenepollinastanol, and the mixed 24-methylpollinastanols each retained two ^2H atoms only when [*Me*-$^2H_3$]methionine was supplied as precursor. Furthermore, when (4*R*)-[5-^{14}C,4-$^3H_1$]mevalonic acid was supplied to the plant, the ^3H:^{14}C atomic ratios in the sterols that were formed were consistent with the biosynthesis of each of the diastereoisomeric 24-methylpollinastanols *via* the Δ^{24}-sterol (94), as summarized in Scheme 8.[177]

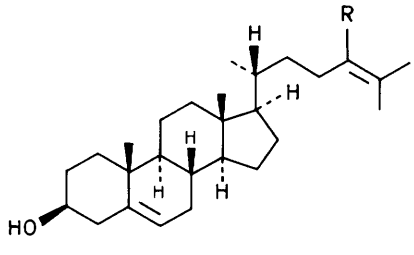
Microsomal extracts from seedlings of maize are capable of



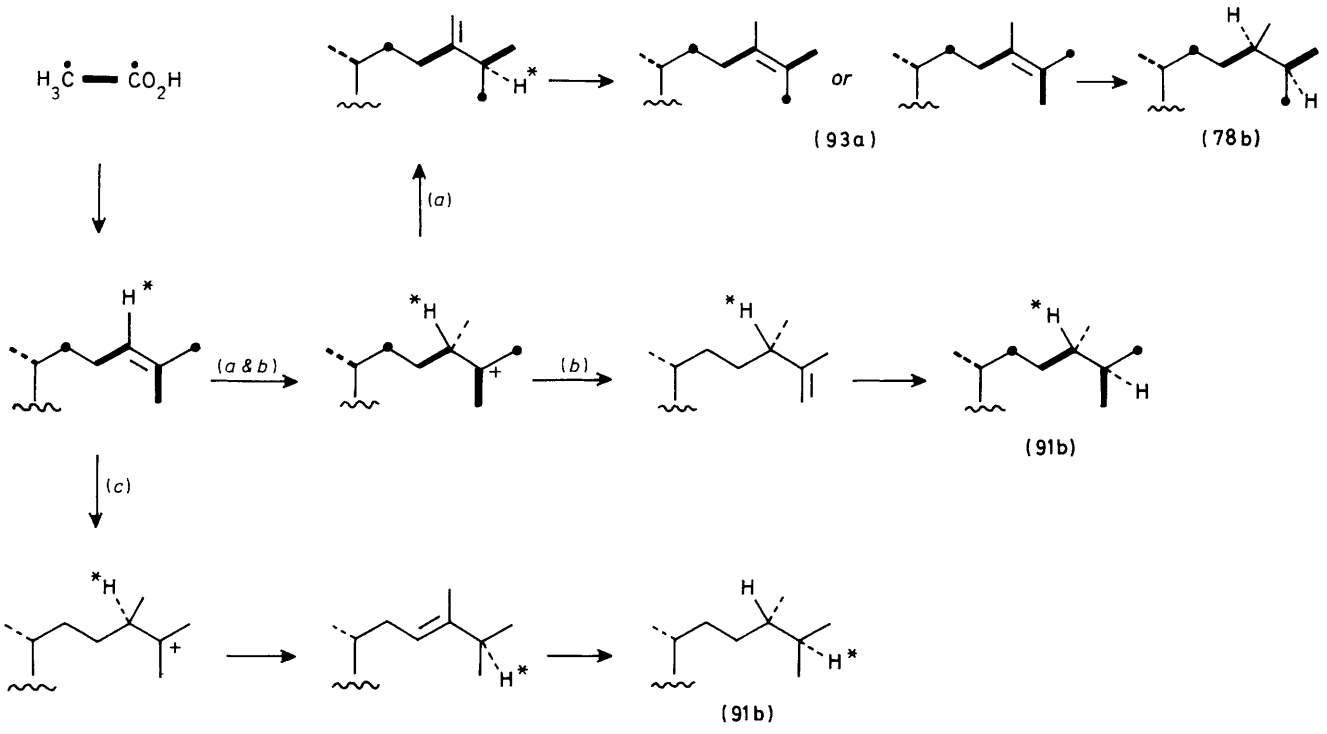
Scheme 6



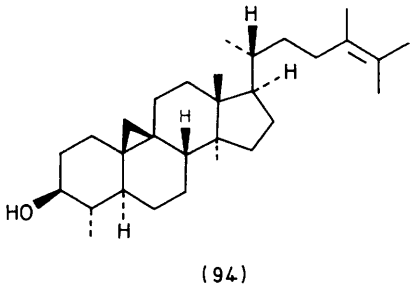
(92) a; R^1 = H, R^2 = Me
b; R^1 = Me, R^2 = H

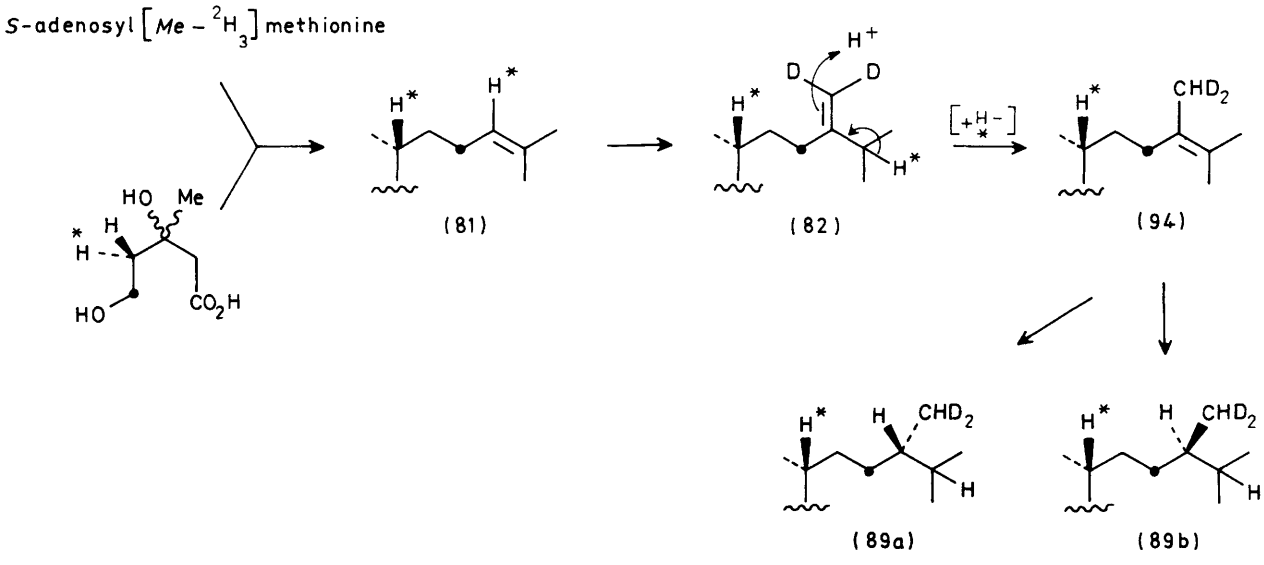


(93) a; R = Me
b; R = H



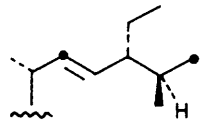
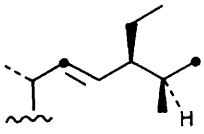
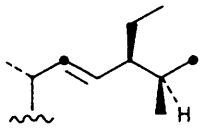
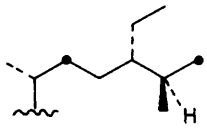
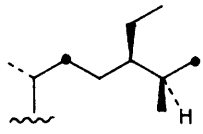
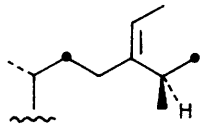
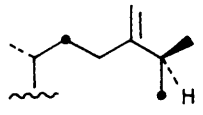
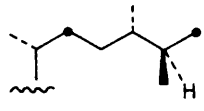
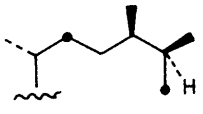
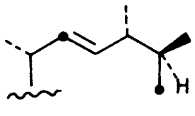
Scheme 7





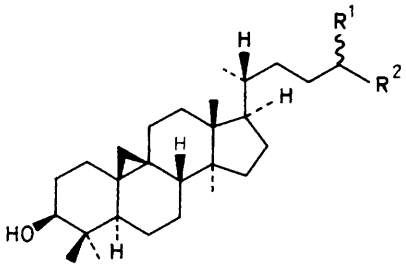
Scheme 8

Table 1 Biosynthetic origins of the 25-methyl groups of phytosterols

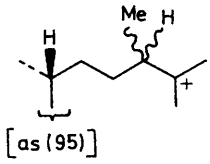
Sterol	Labelling pattern of side-chain (● = derived from C-2 of mevalonate)	Species	Reference
Poriferasterol (90a)		*Ochromonas malhamensis*	169
Stigmasterol (79a)		*Physalis peruviana* *Dioscorea tokoro* *Bupleurum falcatum*	170 170 170
α-Spinasterol (118)		*Bupleurum falcatum*	170
Clionasterol (91a)		*Bupleurum falcatum*	170; *cf.* 172
β-Sitosterol (78a)		*Physalis peruviana* *Dioscorea tokoro* *Isodon japonicus*	170; *cf.* 172 170; *cf.* 172 170; *cf.* 172
Isofucosterol (80a)		*Physalis peruviana* *Pinus pinea*	170; *cf.* 172 171
24-Methylene-cholesterol (80b)		*Physalis peruviana*	173; *cf.* 170
22,23-Dihydro-brassicasterol (91b)		*Physalis peruviana* *Dioscorea tokoro*	173 173
Campesterol (78b)		*Physalis peruviana* *Dioscorea tokoro*	173 173
Ergosterol (66)		*Saccharomyces cerevisiae* *Claviceps paspali*	173 174

catalysing the alkylation of cycloartenol (81) by *S*-adenosyl-[*Me*-^{14}C]methionine to furnish 24-methylenecycloartanol (82). The 24-transmethylase activity was found to be strongly inhibited by the substrate-like analogues (95a) and (95b), which possess a basic nitrogen atom at C-25.[178] The efficacy of these aza-sterols as inhibitors may be due to the structural and electronic resemblance of their conjugate acids to the C-25 cation (96), which is an intermediate in the biosynthetic conversion of (81) into (82). Significantly, the *N*-oxide (95c), the sulphonium salt (95d), and the arsonium salt (95e) were also potent inhibitors of the transmethylase activity.[178] Similarly, the Δ^{24}-sterol methyltransferase [E.C. 2 . 1 . 1 . 41] of the yeast *Saccharomyces cerevisiae* was inhibited *in vivo* and *in vitro* by the 25-azacholestenols (97a) and (97b).[179] Increased levels of zymosterol (21c) and the formation of the sterols (73) and (74) were observed in yeast cultures that were incubated with these inhibitors.[179]

The biosynthesis of the unusual side-chain of 24-propylidenecholesterol (98) in an unidentified marine crysophyte alga has been studied.[180] The possibility that the propylidene substituent is formed by direct ethylation of 24-methylene-cholesterol (80b), or an equivalent precursor, was precluded by the observations that [*Et*-$^2H_5$]ethionine was not incorporated into (98) in the alga, while [*Me*-^{13}C]methionine furnished (98)

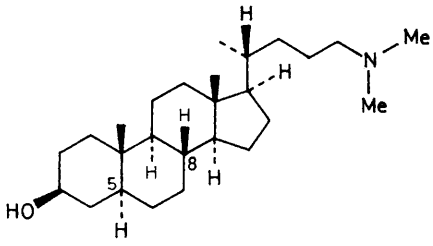


(95) a; R^1 = H, R^2 = NMe_2
b; R^1 = Me, R^2 = NMe_2
c; R^1 = Me, R^2 = $\overset{+}{N}Me_2\bar{O}$
d; R^1 = Me, R^2 = $\overset{+}{S}Me_2$
e; R^1 = Me, R^2 = $\overset{+}{As}Me_3$

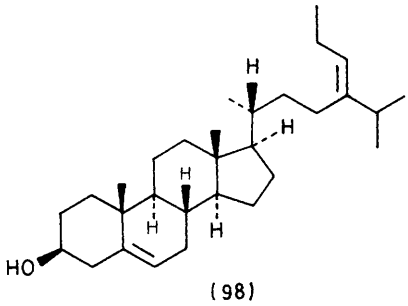


[as (95)]

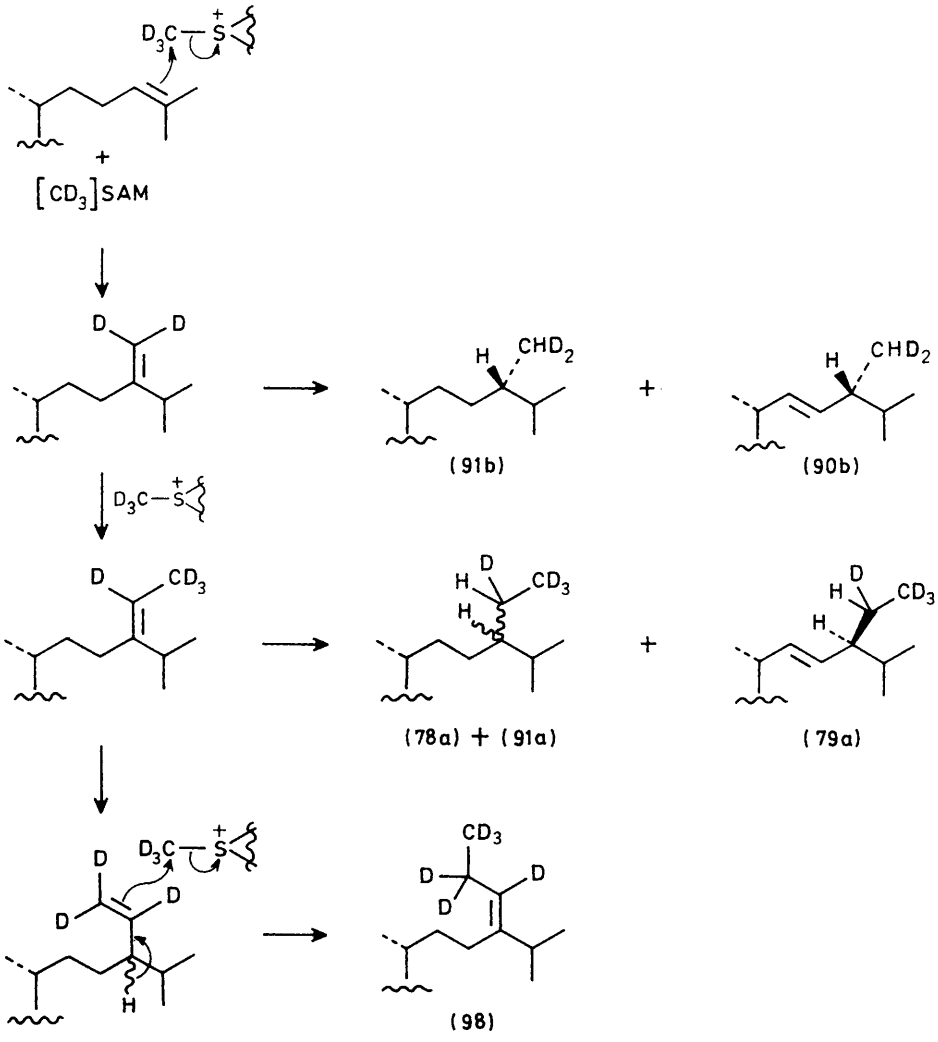
(96)



(97) a; 5,6-didehydro
b; 8,9-didehydro



(98)



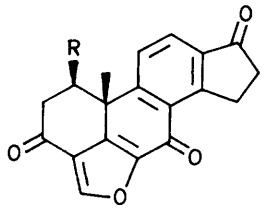
Scheme 9

that was labelled equally at each of the propylidene carbons. Mass spectrometry and ^2H n.m.r. spectroscopy were both used to study the incorporation of label from [*Me*-$^2H_3$]methionine into the 24-methyl-sterols (90b) and (91b), the 24-ethyl-sterols (78a), (91a), and (79a), and 24-propylidenecholesterol; the most plausible interpretation of the results is that the side-chains of these sterols are formed in this alga principally, or exclusively, by the routes that are summarized in Scheme 9.

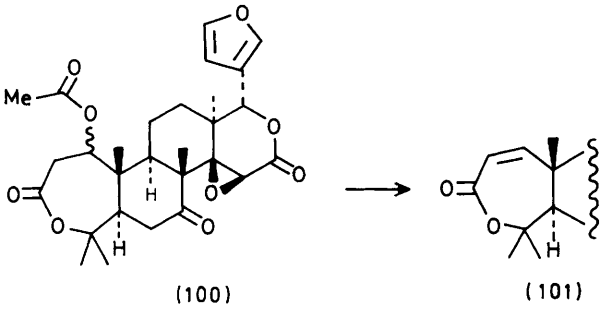
6.4 Further Metabolism of Steroids and Triterpenoids

Biosynthetically labelled [^{14}C]deoxydemethoxyviridin (99a) was incorporated, in 3.9% radiochemical yield, into the antibiotic demethoxyviridin (99b) by the fungus *Nodulisporium hinnuleum*; the putative role of (99a) as a precursor of demethoxyviridin was further supported by its detection in a cold-trap experiment when [1-^{14}C]acetate was fed to the fungus.[181]

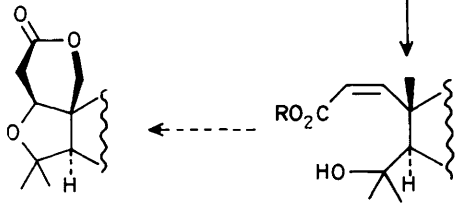
Lemon seedlings (*Citrus limon*) incorporated [1-^{14}C]acetate, [2-^{14}C]mevalonate, and [1-^{14}C]farnesyl diphosphate into the limonoid nomilin (100) in radiochemical yields of 1.5%, 0.5%, and 0.15% respectively.[182] Biosynthetically labelled [^{14}C]-nomilin was metabolized in lemon seedlings to four compounds, one of which was identified as obacunone (101).[183] [^{14}C]Obacunone, which was prepared enzymically from [^{14}C]nomilin, was further converted by the same species into the acid (102a) and limonin (103).[184] The acid (102a) was a poor precursor of limonin; however, this may simply be due to permeability problems, since the unnatural methyl [^{14}C]ester (102b) was metabolized in 50% overall yield to a mixture of products, including limonin.[184] The results of these studies were discussed in terms of the sequence shown in Scheme 10 for the biosynthesis of limonin.



(99) a; R = H
b; R = OH



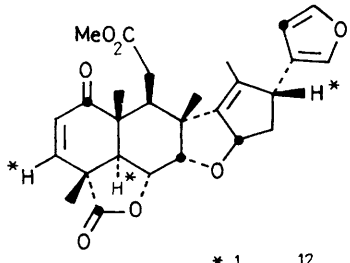
(100) (101)



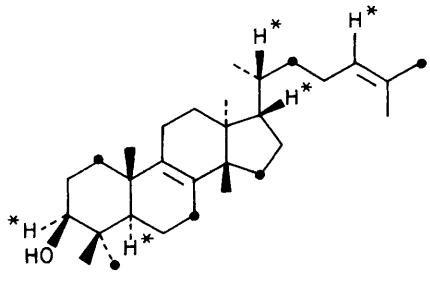
(103) (102) a; R = H
b; R = Me

Scheme 10

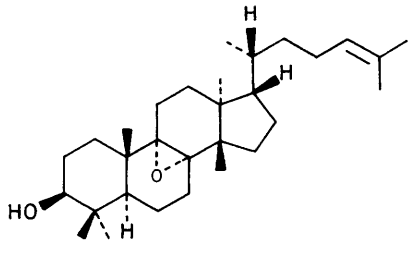
In earlier studies on the biosynthesis of the meliacin nimbolide (104a), the Δ^8-triterpenoid euphol (105a) was a more efficient precursor than were plausible Δ^7-triterpenoids. In more recent work the ^3H-labelled epoxide (106) and the ^3H-labelled diepoxide (107) were incorporated into nimbolide in leaves of *Azadirachta indica* in 5.9% and 4.0% radiochemical yields respectively; under the same conditions ^3H-labelled euphol showed a 2.8% incorporation into nimbolide.[185] These results were taken to mean that a 7,9(11)-diene, derived from an 8α,9α-epoxide, was a more immediate precursor of nimbolide than euphol. The nimbolide that was isolated following the feeding of (4*R*)-[2-^{14}C,4-$^3H_1$]mevalonolactone (^3H:^{14}C atomic ratio of 1:1) had a ^3H:^{14}C atomic ratio of 3:5; this is consistent with the biosynthesis of nimbolide [labelled as (104b)] from endogenous euphol of the assumed labelling pattern (105b).[185] A degradation that purported to demonstrate the presence of tritium label at C-3 [or C-2] of nimbolide (104b) was described.



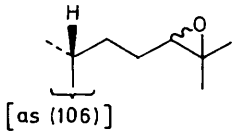
(104) a; H* = ^1H, • = ^{12}C
b; H* = ^3H, • = ^{14}C



(105) a; H* = ^1H, • = ^{12}C
b; H* = ^3H, • = ^{14}C



(106)



[as (106)]

(107)

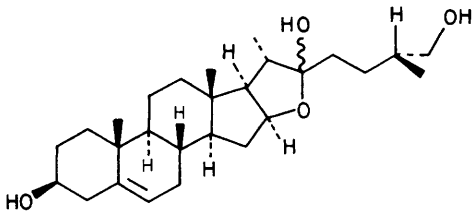
[2-^{14}C]Mevalonolactone was incorporated into the withanolides withaferin A (108a), jaborosalactone A (108b), and jaborosalactone D (109) in leaves and seedlings of *Acnistus breviflorus*. Upon re-feeding it to leaves of the plant, the ^{14}C-labelled jaborosalactone A was incorporated into (108a) and (109) in 2.0% and 1.4% yields, respectively.[186] Partial degradation of the ^{14}C-labelled withaferin A that had been derived biosynthetically from [2-^{14}C]mevalonolactone, surprisingly, showed that a total of only 2% of the ^{14}C label resided at carbons 25, 26, and 27.[187] Further, more definitive experiments are clearly desirable.

The labelling patterns that were observed when [$^{13}C_2$]acetate was incorporated into the spirosapogenins of cell cultures of *Dioscorea tokoro* were reported earlier; the full paper includes data also on the intermediate furostanol glycosides protoneotokorin (110a) and prototokoronin (110b).[188] The furostanol (111) has been isolated from suspension cultures of *Dioscorea deltoidea*.[189] The results of pulse-chase biosynthetic experiments with [1-^{14}C]acetate suggested that (111) is a direct precursor of diosgenin (112) *in vivo*.

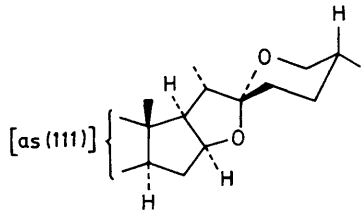
[1,2-$^{14}C_2$]Oxaloacetate was incorporated into scilliroside (113) in *Scilla maritima*; the results of partial degradation were interpreted to mean that C-1, C-2, and C-3 of the precursor furnished C-24, C-23, and C-22, respectively, of the metabolite (113).[190] However, the high degree of randomization of label that was observed in this study, as might be anticipated with such a precursor, renders the result equivocal.

A cytochrome-*P*-450-dependent hydroxylase that converts the cardenolide glycoside digitoxin into its 12β-hydroxy-derivative digoxin has been isolated from cell cultures of *Digitalis lanata*.[191] The biosynthesis of digitoxin in cultures of *Digitalis purpurea* has been studied.[192]

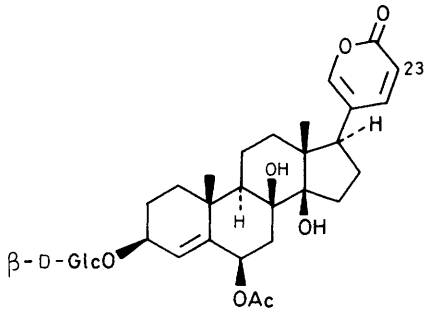
Reviews have been published on the biosynthesis of cucurbitacins and limonoids,[193] steroidal glyco-alkaloids,[194] and sterol glycosides.[195]



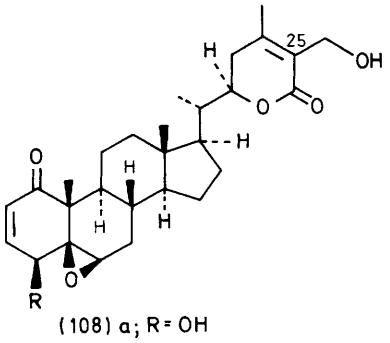
(111)



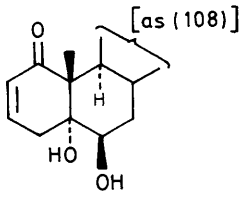
(112)



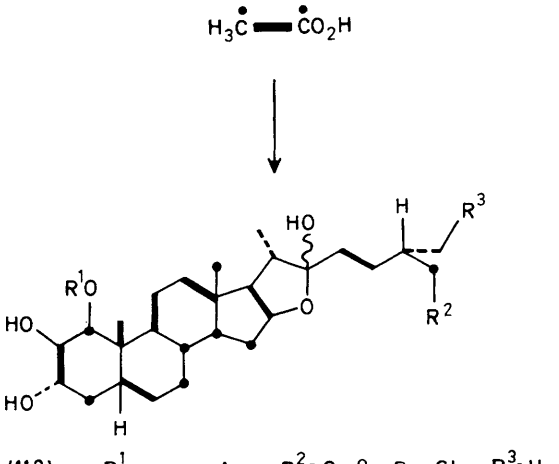
(113)



(108) a; R = OH
b; R = H



(109)



(110) a; R^1 = α-L-Ara, R^2 = O-β-D-Glc, R^3 = H
b; R^1 = α-L-Ara, R^2 = H, R^3 = O-β-D-Glc

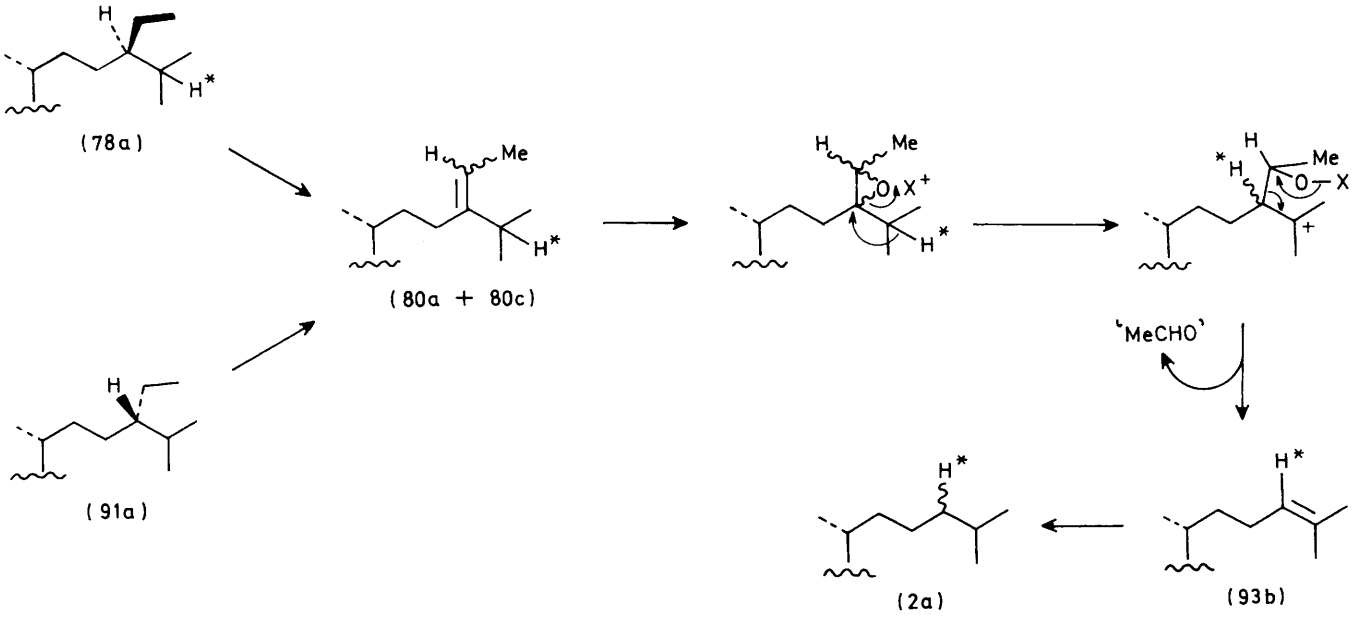
7 Triterpenoids and Steroids in Invertebrates

7.1 Insects

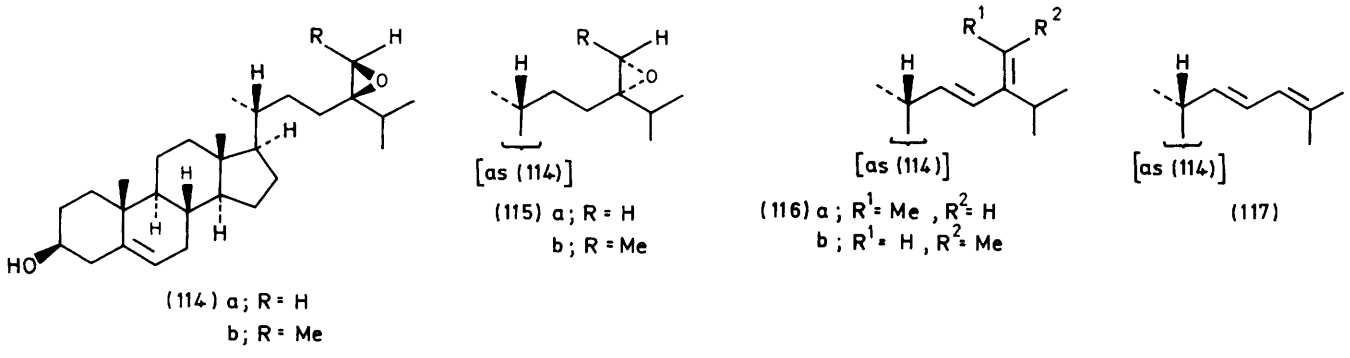
Insects appear to be incapable of biosynthesizing sterols *de novo*; the cholesterol requirement of some phytophagous insects is satisfied by the degradation of dietary phytosterols by the route that is illustrated for β-sitosterol (78a) and clionasterol (91a) in Scheme 11. The dehydrogenation of β-sitosterol is non-stereospecific; a mixture of fucosterol (80c) and isofucosterol (80a) is formed in the first step. The stereochemistry of the dehydrogenation of clionasterol has now been investigated by feeding a mixture of [7,7-$^3H_2$]clionasterol and [4-^{14}C]-β-sitosterol to larvae of *Tenebrio molitor*, in the presence of unlabelled fucosterol or isofucosterol (to act as isotopic traps); the ratios of ^{3}H to ^{14}C in the isotopic label that was acquired by the latter compounds demonstrated that clionasterol and β-sitosterol were metabolized to fucosterol with equal efficiency, while isofucosterol was formed less readily from clionasterol than from β-sitosterol.[196] Phytosterols with a C_9 side-chain can also satisfy the sterol requirements of *T. molitor*. The diastereoisomeric compounds (24*R*)- and (24*S*)-24,28-epoxy-[23,23,25-$^3H_3$]ergost-5-en-3β-ol [(114a) and (115a), respectively] have now been tested as precursors of cholesterol, each in competition with [4-^{14}C]-β-sitosterol; it was clear from the ^{3}H:^{14}C atomic ratios of the metabolites in these experiments that only the (24*R*)-epoxide (114a) was converted into cholesterol.[197] This high degree of stereospecificity is in striking contrast to the relative lack of discrimination between the two epoxides (114b) and (115b) of fucosterol that was previously reported to have been displayed by the same species.

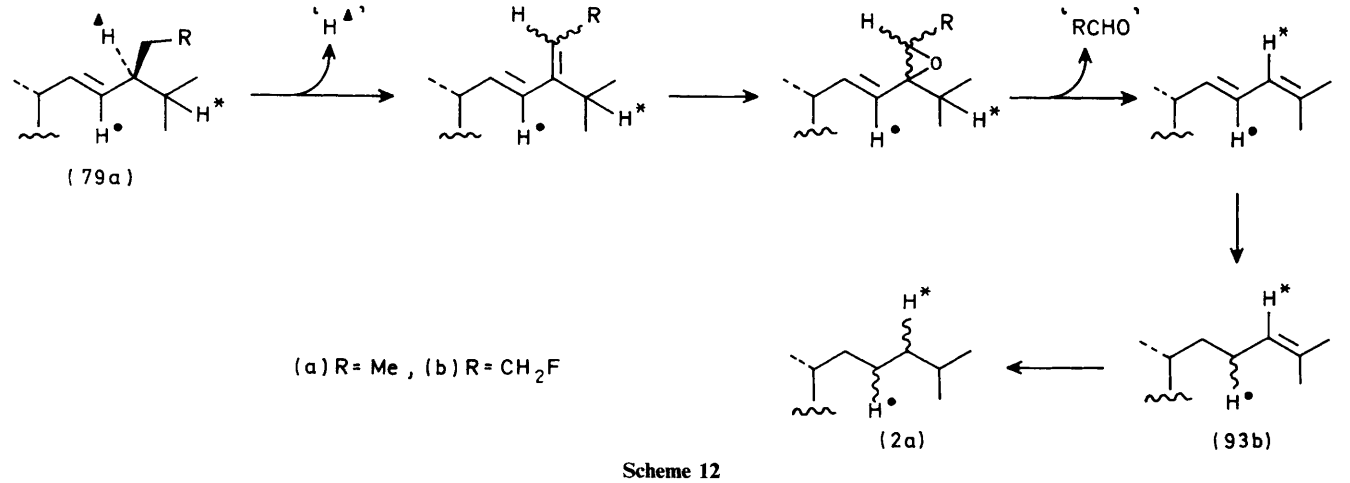
A noteworthy feature of Scheme 11 is the hydride migration, from C-25 to C-24, that is observed during the conversion of β-sitosterol into desmosterol (93b) and cholesterol (2a). Indirect evidence has been furnished for the equivalent hydride

migration in the dealkylation of stigmasterol (79a) by larvae of the silkworm *Bombyx mori*, as summarized in Scheme 12(a); in particular, mass spectrometry indicated that the deuterium label was retained in the side-chain of samples of desmosterol and of cholesterol that were formed from [23-^{2}H]- or [25-^{2}H]-stigmasterol (79a), while unlabelled C_{27}-sterol metabolites were formed when [24-$^2H_1$]stigmasterol was supplied as precursor.[198] The diastereoisomeric ergostatrienols (116a) and (116b) were each converted into cholesterol, *via* the cholestatrienol (117), by *Bombyx mori*.[198] The unlabelled 24α-phytosterols β-sitosterol (78a), stigmasterol (79a), campesterol (78b), and crinosterol (79b) and their 24β-epimers each satisfied the sterol requirement of silkworm larvae, and were converted into cholesterol with similar efficiencies.[199] The Δ^5-sterols β-sito-



Scheme 11





Scheme 12

sterol and campesterol, several Δ^7-sterols [such as spinasterol (118)], and several fully saturated sterols [*e.g.* campestanol] supported growth of the lepidopteran *Heliothis zea*; in each case, dealkylation of the steroid side-chain occurred but the steroid nucleus was mainly unaltered by this insect.[200]

The putative fucosterol-epoxide lyase may be assayed rapidly in cell-free homogenates of the tobacco hornworm (*Manduca sexta*) by monitoring the release of water-soluble tritium, as [^{3}H]acetaldehyde (see Scheme 11) or its metabolic equivalent, from the (24*R*,28*R*)-[24-^{14}C,29-^{3}H]epoxide (114b). Surprisingly, the (24*S*,28*S*)-epoxide (115b) was not a substrate in this enzyme system.[201] An alternative assay for the same lyase has been described.[202]

Triadimefon and a series of related fungicides strongly inhibited the dealkylation of β-sitosterol (78a) and of campesterol (78b) by larvae of the cotton leafworm (*Spodoptera littoralis*); these fungicides also inhibited the conversion of a mixture of the epoxides (114b) and (115b) of [6-^{3}H]fucosterol into cholesterol by a cell-free enzyme system from the same species.[203] Larvae of the tobacco hornworm (*Manduca sexta*) converted 29-fluoro[29-^{3}H]stigmasterol (79c), in 0.01% radiochemical yield, into (2*R*,3*R*)-2-fluoro[2-^{3}H]citric acid; the latter compound, which accounts for the toxicity of the fluorostigmasterol, is formed from the two-carbon fragment that is released during dealkylation of (79c) according to Scheme 12(b).[204]

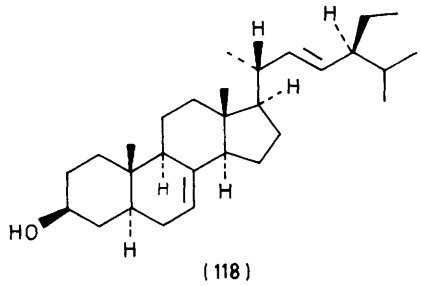
Some phytophagous insects are not able to dealkylate phytosterols; these include the cactophilic species *Drosophila mettleri* and *D. mojavensis*.[205] The sterol composition of the heteropteran *Dysdercus fasciatus*, *i.e.* 95% β-sitosterol and 5% campesterol, closely resembled that of its diet of cotton seed, in confirmation of the observation that this insect is unable to dealkylate β-sitosterol.[206]

Moulting in insects is controlled by steroidal moulting hormones, the ecdysteroids. The sites of biosynthesis of ecdysone (119a) by *Locusta migratoria* have been probed with the aid of the 22,23,24,25-$^3H_4$-labelled precursor (119b); the larval prothoracic gland was identified as the tissue which was the most efficient in the biosynthesis of ecdysone.[207] It has been reported that the introduction of the 2β-hydroxyl group occurs with retention of configuration at C-2 during the biosynthesis of ecdysone in ovaries of the desert locust (*Schistocerca gregaria*).[208] This conclusion was reached following examination of the ^{3}H:^{14}C atomic ratios in the ecdysone (119a) and 2-deoxyecdysone (119c) that had incorporated label from a mixture of [^{3}H]cholesterol (containing label predominantly but not exclusively at the 1α- and 2α-positions) and [4-^{14}C]cholesterol.

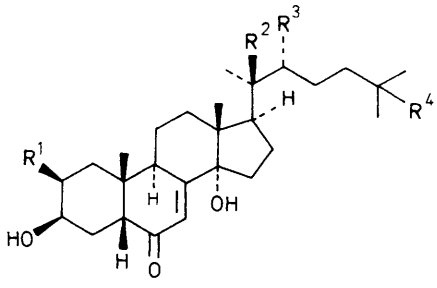
It is generally accepted that the true moulting hormone is 20-hydroxyecdysone (119d), which is biosynthesized from ecdysone in many insect tissues. The properties of ecdysone 20-mono-oxygenase [E.C 1 . 14 . 99 . 22],[209] which is a cytochrome-*P*-450-based mixed-function oxidase, and its distribution in the bodies of larvae of *Schistocerca gregaria* have been studied.[210] Microsomal and mitochondrial versions of the enzyme have been identified in larvae of the tobacco hornworm (*Manduca sexta*).[211] The enzyme ecdysone oxidase [E.C. 1 . 1 . 3 . 16] catalyses the oxidation of ecdysone by molecular oxygen to furnish the dehydroecdysone (120a) and hydrogen peroxide.[212]

Ecdysone oxidase and two NADPH-dependent dehydroecdysone reductases have been investigated in tissue extracts of the lepidopteran *Pieris brassicae*.[213] The two reductases have complementary specificities; the new reductase catalyses the reduction of (120a) by NADPH to furnish ecdysone while the other (known) reductase catalyses the reduction of the same substrate to give 3-*epi*-ecdysone (120b).[213] It is possible that the enzyme activity that had previously been known as ecdysone 3-epimerase[214] arises from the sequential operation of ecdysone oxidase and the latter reductase.[212]

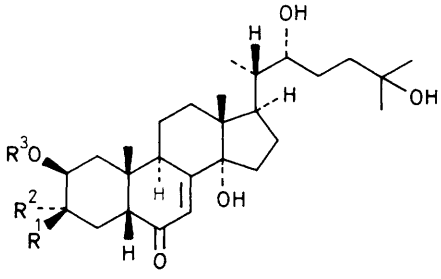
High concentrations of phosphate esters of ecdysteroids, such as ecdysone 22-*O*-phosphate (119e), are found in newly laid eggs of *Schistocerca gregaria*. This phosphate can be used by the



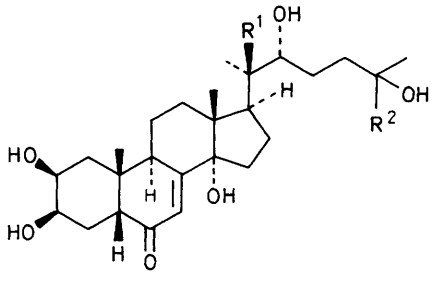
(118)



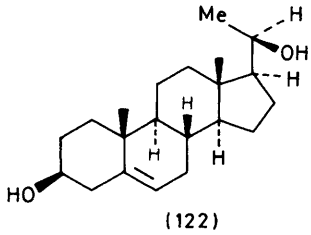
(119) a; $R^1 = R^3 = R^4 = OH$, $R^2 = H$
b; $R^1 = R^2 = R^3 = R^4 = H$
c; $R^1 = R^2 = H$, $R^3 = R^4 = OH$
d; $R^1 = R^2 = R^3 = R^4 = OH$
e; $R^1 = R^4 = OH$, $R^2 = H$, $R^3 = O$Ⓟ



(120) a; $R^1R^2 = O$, $R^3 = H$
b; $R^1 = R^3 = H$, $R^2 = OH$
c; $R^1 = OAc$, $R^2 = H$, $R^3 =$ Ⓟ



(121) a; $R^1 = H$, $R^2 = CO_2H$
b; $R^1 = OH$, $R^2 = CO_2H$
c; $R^1 = H$, $R^2 = CH_2O$Ⓟ



(122)

organism as a source of ecdysone, and may serve as a storage compound.[215] The acetyl-phosphate (120c) has been identified as an end product of the metabolism of ecdysone in eggs of the same species;[216] other metabolites of [23,24-^{3}H]ecdysone in larvae of this organism included the hormone 20-hydroxyecdysone (119d) and the carboxylic acids (121a) and (121b).[217] The latter carboxylic acids were formed as major metabolites from [23,24-^{3}H]ecdysone and 20-hydroxyecdysone, respectively, by the lepidopteran *Pieris brassicae*, by the blowfly *Calliphora vicina*, and by *Locusta migratoria*.[218] The metabolism of ecdysone and 20-hydroxyecdysone has been studied also in the flesh-fly *Sarcophaga peregrina*[219] and in the tick *Ornithodoros moubata*,[220] while the metabolism of 20-hydroxyecdysone in *Drosophila melanogaster* has been investigated.[221] [^{3}H]Ecdysone was converted into 20-hydroxyecdysone and other metabolites in cell cultures of *Bombyx mori*.[222]

Pupae of *Manduca sexta* incorporated [4-^{14}C]cholesterol into the 26-phosphate (121c) of 26-hydroxyecdysone; another labelled metabolite was shown to be a glycoside of the pregn-5-ene-3β,17-diol (122).[223] The biosynthesis and metabolism of ecdysteroids has been reviewed.[224] Reviews have been published also on the biosynthesis and metabolism of sterols, with particular reference to insects.[225]

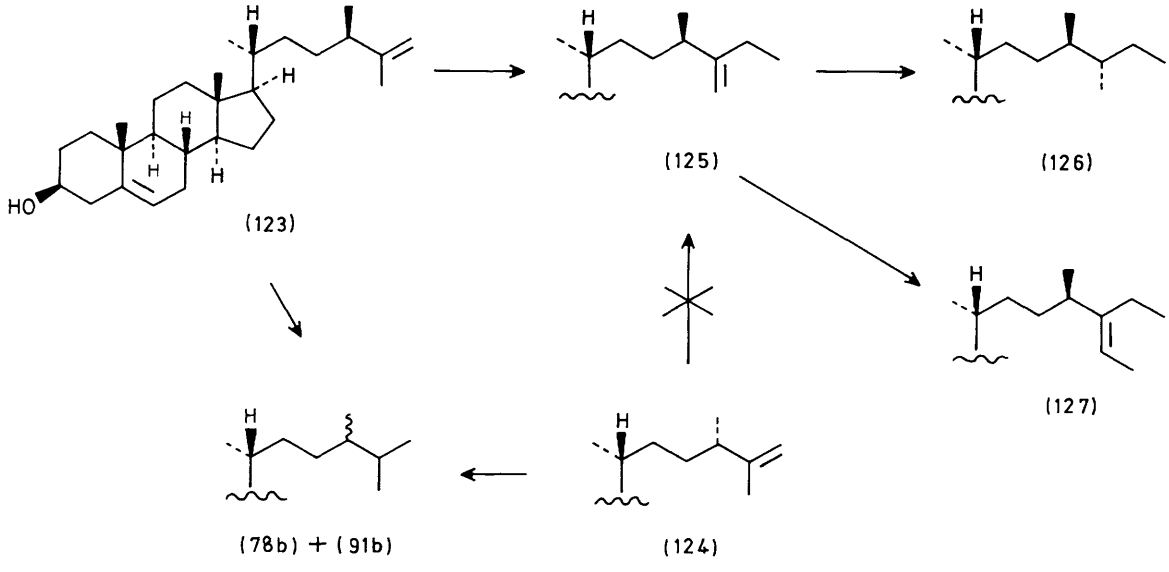
7.2 Other Invertebrates

Incorporation studies with [1-^{14}C]acetate have shown that the soil amoeba *Acanthamoeba polyphaga* is capable of biosynthesizing sterols *de novo*.[226] Surprisingly, the major sterols of the organism were 24-alkyl-24β-sterols, such as ergosterol (66) and poriferasterol (90a), which are more typical of algae; furthermore, cycloartenol (81), 24-methylenecycloartanol (82), and cycloeucalenol (83) were present while lanosterol was not detected. This protozoon also furnished some unusual aromatic sterols.[226] The 14α-demethylation of sterols by *Leishmania mexicana* was inhibited by treatment with ketoconazole;[227] the biosynthesis of sterols from [2-^{14}C]mevalonate was also blocked by the drug SF86-327, which led to the accumulation of [^{14}C]squalene.[228]

Like insects, nematodes are incapable of biosynthesizing sterols, for which they have a nutritional requirement. The free-living nematode *Caenorhabditis elegans* not only has the ability to remove the 24-ethyl group from dietary β-sitosterol but is also capable of altering the steroid nucleus; the most remarkable transformation that has been observed was the introduction of a 4α-methyl group.[229,230] Thus [4-^{14}C]-β-sitosterol was metabolized to cholesta-5,7-dien-3β-ol (35) as the major product together with cholesterol (2a), cholest-7-en-3β-ol (34), and 4α-methylcholest-8(14)-en-3β-ol (92b), amongst other sterols. [26-^{14}C]Desmosterol (93b) was metabolized to a similar range of compounds,[230] as was unlabelled stigmasterol (79a) and other phytosterols.[231] It was suggested that the dealkylation of the side-chains of β-sitosterol[230] and of stigmasterol[231,232] may occur *via* the routes [see Schemes 11 and 12(a), respectively] that have been established for phytophagous insects. In keeping with this suggestion, β-sitosterol was metabolized principally to Δ^{24}-sterols, such as cholesta-5,7,24-trien-3β-ol (73), when it was incubated with the nematode in the presence of the hydrochloride of 25-azacoprostane, which is a potent inhibitor of the reduction of the 24–25 double-bond of desmosterol in insects.[230] *NN*-Dimethyldodecanamine and related nematocides also blocked the putative Δ^{24}-reductase of *C. elegans*.[232,233]

Marine invertebrates continue to attract attention. Conventional sterols such as cholesterol (2a), epicodisterol (123), and codisterol (124) co-occur in the Pacific sponge *Aplysina fistularis* with sterols that possess unusual side-chains, exemplified by the main sterol 25,26-didehydroaplysterol (125) and the related compounds aplysterol (126) and verongulasterol (127).[234] [26-^{14}C]Epicodisterol was incorporated, in 11% radiochemical yield, into 25,26-didehydroaplysterol and in significant yield into aplysterol, verongulasterol, and the mixed 24-methyl-sterols campesterol (78b) and 22,23-dihydrobrassicasterol (91b); on the other hand, [26-^{14}C]codisterol was incorporated only into the mixed 24-methylcholesterols. These data are consistent with the proposed biosynthetic route shown in Scheme 13. However, the source of epicodisterol remains a puzzle, since this sponge is known to be incapable of biosynthesizing sterols *de novo*. [28-^{14}C]-24-Methylenecholesterol (80b) and [4-^{14}C]cholesterol were only incorporated to a significant extent into the mixed 24-ethyl-sterols [(78a)+(91a)] and into 5α-cholestan-3β-ol, respectively.[234]

Cell-free enzyme preparations from zooxanthellae that had been isolated from the gorgonians *Pseudoplexaura porosa*, *P. flagellosa*, *P. wagenaari*, and *Pseudopterogorgia americana* were able to convert [^{14}C]farnesyl diphosphate into squalene, but not into sterols.[235] It was concluded that the biosynthesis of sterols in these symbiotic systems is completed by the respective gorgonians.



Scheme 13

The $\Delta^{9(11)}$-sterols (128a) and (128b) have been isolated from a sea cucumber.[236] It was suggested that squalene epoxide is cyclized directly to 5α-lanosta-9(11),24-dien-3β-ol (128c) in members of the Class Holothuroidea. Liver homogenates from the sea star *Asterias amurensis* incorporated [4-^{14}C]cholesterol into the pregnane aglycon (129) of an asterosaponin in 0.0002% radiochemical yield; other putative precursors of this aglycon gave lower incorporations.[237] The metabolism of pregnenolone (36a) and progesterone (38a) by the lobster *Homarus americanus* has been investigated.[238] The metabolism of ecdysteroids in the crab *Carcinus maenas* has been studied.[239]

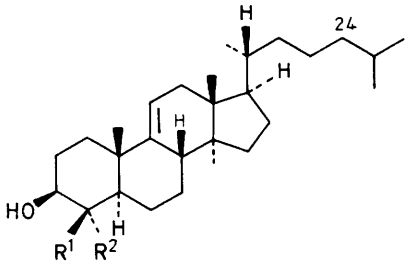
8 The Biosynthesis of Carotenoids

In most species that synthesize carotenoids, *cis*-phytoene (130a) is converted, in a series of discrete dehydrogenation steps, into all-*trans*-lycopene [ψ,ψ-carotene] (131), which is a precursor of the typical cyclic carotenoid β-carotene [β,β-carotene] (132a). Further studies have been reported on the solubilization of enzymic activity for the conversion of [2-^{14}C]mevalonate into *cis*- (130a) and *trans*-phytoene (130b) in a phytoene-accumulating strain of *Phycomyces blakesleeanus*; a soluble enzyme extract that incorporated [2-^{14}C-]mevalonate into both phytoene and β-carotene was also prepared from a β-carotene-forming strain of this fungus.[240] The biosynthesis of β-carotene from [2-^{14}C]mevalonate or [1-^{14}C]isopentenyl diphosphate was inhibited when the latter cell-free enzyme system was incubated with bleaching herbicides, among which fluridone and difunon led to the accumulation of phytoene while amitrole and J852 caused the accumulation of acyclic carotenes.[241] Mutants of *P. blakesleeanus* that are blocked in the formation of carotenoids have been reviewed.[242] A gene (*carC*) that appears to be involved in the regulation of the biosynthesis of carotenoids in *P. blakesleeanus* has been identified in studies of such mutants.[243] The biosynthesis of β-carotene was stimulated by high concentrations of inorganic phosphate in the related fungus *Blakeslea trispora*.[244] Reviews have been published on the regulation of the biosynthesis of carotenoids by tertiary amines in the latter species[245] and on the biosynthesis of carotenoids in members of the Order Mucorales[246] and by *Neurospora crassa*.[247]

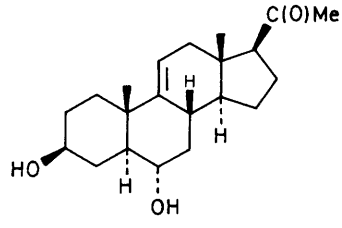
β-Cyclocitral (133a) and the hydroxy-derivative (133b) are released when cells of the cyanobacterium *Microcystis* PCC 7806 are ruptured by freezing.[248] It was shown that these aldehydes arose from oxidative degradation of endogenous β-carotene (132a) and zeaxanthin (132b), respectively, catalysed by a membrane-bound oxygenase, and that crocetindial (134) is also formed, as shown in Scheme 14. [$^{18}O_1$]-β-Cyclocitral and unlabelled crocetindial were isolated when cells of this organism that had grown in an atmosphere of [$^{18}O_2$]oxygen were disrupted.[248]

Bleaching herbicides and a range of 3-phenoxybenzamide derivatives blocked the incorporation of [3-^{14}C]geranylgeranyl diphosphate into β-carotene (132a) in a cell-free enzyme system prepared from the cyanobacterium *Aphanocapsa* PCC 6714. Most of the inhibitors that were tested led to the accumulation of ^{14}C-labelled phytoene and phytofluene (130c).[249] Neither [2-^{14}C]mevalonate nor [1-^{14}C]isopentenyl diphospate was incorporated into carotenoids by the cell-free homogenate from the strain of *Aphanocapsa* while [3-^{14}C]geranylgeranyl diphosphate was a poor precursor of β-carotene. However, the enzymes of this strain of *Aphanocapsa* efficiently incorporated isotopic label into β-carotene when [^{14}C]phytoene was generated *in situ*, from [2-^{14}C]mevalonate, by co-incubation with a cell-free enzyme preparation from a phytoene-accumulating strain of *P. blakesleeanus*.[250] A similar 'coupled enzyme system' was utilized to observe the biosynthesis of β-cryptoxanthin [β,β-caroten-3-ol] (135a) and the derived ketone (135b) from [^{14}C]phytoene by a broken-cell preparation of *Aphanocapsa* that retained intact membranes.[251] No incorporation of [^{14}C]phytoene into zeaxanthin (132b) was observed, and little into echinenone [β,β-caroten-4-one] (135c) and into the carotenoid glycoside myxoxanthophyll, despite the fact that these were major carotenoids in intact *Aphanocapsa*.

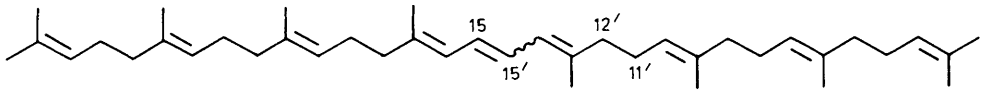
A chromoplast membrane preparation from fruits of *Capsicum annuum* incorporated [15,15′-^{3}H]lycopene (131) into β-carotene (132a) without the accumulation of γ-carotene [β,ψ-carotene].[252] The requisite enzyme activity was tightly mem-



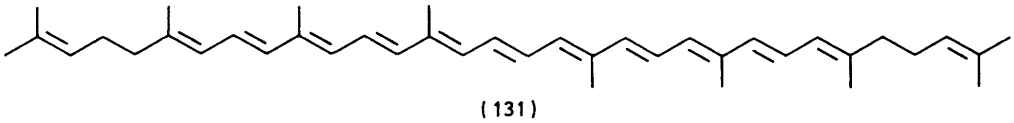
(128) a; R^1 = H, R^2 = Me
b; R^1 = R^2 = H
c; R^1 = R^2 = Me ; 24,25-didehydro



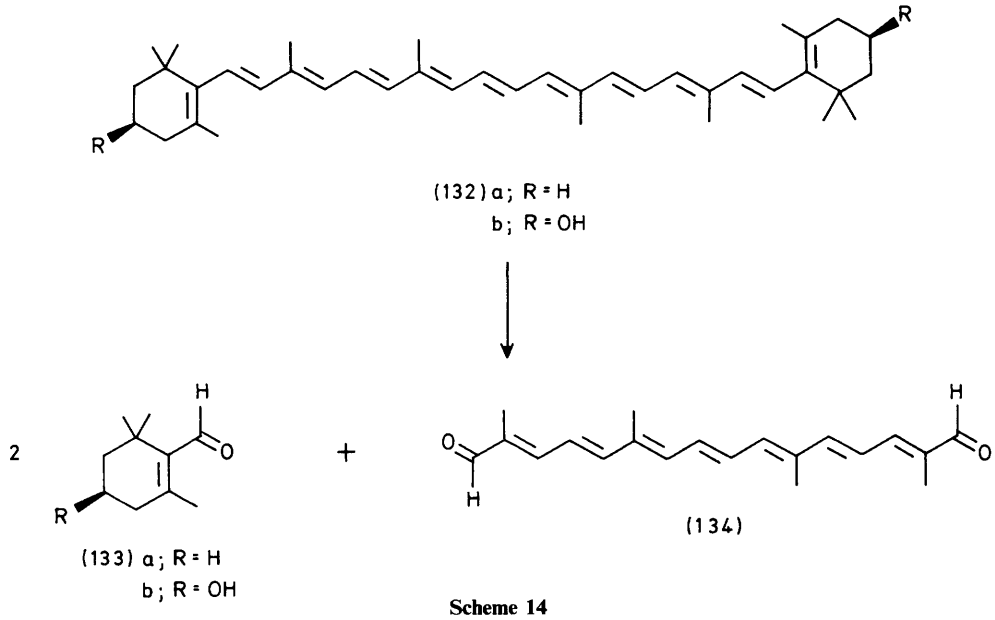
(129)



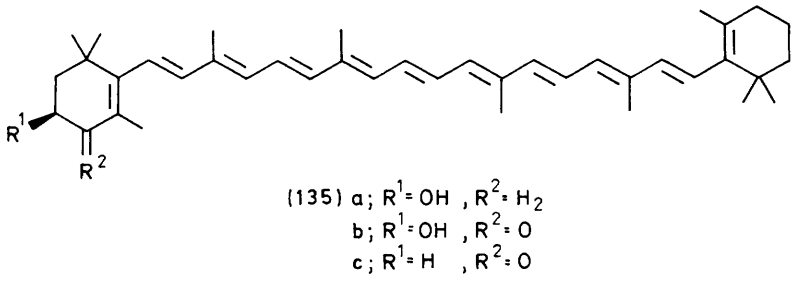
(130) a; 15,15′-*cis*
b; 15,15′-*trans*
c; 15,15′-*cis* or *trans*; 11′,12′-didehydro



(131)



Scheme 14



brane-bound and exhibited a pH optimum at 6.8. The cyclization of lycopene was inhibited by reagents that react with thiol groups,[252] and also by 2-aza-2,3-dihydrosqualene.[253] Practical accounts have been published on the biosynthesis of carotenoids by chromoplasts of *Capsicum annuum*[254] and of daffodil[255] and by chloroplasts of spinach[255] and on the biosynthesis of phytoene by enzyme preparations from tomato.[256]

The biosynthesis of phytoene and acyclic carotenoids was found to be stimulated by red light in radish plants (*Raphanus sativus*) that had been grown in the dark in the presence of the herbicides SAN 6706, amitrole, or J852.[257] The biosynthesis of β-carotene was enhanced by illumination in the yeast *Rhodosporidium diobovatum*[258] and in *Phycomyces blakesleeanus* by blue light.[259] Unusually, the rate of biosynthesis of carotenoids by the fungus *Trichophyton mentagrophytes* was reduced by visible light.[260]

It is generally accepted that animals lack the ability to synthesize carotenoids *de novo*. The wide variety of carotenoids that are found in animals arise from the modification of dietary carotenoids.[261] On the basis of isolation studies only, pathways have been postulated for the metabolism of carotenoids in ten species of sea squirt (Tunicata),[262] in the spindle shell *Fusinus perplexus*,[263] and in some marine fish.[264]

Reviews have been published on the biosynthesis of carotenoids,[265] the enzymes of carotenoid biosynthesis,[266] and the metabolism of carotenoids in fish.[267] The proceedings of the 7th international symposium on carotenoids contain, *inter alia*, papers on carotenogenic enzymes from *Neurospora crassa*,[268a] *Phycomyces blakesleeanus*,[268b] and chromoplasts of *Capsicum annuum*;[268c] other reviews describe the metabolism of carotenoids in animals,[268d] the use of stable isotopes in biosynthetic studies,[268e] and the photoregulation of the biosynthesis of carotenoids.[268f]

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