The Biosynthesis of Triterpenoids, Steroids, and Carotenoids

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Reviewing the literature published between January 1986 and December 1987
(Continuing the coverage of literature in *Natural Product Reports*, 1988, Vol. 5, p. 387)

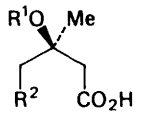
1 Introduction

This Report surveys the literature on the biosynthesis of steroids, triterpenoids, and carotenoids for the years 1986 and 1987, and follows the style that was adopted previously.[1] The papers that are cited were chosen following perusal of *Chemical Titles*, and of the major chemistry and biochemistry journals, for the appropriate period. As in the earlier Reports, the term 'biosynthesis' has been interpreted broadly to include in some instances studies on the inhibition, biochemical control, and characterization of the enzymes of biosynthesis; these aspects of biosynthesis have been treated in an illustrative rather than a comprehensive fashion.

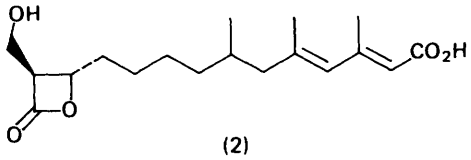
2 Mevalonic Acid

The condensation of acetyl-coenzyme A with acetoacetyl-coenzyme A to form (3*S*)-3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) (1a) is catalysed by the enzyme HMG-CoA synthase [E.C. 4.1.3.5]. This synthase has been isolated from the cytoplasmic fraction of rat liver and has been purified to homogeneity.[2] The enzyme was identified as a dimer, with identical subunits of molecular weight 53000. The β-lactone (2), and related compounds, were potent inhibitors of the synthase from rat liver; other enzymes involved in the biosynthesis of cholesterol (3a) were unaffected.[3]

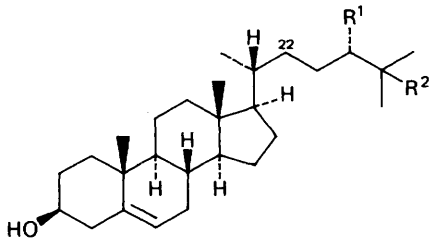
Mevalonic acid (1b) is formed by the reduction of HMG-CoA with NADPH, catalysed by the enzyme HMG-CoA reductase (NADPH) [E.C. 1.1.1.34]. The mammalian reductase is subject to complex regulatory controls, some of which will be discussed below, and is generally regarded as the rate-limiting enzyme in the biosynthesis of cholesterol. Consequently, the reductase is an important target in the search for hypocholesterolemic agents. The fungal metabolite compactin (4a) is one such agent; some natural[4] and synthetic[5] compounds related to compactin also show useful hypocholesterolemic



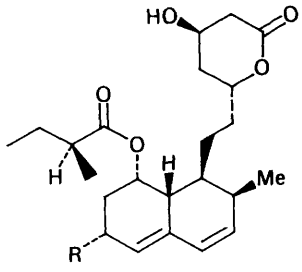
(1) a; R^1 = H, R^2 = C(O)S–CoA
b; R^1 = H, R^2 = CH_2OH
c; R^1 = H, R^2 = CH_2O(PP)
d; R^1 = (P), R^2 = CH_2O(PP)



(2)



(3) a; R^1 = R^2 = H
b; R^1 = H, R^2 = OH
c; R^1 = Et, R^2 = H, Δ^{22}
d; R^1 = R^2 = H, Δ^{24}



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

activity, and many other 5-substituted 3,5-dihydroxypentanoic acid derivatives[6] have been synthesized and assayed as inhibitors of HMG-CoA reductase. Several lines of compactin-resistant hamster cells are known that greatly overproduce both HMG-CoA reductase and HMG-CoA synthase; the base-sequence of the cDNA for the HMG-CoA synthase of such a cell culture has been determined.[7] It has long been known that the

mammalian reductase requires the presence of a thiol for activity when assayed *in vitro*; a unique protein, 'HMG-CoA reductase activating protein', that was isolated from rat liver cytosol, reactivates the oxidized form of the reductase in a time-dependent manner in a reaction requiring NADPH.[8]

The activity of mammalian HMG-CoA reductase is subject to rapid modulation by means of a reversible phosphorylation–dephosphorylation cycle. The reductase is converted into its inactive (phosphorylated) form by an ATP-dependent kinase, the activity of which is also mediated by reversible phosphorylation.[1,9] It has been shown that low density lipoprotein, 25-hydroxycholesterol (3b), and mevalonolactone, all suppress the activity of the reductase of cultured human cells by a mechanism that involves reversible phosphorylation.[10] Experiments with a hamster cell line[11] and with rat liver cells[12] suggested that the proteolytic degradation of the reductase *in vivo* may be promoted by phosphorylation. In the latter case the reductase was believed to exist as a monomer (mol. wt. 97000) or a carbohydrate-linked dimer (mol. wt. 200000); only the monomer was phosphorylated, and subsequently degraded, upon incubation of cells with mevalonate.[12] Notwithstanding the previous work, it has been reported that the native reductase of rat liver microsomes is an unglycosylated monomer of mol. wt. 100000.[13] A highly purified protein kinase from rat liver catalysed the phosphorylation and inactivation of both HMG-CoA reductase and acetyl-coenzyme A carboxylase, in the presence of ATP and Mg^{2+} ions;[14] both effects were stimulated in an identical manner by AMP, and were greatly reduced by prior treatment of the kinase with a protein phosphatase. These results support the view that a common phosphorylation–dephosphorylation cycle regulates the biosynthesis both of steroids and fatty acids.[14]

It has been reported that the yeast *Saccharomyces cerevisiae* contains two functional genes that code for HMG-CoA reductase.[15] Chemical modification of the reductases of both yeast and rat liver has provided evidence for essential cysteine and histidine residues.[16]

Further studies have been reported on the HMG-CoA reductase (NADPH) from seedlings of radish, *Raphanus sativus*; the enzyme was described as a tetramer, of mol. wt. 180000, and contained free, essential thiol groups. Antibodies raised to the reductase of rat liver or yeast did not bind to that of radish.[17] The reductase of leaves of *Parthenium argentatum* also contained an essential thiol group, and required the presence of thiols to prevent loss of activity. The enzyme that was associated with the chloroplast had a pH optimum of 7.5, while the cytosolic enzyme had a pH optimum of 7.0.[18]

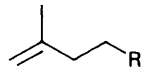
The rate of biosynthesis of poriferasterol (3c) was reduced initially by over 90% when the chrysophyte alga *Ochromonas malhamensis* was grown in the presence of a 10 μM solution of the reductase inhibitor mevinolin (4b), but had returned to normal levels within 2 days. After removal of the inhibitor, it was shown that the level of HMG-CoA reductase activity had increased temporarily 10–15-fold, in a manner that is reminiscent of the response of some mammalian cells to inhibitors of this type.[19] Mevinolin also acted as a competitive inhibitor of the reductase of the bacterium *Halobacterium halobium*, leading to the accumulation of intracellular HMG-CoA, and blocking the growth of the organism; however, in contrast to the effect observed with eukaryotes, referred to above, subsequent assay in the absence of mevinolin revealed no increase in HMG-CoA reductase activity.[20]

3 The Biosynthesis of Squalene from Mevalonic Acid

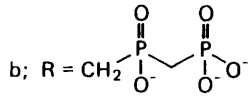
The enzymes mevalonate kinase [E.C. 2.7.1.36] and phosphomevalonate kinase [E.C. 2.7.4.2], which catalyse the two sequential ATP-Mg^{2+} dependent steps in the conversion of mevalonate into mevalonic acid diphosphate (1c), have been investigated in extracts from green leaves of the lemon grass *Cymbopogon citratus*. Both enzymes were found exclusively in the cytosol and both required the presence of thiols for maximum catalytic efficiency.[21] The former two kinases and diphosphomevalonate decarboxylase [E.C. 4.1.1.33] have been isolated by an efficient route from pig liver; the three enzymes were separated conveniently at a late stage in their purification.[22] The decarboxylase had a mol. wt. of 88000 and appeared to be a dimer but, unlike the avian enzyme, Mg^{2+} was clearly superior as a co-factor to other metal di-cations, and the enzyme had a broad optimum pH range (5.0–8.0).[22] The enzyme was apparently activated by ATP,[22] as observed earlier for the two kinases, and also for the avian decarboxylase.[23]

In the presence of Mg^{2+} ions, diphosphomevalonate decarboxylase catalyses the reaction between diphosphomevalonate and ATP to furnish isopentenyl diphosphate (5a), carbon dioxide, ADP, and inorganic phosphate, possibly *via* the intermediate triphosphate (1d). The stereochemistry of the phosphorylation step has been investigated, for the reaction catalysed by the avian decarboxylase, by utilizing (S_p)-adenosine-5′-*O*-(3-thio[γ-$^{17}O_2$,$^{18}O_1$]triphosphate)-Mn^{2+} as a substrate in place of ATP-Mg^{2+}; it was reported that the labelled thiophosphate ion that was subsequently released had undergone inversion of configuration, in keeping with the mechanism summarized in Scheme 1.[24]

The conversion of mevalonate (but not of isopentenyl diphosphate) into cholesterol by rat liver homogenates was inhibited by the fluoromevalonates (6a), (6b), (6c), and (6d).[25] In each case 5-phospho- and 5-diphosphomevalonates accumulated. For the 6-fluoro- and 6,6,6-trifluoromevalonates, (6a) and (6c) respectively, it was confirmed that the enzyme diphosphomevalonate decarboxylase was the site of inhibition.

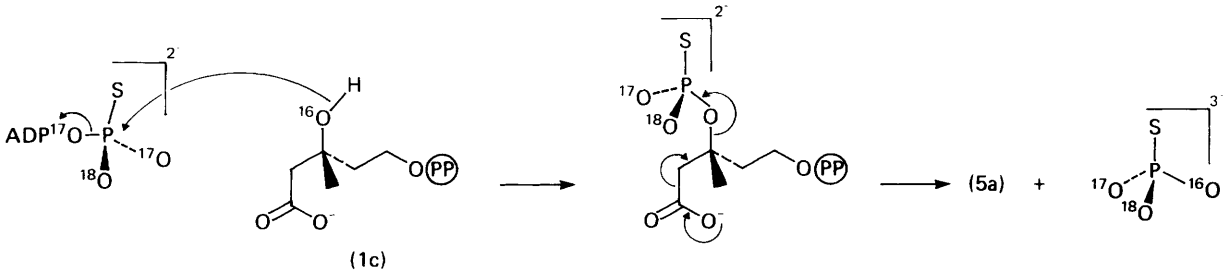


(5) a; R = O(PP)



b; R = CH_2

c; R = CH_2O(PP)

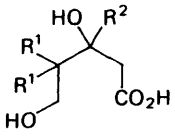


(1c)

Scheme 1

These results confirm and extend those reported earlier by other workers.[1] Aspects of the biosynthesis and compartmentation of acetyl-coenzyme A and of isopentenyl diphosphate (5a) in plants have been reviewed.[26]

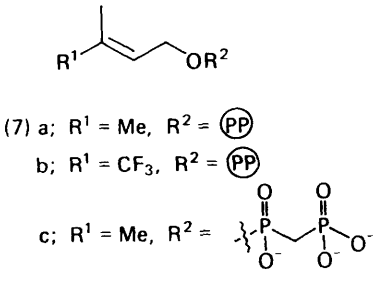
Isopentenyl diphosphate Δ-isomerase [E.C. 5.3.3.2] catalyses the equilibration between isopentenyl diphosphate (5a) and dimethylallyl diphosphate (7a) in the presence of Mg^{2+} ions. Details have now been published on the purification of the Δ-isomerase from the yeast *Saccharomyces cerevisiae*.[27] The enzyme was isolated as a monomer, of mol. wt. 40000, which contained an essential thiol group and displayed a broad pH-activity profile. The trifluoro-derivative (7b) of dimethylallyl diphosphate was an extremely poor substrate, and the ammonium salt (8) inactivated the enzyme irreversibly, without covalent modification. Both observations are consistent with a carbocation intermediate in the isomerization of isopentenyl diphosphate by the enzyme.[27] The Δ-isomerase of *Claviceps* SD58 has also been purified to near homogeneity. The enzyme was a monomer, of mol. wt. 35000, had a broad pH optimum (6.0—8.5), and contained an essential thiol group.[28]



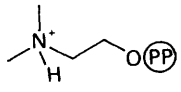
(6) a; $R^1 = H$, $R^2 = CH_2F$
b; $R^1 = H$, $R^2 = CHF_2$
c; $R^1 = H$, $R^2 = CF_3$
d; $R^1 = F$, $R^2 = CH_3$

The Δ-isomerase (monomer, mol. wt. 33500) and geranylgeranyl diphosphate synthase have been isolated from *Capsicum* chromoplasts and purified to homogeneity by affinity chromatography.[29] The synthase was described as a dimer (mol. wt. 74000) of identical subunits, and was reported to catalyse the condensations of isopentenyl diphosphate with dimethylallyl diphosphate to furnish geranyl diphosphate (9a), (*i.e.* [E.C. 2.5.1.1] activity), of isopentenyl diphosphate with geranyl diphosphate to furnish farnesyl diphosphate (10), (*i.e.* [E.C. 2.5.1.10] activity), and of isopentenyl diphosphate with farnesyl diphosphate to furnish geranylgeranyl diphosphate (*i.e.* [E.C. 2.5.1.30] activity).[29] A cholesterol-repressible enzyme of rat liver, that was tentatively identified as a 'prenyl transferase' (presumably [E.C. 2.5.1.1] and [E.C. 2.5.1.10]), has been cloned; the cDNA coded for a protein of mol. wt. 39615, containing 344 amino-acid residues.[30]

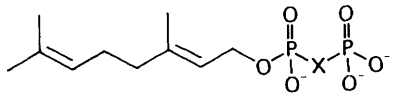
It has been shown, with the aid of appropriately deuterium-labelled substrates, that the formation of farnesyl diphosphate (10) catalysed by the synthase isolated from pumpkin involves loss of the 2(*pro*-2*R*)-proton of isopentenyl diphosphate, and alkylation at the 4*Si* face of the double bond of the same substrate, in agreement with the results obtained previously for the synthase from pig liver.[31] It is instructive to compare these results with those reported for the 'prenyl transferases' of *Bacillus subtilis*. Thus the enzyme heptaprenyl diphosphate synthase utilized the same stereochemical pathway as described above in catalysing the stepwise condensation of four molecules of isopentenyl diphosphate with farnesyl diphosphate to yield the all-*trans* product (11).[32] On the other hand the undecaprenyl diphosphate synthase [E.C. 2.5.1.31] of the same organism catalysed the sequential condensation of farnesyl diphosphate with eight molecules of isopentenyl diphosphate, to form the '*poly-cis*' product (12), by alkylation at the 4*Si* face of the double bond of isopentenyl diphosphate but with loss of the 2(*pro*-2*S*)-proton of the latter substrate (Scheme 2). It is



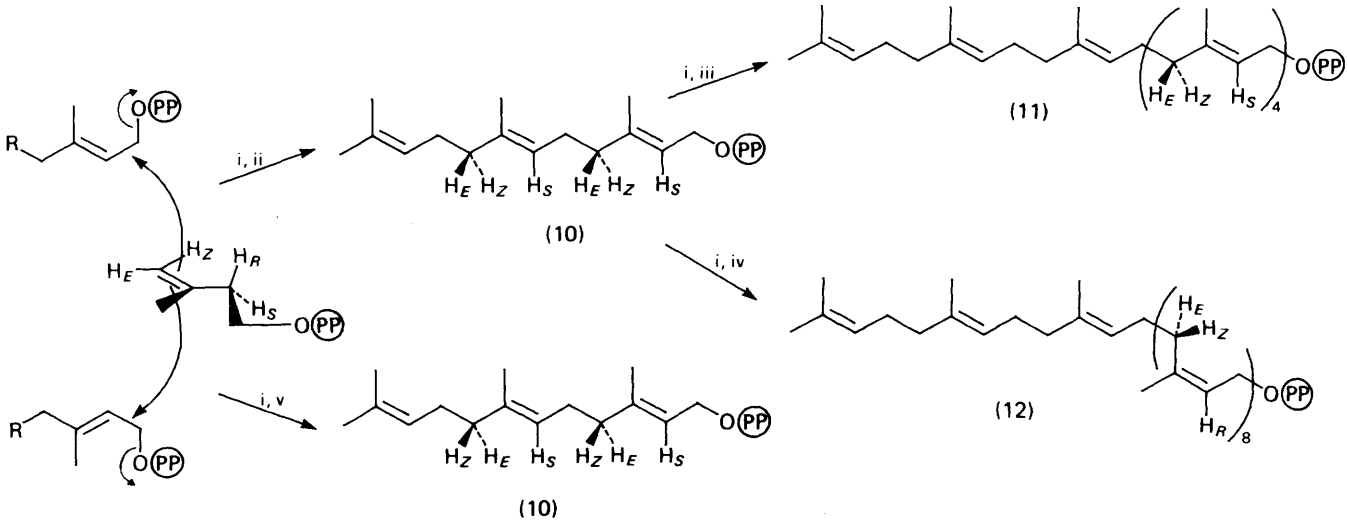
(7) a; $R^1 = Me$, $R^2 = OPP$
b; $R^1 = CF_3$, $R^2 = OPP$
c; $R^1 = Me$, $R^2 =$



(8)



(9) a; X = O
b; X = CH_2
c; X = CF_2



(10)

(11)

(12)

(10)

Reagents: i, isopentenyl diphosphate; ii, farnesyl diphosphate synthase of pig liver or pumpkin; iii, heptaprenyl diphosphate synthase of *Bacillus subtilis*; iv, undecaprenyl diphosphate synthase of *Bacillus subtilis*; v, farnesyl diphosphate synthase of pea (in presence of ICH_2CONH_2)

Scheme 2

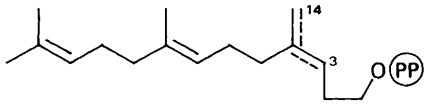
significant that in each of the above cases the double bond of isopentenyl diphosphate is alkylated on the 4*Si* face, and that the alkylation and deprotonation steps occur in a suprafacial manner.[32] Surprising results have been reported for the farnesyl diphosphate synthase of the pea, *Pisum sativum*. Normally the synthase catalysed the alkylation of the 4*Si* face of the double bond of isopentenyl diphosphate but, after being treated with iodoacetamide, the modified enzyme catalysed the alkylation of the 4*Re* face,[33,34] with antarafacial elimination of the 2(*pro*-2*R*)-proton (Scheme 2).[34] No such aberrant change in stereoselectivity was observed for the synthases of pig liver or pumpkin, when they were incubated with iodoacetamide.[33]

The significance of the diphosphate moiety, in substrates for farnesyl diphosphate synthase, has been probed with the aid of the dimethylallyl- and geranyl-diphosphonates (7c) and (9b) respectively.[35,36] These compounds were less good as substrates for the synthase than were the corresponding diphosphates, despite similar K_m values, owing to a 50-fold reduction in the V_{max} for their condensation with isopentenyl diphosphate. The difluoro-derivative (9c) was a better substrate than (9b), for the avian synthase, owing to improvement in the leaving group ability of the diphosphonate unit.[36] These compounds have potential as substrate analogues in kinetic studies because they are resistant to phosphatases which are frequent contaminants of crude enzyme systems. The phosphonylphosphinate (5b) was a good inhibitor of the condensation of dimethylallyl diphosphate with isopentenyl diphosphate, catalysed by the same synthase.[37]

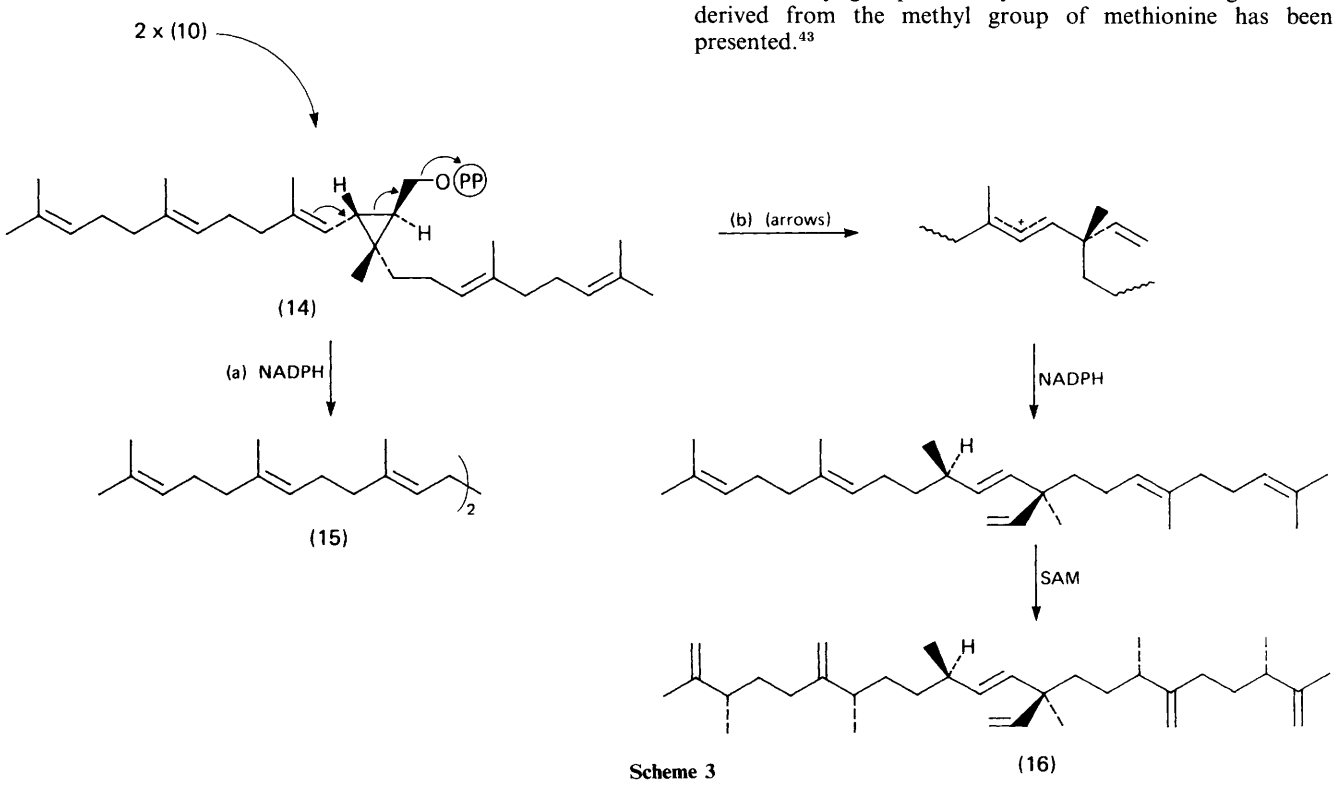
It was shown earlier[1] that the (3*Z*,7*E*)-homo-farnesyl diphosphate (13a) was formed when geranyl diphosphate was condensed with the unnatural substrate (5c), catalysed by the farnesyl diphosphate synthase of pig liver. It has now been reported that when the same condensation is performed in the presence of Ni^{2+} or Co^{2+} ions, in place of Mg^{2+}, the isomeric product (13b) is formed.[38] The ability of the synthase to accept unnatural substrates has been exploited in a synthesis of 4-methyl-juvenile hormone 1.[39]

Squalene synthetase [farnesyl-diphosphate farnesyltransferase (E.C. 2.5.1.21)] has been isolated in soluble form from yeast and purified 50-fold. It was shown that the enzyme was a single protein of mol. wt. 53000, which catalysed both the condensation reaction between two molecules of farnesyl diphosphate to give presqualene diphosphate (14) and the subsequent reductive elimination of diphosphate to furnish squalene (15) (Scheme 3a).[40] Theoretical calculations have been performed on model compounds in an attempt to clarify the mechanisms for the biosynthesis of presqualene diphosphate and of squalene.[41]

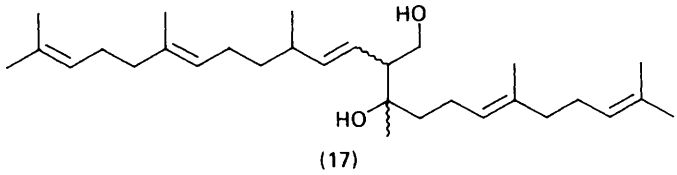
The absolute stereochemistry of botryococcene (16) has been determined. It was suggested that the biosynthesis of this fascinating C_{34}-hydrocarbon, in the green alga *Botryoccus braunii*, involves an alternative mode of ring-opening of presqualene diphosphate (Scheme 3b).[42] Evidence that the 'extra' methyl groups of botryococcene and its congenors are derived from the methyl group of methionine has been presented.[43]



(13) a; 3(4)-double bond, *cis*
b; 4(14)-double bond



Scheme 3



(17)

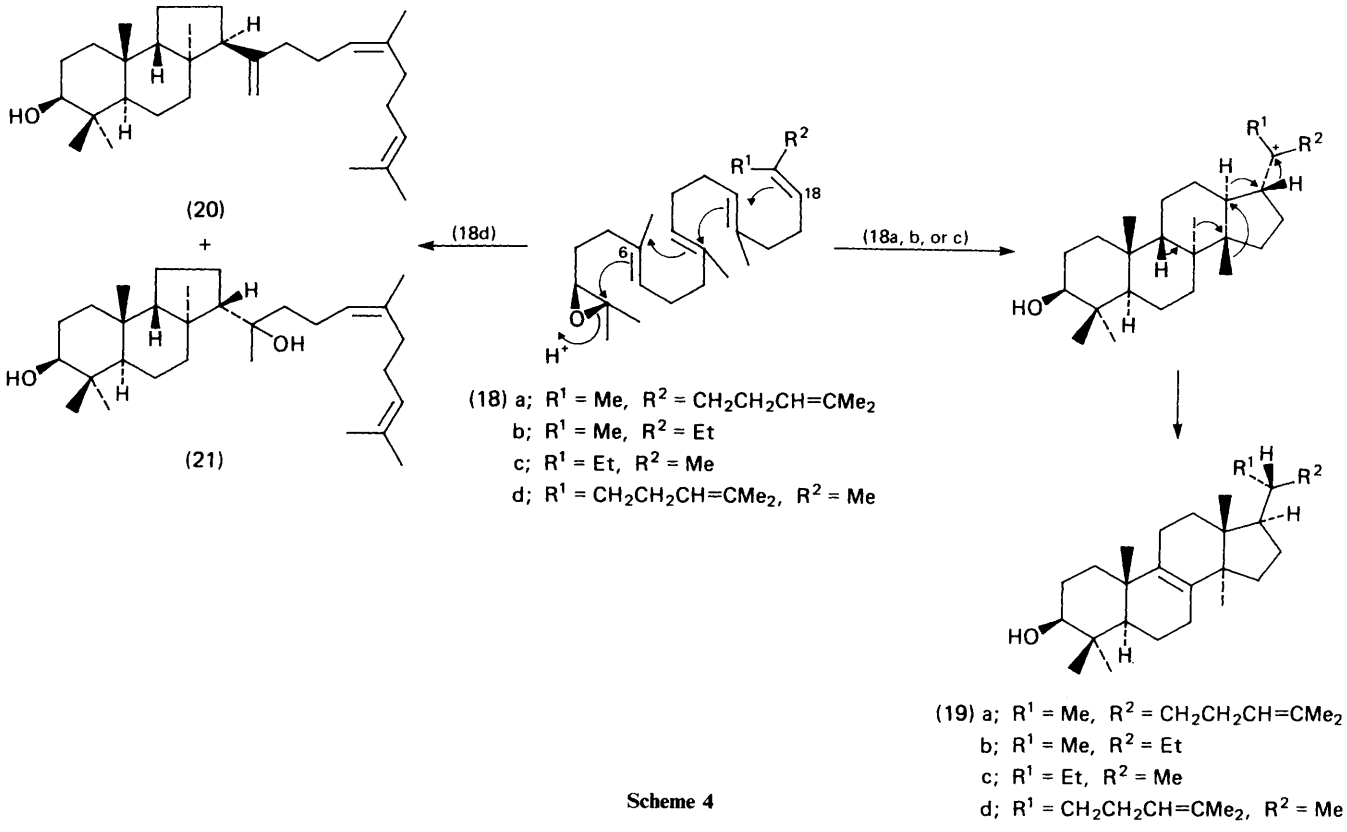
The mixed diols (17), labelled with tritium, showed a 0.16% incorporation into squalene in a cell-free extract from *Rhizopus arrhizus*. This incorporation was said to be negligible, since in parallel experiments [^{14}C]mevalonate gave an 11% incorporation into squalene, and it was concluded that the diol or its phosphorylated derivatives were unlikely to be intermediates in the biosynthesis of presqualene diphosphate.[44] It has been reported that only 12 of the carbons of squalene (carbons 1, 3, 5, 7, 9, 11 *etc.*) were isotopically labelled when [2-^{13}C]acetate was incorporated into squalene in the bacterium *Halobacterium cutirubrum*; it is not possible, on the basis of the data presented, to assess the significance of this surprising observation.[45]

4 The Biosynthesis of Triterpenoids from Squalene

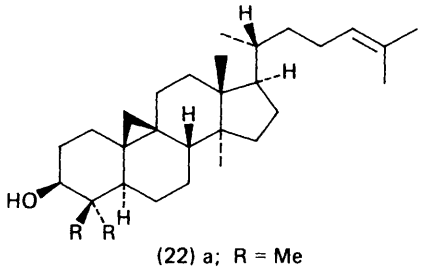
The biosynthesis of triterpenoids in eukaryotes generally involves the oxidation of squalene to furnish (3*S*)-squalene 2,3-epoxide (18a), which subsequently undergoes proton-initiated cyclization. The squalene epoxidase [squalene mono-oxygenase (E.C. 1.44.99.7)] of the yeast *Saccharomyces cerevisiae* could not be assayed satisfactorily in crude enzyme preparations owing to the poor solubility of exogenously supplied squalene. A new assay, which relied on the conversion *in situ* of [^{14}C]farnesyl diphosphate into [^{14}C]squalene, has been described.[46] It was shown, using this assay, that the epoxidase of yeast was induced by oxygen, and was not inhibited by lanosterol or ergosterol.[46]

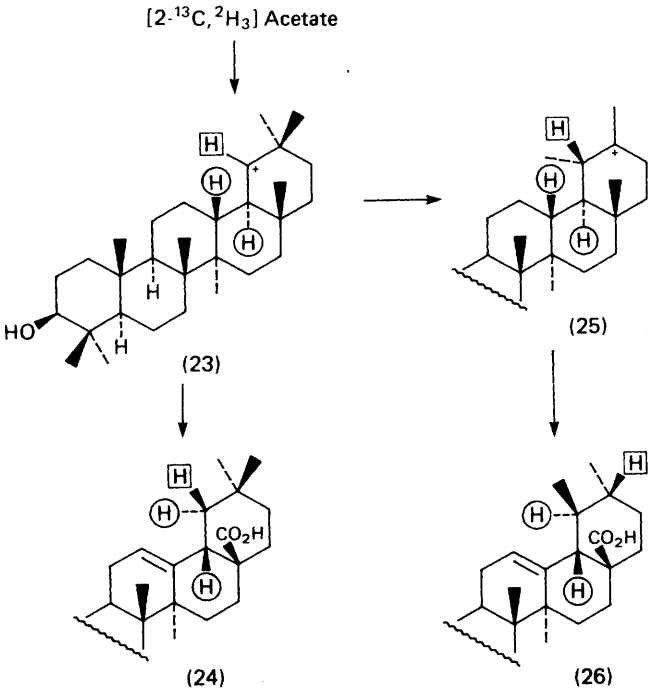
Interest continues in the mechanism of mammalian lanosterol synthase [E.C. 5.4.99.7], which catalyses the sequential cyclization and rearrangement of squalene epoxide (Scheme 4) to furnish lanosterol (19a). It was reported earlier that the diastereoisomeric tetranorsqualene epoxides (18b) and (18c) were cyclized by lanosterol synthase to the epimeric tetranorlanosterol analogues (19b) and (19c) respectively.[47] In contrast when the (18*Z*) isomer (18d) of squalene epoxide was submitted to the lanosterol synthase of hog liver, the tricycles (20) and (21) were formed. No tetracyclic product, such as the 20-epimer (19d) of lanosterol, could be found.[48] This result reopens the question of whether the enzymatic cyclization of squalene epoxide occurs *via* an intermediate tricyclic carbocation with a 5-membered ring C. The results of biomimetic polyene cyclization reactions have demonstrated the efficacy of carbocation stabilizing substituents. It was suggested that lanosterol synthase may catalyse the cyclization of squalene epoxide, in part, by the placing of negative charge on the β-face near C-6 and on the α-face near C-10 and C-14.[49]

A cell culture of gardenia (*Gardenia jasmoides*) that had been supplied with [1-^{13}C]-, [2-^{13}C]-, or [1,2-$^{13}C_2$]acetate furnished cycloartenol (22a) with the expected labelling pattern in each case.[50] In the course of this study, some of the resonances in the ^{13}C-NMR spectrum of cycloartenol were reassigned.



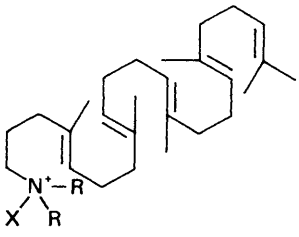
Scheme 4



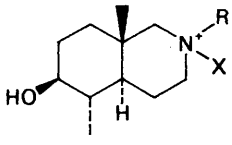


The symbols [H] and (H) represent ^{2}H-label. For the sake of clarity, only ^{2}H that is involved in migration is shown in the scheme

Scheme 5



(27) a; R = Me, X = H
b; R = Me, X = O$^-$
c; R = Et, X = H

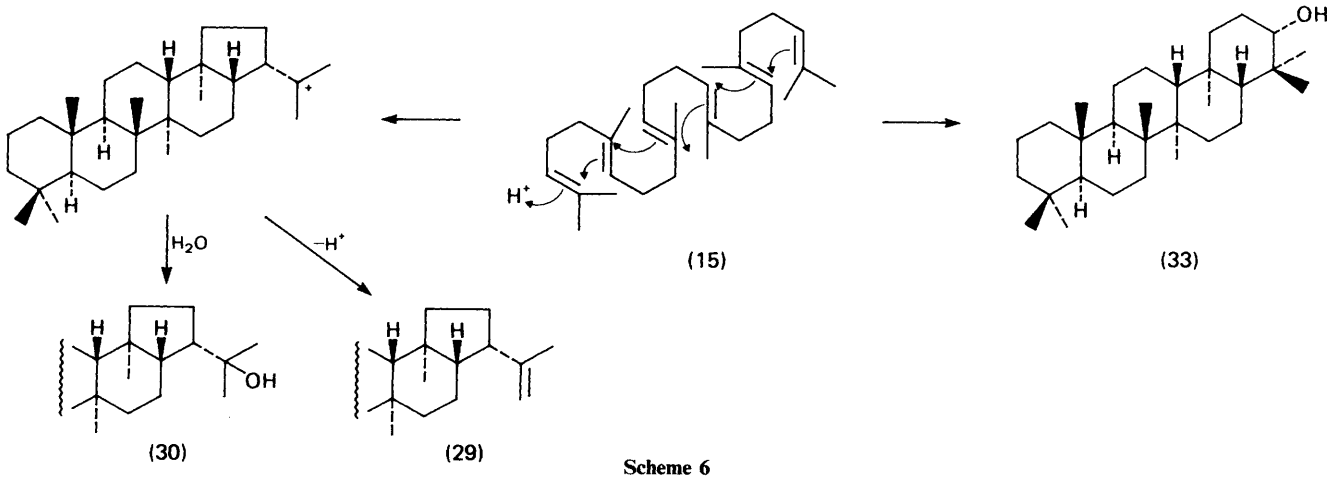


(28) a; R = CHMe(CH$_2$)$_3$CHMe(CH$_2$)$_3$CHMe$_2$, X = H
b; R = CH$_2$Ph, X = O$^-$

The biosynthesis of oleanolic acid (24) from squalene epoxide is expected to involve two sequential 1,2-hydride migrations in the intermediate cation (23), while that of ursolic acid (26) is known[1] to involve three sequential hydride migrations in the intermediate carbocation (25). These hydride shifts have been observed by ^{13}C-NMR, utilizing the β-deuterium isotope effect, on the methyl esters of oleanolic acid and ursolic acid that had been isolated from suspension cultures of *Rabdosia japonica* (formerly *Isodon japonicus*) which had been supplied with sodium [2-^{13}C, 2-^{2}H$_3$]acetate (Scheme 5).[51] Much of our current knowledge of the biosynthesis of sterols and triterpenoids arose from feeding experiments with specifically tritium-labelled mevalonates. The early work on the mode of incorporation of the C-2 hydrogen atoms of mevalonate into steroids and triterpenoids has been reviewed.[52]

2-Aza-2,3,-dihydrosqualene, presumably as the derived ammonium cation (27a), is known to inhibit both the lanosterol synthase of rat liver and the cycloartenol synthase of higher plants. It has now been reported that (27a) is an effective inhibitor of the lanosterol synthase of the pathogenic yeast *Candida albicans*, and also of the squalene epoxidase of the latter species and of rat liver.[53] Moreover, the bicyclic ammonium cation (28a) is a potent inhibitor of the cycloartenol synthase of maize seedlings, bramble, pea, and of the lanosterol synthase of rat liver.[54] These two inhibitors are believed to act by mimicing the carbocation-like intermediates that would formally arise if squalene epoxide were allowed to cyclize in a stepwise manner. The design of such inhibitors of 'squalene epoxide cyclases'[55,56] and of inhibitors of carbocation intermediates in other stages in sterol biosynthesis[56] has been reviewed.

A membrane-bound squalene cyclase has been isolated from the thermophilic bacterium *Bacillus acidocaldarius*, and purified 270-fold[57] and 670-fold[58] respectively by two separate research groups. The enzyme contained essential cysteine and histidine residues,[57] and catalysed the cyclization of squalene to diploptene (29) and diplopterol (30), in the ratio of 86:14, at 54 °C (Scheme 6). The enzyme also accepted several unnatural substrates; for example the homofarnesol (31) furnished the tricyclic ether (32) when incubated with the enzyme.[58] The squalene epoxide cyclase inhibitors 2-aza-2,3-dihydrosqualene (27a), the corresponding *N*-oxide (27b), and the diethylamino-compound (27c) were also potent inhibitors of the cyclization of squalene to diploptene and diplopterol in cell-free enzyme preparations from the bacteria *Acetobacter pasteurianus* subspecies *pasteurianus*, *Methylobacterium organophilum*, and *Zymomonas mobilis*.[59] A cell-free enzyme system from the protozoon *Tetrahymena pyriformis*, that catalysed the cyclization of squalene to tetrahymanol (33), was also active in the cyclization of all-*trans*-pentaprenyl and hexaprenyl methyl



Scheme 6

ethers; for example, the methyl ether (34) was converted to the tetracycle (35) when it was incubated with the crude enzyme preparation.[60]

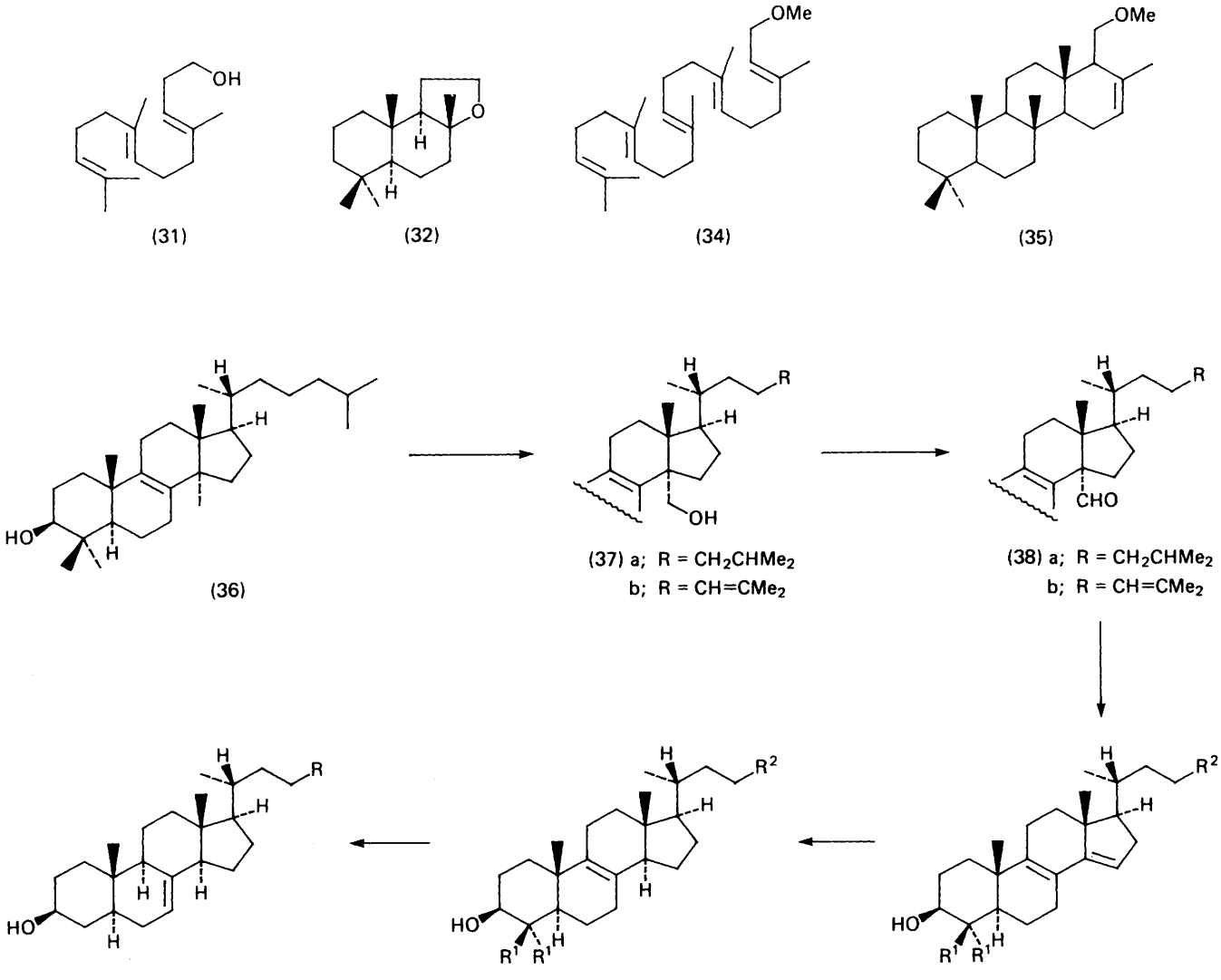
5 The Formation of Sterols in Vertebrates

5.1 The Biosynthesis of Cholesterol

The biosynthesis of cholesterol (3a) from lanosterol (19a), in mammals, follows a multitude of pathways which vary according to the timing of the reduction of the Δ^{24}-double bond. Scheme 7 summarizes the early stages of the generally accepted route for the biosynthesis of cholesterol from 24,25-dihydrolanosterol (36). The large scale incubation of [24,25-^{3}H]dihydrolanosterol with rat liver microsomes permitted isolation and rigorous proof of structure of the intermediate primary alcohol (37a) and the derived aldehyde (38a) (when the NADPH supply was limited) and of the primary demethylation product (39a) and the Δ^8-sterol (40a) (when the incubation was performed in the presence of sodium cyanide).[61] It is noteworthy that no $\Delta^{8(14)}$-sterol was detected, nor was the $\Delta^{7,14}$-isomer of (39a). The primary alcohol (37a) and the aldehyde (38a) could also be isolated, in a constant 1:3 ratio, when short incubation times, limiting enzyme concentrations, high concentrations of dihydrolanosterol, or high pH were employed during these studies.[62] The conversion of dihydrolanosterol to the $\Delta^{8,14}$-steroid (39a) was subject to competitive inhibition by lanosterol, which was a better substrate for the demethylase of intact microsomes than was dihydrolanosterol.[62]

The cytochrome P-450 component that is responsible for the 14α-demethylation of lanosterol or dihydrolanosterol has been purified to homogeneity, to give a heme-containing protein of mol. wt. 51000.[63] The addition of NADPH-cytochrome c reductase, dilauryl phosphatidyl-choline, NADPH, and oxygen, restored the ability of this species of cytochrome P-450 to remove the 14α-methyl group of dihydrolanosterol *via* the sequence (36) to (37a) to (38a) to (39a). No other oxygenated intermediate was detected.[63]

The membrane-bound sterol Δ^8-Δ^7 isomerase of rat liver microsomes has been solubilized and partially purified.[64] The enzyme catalysed the isomerization of cholesta-8,24-dienol (40b) to cholesta-7,24-dienol (41a), and the isomerization of cholest-8-enol (40c) to its Δ^7-isomer (41b). The former pair of



Scheme 7

steroids were significantly better substrates for the isomerase than were the latter two; in each series the ratio of Δ^8-sterol to Δ^7-sterol was about 1:19 at equilibrium. The isomerase did not catalyse the isomerization of cholesta-8,14-dien-3β-ol (39b) nor did it accept cholest-8(14)-ene-3β-ol as a substrate.[64]

The sub-cellular location of the lanosterol 14α-demethylase, the sterol Δ^{14}-reductase, and the sterol Δ^8-Δ^7 isomerase, have been studied in rat liver.[65] The primary structure of rat liver 'sterol carrier protein-2', which is involved in the later stages of the biosynthesis of cholesterol, has been determined.[66]

The antimycotic agents miconazole and ketonazole function by inhibiting the 14α-demethylase of cholesterol biosynthesis. Low concentrations of these inhibitors reduced the HMG-CoA reductase activity of a hamster cell line, but the reductase activity was restored with higher concentrations of the same inhibitors.[67] It was shown that the inhibition of HMG-CoA reductase activity was mediated by the aldehyde (38a), which accumulated in the cell culture only if the 14-demethylase was partially (but not totally) inhibited.[67] Addition of mevalonate to another hamster cell line, at a concentration sufficient to reduce HMG-CoA reductase activity by about 90%, led to the accumulation of the inhibitory sterols (37b) and (38b).[68] Many synthetic 15-oxygenated steroids are potent inhibitors of the biosynthesis of cholesterol in cell-free enzyme systems or in cultured mammalian cells.[69] The regulation of HMG-CoA reductase activity by oxygenated sterols and lanosterol derivatives has been studied.[70] Other accounts have been published on the inhibition of the biosynthesis of cholesterol.[71]

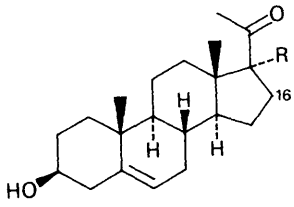
The resonances due to the diastereotopic 25-methyls in the ^{1}H-NMR spectrum and in the ^{13}C-NMR spectrum of cholesterol have been assigned with the aid of stereospecifically deuteriated samples of cholesterol.[72] The ^{13}C-NMR assignments provide further confirmation for those made previously on the basis of biosynthetic experiments.[cf. 47]

5.2 The Biosynthesis of Steroidal Hormones

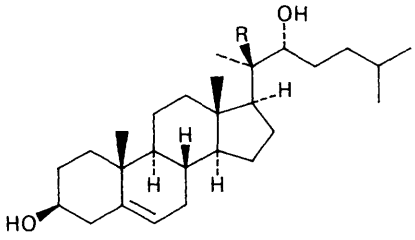
The first committed step in the biosynthesis of the steroidal hormones is the oxidative cleavage of the side chain of cholesterol to furnish pregnenolone (42a) and 4-methylpentanal. This transformation involves the transient formation of (22*R*)-22-hydroxycholesterol (43a) and (22*R*)-20,22-dihydroxycholesterol (43b), and is catalysed by the NADPH-requiring mixed-function oxidase cholesterol mono-oxygenase (side-chain cleaving) [E.C. 1.14.15.6]. Further study has been reported on the mode of binding of cholesterol and its hydroxylated derivatives (43a) and (43b) to the cytochrome P-450 component of the enzyme system (cytochrome P-450$_{scc}$),[73] and on the access of water molecules to the active site of the latter.[74] The primary structure of the cytochrome P-450$_{scc}$ from bovine adrenocortical mitochondria has been confirmed by protein sequencing techniques,[75] and the cysteine residue whose thiol group is coordinated to the heme unit has been identified.[76] The human gene that codes for cytochrome P-450$_{scc}$ has been studied.[77]

The biosynthetic conversions of pregnenolone (42a) to progesterone (44a), or of dehydroepiandrosterone (45) to androst-4-ene-3,17-dione, both involve a 3-keto-Δ^5 intermediate and each conversion is catalysed, for example, by the NAD$^+$-dependent enzyme 3β-hydroxysteroid dehydrogenase [E.C. 1.1.1.145]. This dehydrogenase has been isolated and purified to homogeneity from both the adrenal[78] and testis[79] of the rat. The enzymes isolated from the two sources were closely similar, and had the same mol. wt. (46500). Both enzymes displayed steroid Δ-isomerase activity, [E.C. 5.3.3.1] only in the presence of NAD$^+$ or NADH (presumably as an allosteric effector),[78,79] unlike the more intensively-studied steroid Δ-isomerase of the bacterium *Pseudomonas testosteroni*[80] which requires no coenzyme.

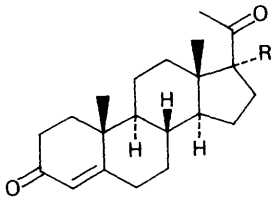
The biosynthesis of the C_{19}-steroidal hormones involves hydroxylation of pregnenolone (42a) or progesterone (44a)



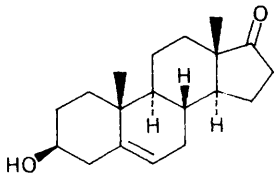
(42) a; R = H
b; R = OH



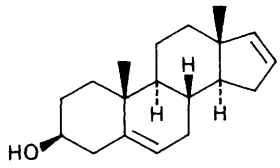
(43) a; R = H
b; R = OH



(44) a; R = H
b; R = OH
c; R = OCOCH$_2$Br



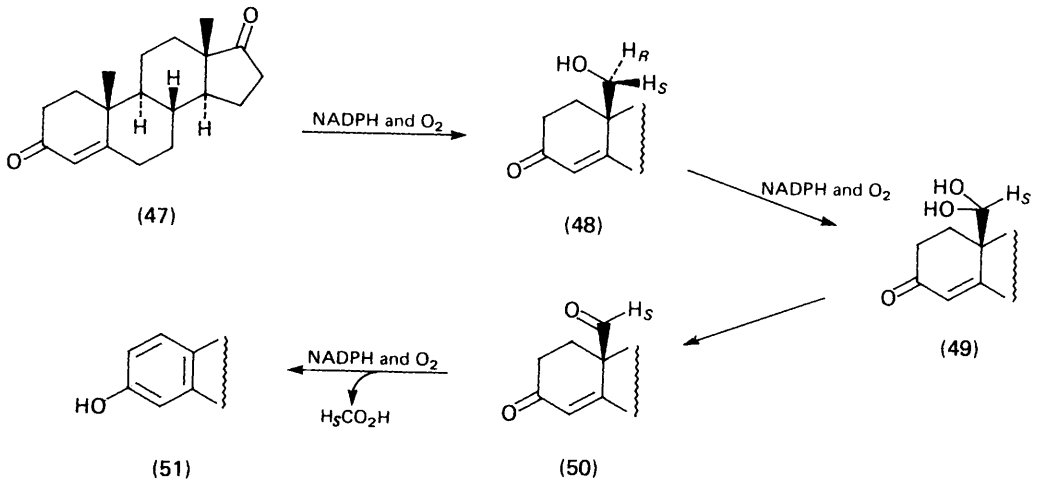
(45)



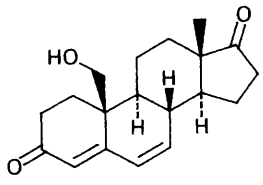
(46)

[catalysed by the enzyme C_{21}-steroid-17α-hydroxylase (E.C. 1.14.99.9)] to furnish (42b) or (44b), and subsequent cleavage of the C_2-side chain of the latter compounds (catalysed by a 17,20-lyase) to give dehydroepiandrosterone (45) or androst-4-ene-3,17-dione respectively. Both enzyme activities are believed to reside at the same active site of a cytochrome P-450 based hydroxylase (cytochrome P-450$_{c17}$). It was shown that the drugs ketoconazole and etomidate inhibited the biosynthesis of androstenedione from 17α-hydroxyprogesterone in a purified and reconstituted 17α-hydroxylase-17,20-lyase from pig testis.[81] Both activities of this enzyme were irreversibly destroyed, at the same rate, upon incubation with 17-bromoacetoxy-progesterone (44c); it was shown that the inactivation of the enzyme depended upon modification of an essential cysteine residue.[82] Evidence has been presented to show that the cytochrome P-450$_{c17}$ of human testis and adrenal are identical.[83]

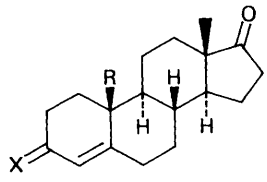
An alternative fat of pregnenolone in boar testis is the biosynthesis of androsta-5,16-dienol (46). The results of feeding



Scheme 8



(52)



(53) a; R = Et, X = O
b; R = MeCO, X = O
c; R = MeCH(OH), X = O
d; R = Me, X = CH_2
e; R = CHF_2, X = O

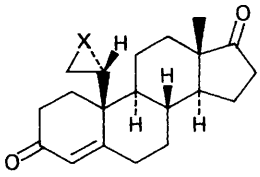
experiments with (16*R*)- and (16*S*)-[16-$^2H_1$]pregnenolone, with subsequent analysis of metabolites by GCMS, have revealed that the 16α-proton of pregnenolone is lost and that the 16β-proton is retained during the formation of the androstadienol (46).[84]

The biosynthesis of oestrogens from androgens remains an important topic of research, not least because of the involvement of oestrogens in the pathology of breast cancer. It is known that the biosynthesis of oestrone from androst-4-ene-3,17-dione (47) involves three sequential oxidative steps, each requiring molecular oxygen and NADPH, which are catalysed by the placental enzyme system 'aromatase' (Scheme 8). It was reported earlier that the first step in this sequence, namely hydroxylation of (47) to yield the primary alcohol (48), proceeds with retention of configuration at C-19.[47] Details have now been published of the syntheses of (19*R*)- and (19*S*)-3β-hydroxy[19-$^2H_1$, 19-$^3H_1$]androst-5-ene-17-ones,[85] and of the biosynthetic study, in which they were used as substrates for a crude enzyme system from human placentae.[86]

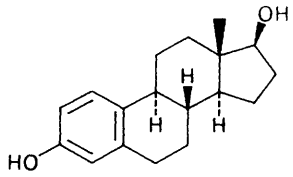
In the second oxidative step (Scheme 8) the 19(*pro*-19*R*) proton of (48) is replaced by a hydroxy-group, to furnish the geminal diol (49), and thence the aldehyde (50). In the course of the third oxidative step, the 19-carbon of the androgen is eliminated as formic acid to furnish oestrone (51). It was suggested earlier that the retention in formic acid of the 19(*pro*-19*S*) proton of (48), during the oxidation of the latter by aromatase, might be simply the result of a conformational preference of the substrate. However, the results of molecular mechanics calculations, performed on (48), revealed that conformational energy differences alone are insufficient to account for the observed stereoselectivity. Furthermore, it was shown that the unnatural substrate (52) was hydroxylated by aromatase with the same stereochemical result, despite the prediction of even greater conformational flexibility for the hydroxymethyl group in this compound.[87]

The unnatural androgens (53a), (53b), and (53c), were shown to be competitive inhibitors of aromatase in human placental microsomes.[88] Of these compounds, neither the 10-ethyl-steroid (53a) nor the 10-acetyl analogue (53b) were metabolized by aromatase (though both were reduced, in part, to their 17β-hydroxy-derivatives, owing to the intervention of a microsomal dehydrogenase). Surprisingly, both of the epimeric alcohols (53c) were oxidized by aromatase to the acetyl derivative (53b); the latter product was significantly labelled with ^{18}O (70%) when the (19*R*)-alcohol (53c) [but not its (19*S*)-epimer] was incubated with the same enzyme system in the presence of NADPH and $^{18}O_2$.[88] It was suggested in the same paper that the third monooxygenation step in the biosynthesis of oestrones is initiated by the abstraction of the 1β-hydrogen of the geminal diol (49), followed by homolytic cleavage of the C(10)-C(19) bond.[88] A chemical model for an alternative mechanism, suggested originally by Akhtar, has been tested with inconclusive results.[89]

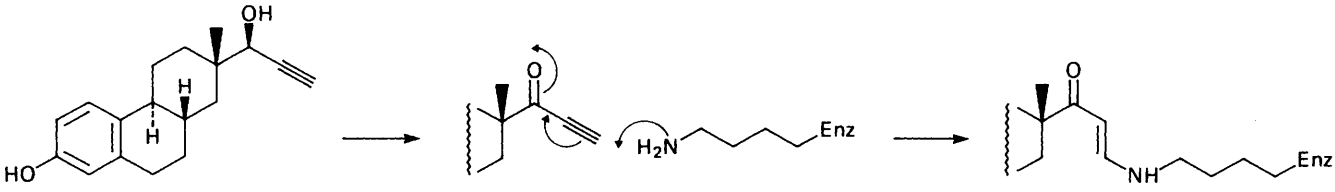
Interest continues in the purification and study of the cytochrome P-450 component (cytochrome P-450_{AROM}) of human placental aromatase. It has been claimed that a reconstituted aromatase, containing *inter alia* cytochrome P-450_{AROM} that had been purified by a combination of adsorption and affinity chromatography, converted [1,2-^3H]androst-4-ene-3,17-dione (47) into oestrone (51) with loss of the 1α- and 2β-protons.[90] This claim has been refuted by other workers, who have confirmed the generally accepted loss of the 1β- and 2β-protons of the same substrate during its conversion to oestrone by a purified and reconstituted aromatase system.[91] Other descriptions of the isolation and purification of cytochrome P-450_{AROM}, and of the preparation of reconstituted aromatase, have been published.[92] A model has been proposed for the active site of aromatase.[93] The amino-acid sequence of the *N*-terminal region of cytochrome P-450_{AROM} has been determined.[94]



(54) a; X = O
b; X = S

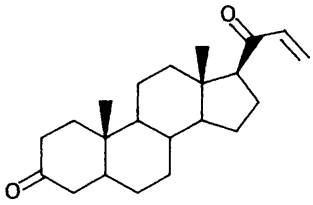


(55)

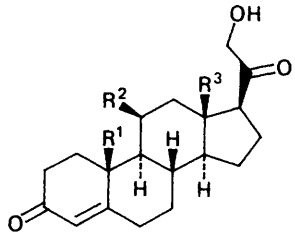


(56)

Scheme 9



(57)



(58) a; $R^1 = R^3 = Me$, $R^2 = OH$
b; $R^1 = R^3 = Me$, $R^2 = H$
c; $R^1 = Me$, $R^2 = H$, $R^3 = CH_2OH$
d; $R^1 = CH_2OH$, $R^2 = H$, $R^3 = Me$
e; $R^1 = CHO$, $R^2 = H$, $R^3 = Me$
f; $R^1 = Me$, $R^2 = OH$, $R^3 = CHO$

It has been reported that the 3-methylene-androstenone (53d) acted as a competitive inhibitor of aromatase, rather than as a substrate, which suggests that a 3-oxo function is essential for hydroxylation of an androgen by aromatase.[95] The 19,19-difluoroandrogen (53e), and its 4-hydroxy-derivative, were both less effective as inhibitors of aromatase than 4-hydroxyandrost-4-ene-3,17-dione.[96] The (19*R*)-oxiran (54a)[97] and the (19*R*)-thiiran (54b)[98] were both powerful competitive inhibitors of aromatase; in each series the (19*S*)-derivatives were less effective as inhibitors in the same assay.[99] Other sulphur-containing androgen analogues have been tested as inhibitors of aromatase.[100] The potential clinical significance of inhibitors of aromatase has been reviewed.[101]

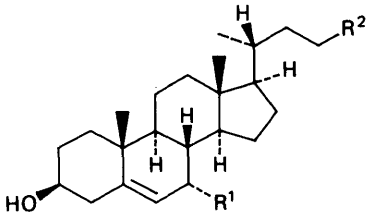
Oestradiol (55) is a far more potent oestrogen than is oestrone (51), and consequently the NAD^+-dependent enzyme oestradiol dehydrogenase [E.C. 1.1.1.62] is a potentially important target for inhibition in clinical practice. The acetylenic seco-oestradiol (56) is a potent mechanism-based inactivator for the purified oestradiol dehydrogenase of human placentae,[102] owing to Michael addition of the ε-amino group of a lysine residue to the ynone metabolite (Scheme 9).[103] Similarly the conjugated enone (57) was an effective and irreversible inhibitor of rat ovarian 20α-hydroxy-steroid dehydrogenase,[104] which catalyses the NADPH-dependent reduction of the 20-keto function of progesterone (44a).

The biosynthesis of corticosterone (58a) involves the hydroxylation of progesterone at carbons 11 and 21, catalysed by cytochrome P-450 dependent mixed function oxidases. The complete amino-acid sequence of the cytochrome p-450 component of the 21-hydroxylase, that was isolated from pig adrenal microsomes, has been reported.[105] 11-Deoxycorticosterone (58b) was converted to corticosterone (58a), and to its 18-hydroxy- (58c) and 19-hydroxy-derivatives (58d), when it was incubated with a purified, reconstituted 11β-hydroxylase; slow conversion of the latter metabolite to the derived aldehyde (58e) was also observed.[106] Two different forms of cytochrome $P\text{-}450_{11\beta}$ have been observed in the adrenal cortex of the rat; only one of these was capable of catalysing the final two steps in the biosynthesis of aldosterone (58f).[107] The cDNA encoding human[108] and bovine[108,109] cytochrome $P\text{-}450_{11\beta}$ has been sequenced.

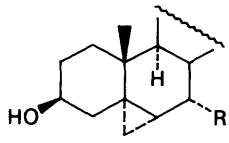
The role of cytochrome P-450-based enzymes in the biosynthesis of steroidal hormones has been reviewed.[110]

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

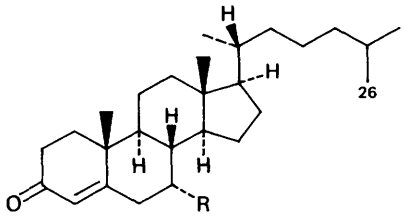
The hydroxylation of cholesterol to furnish 7α-hydroxycholesterol (59a) is normally regarded as the rate-limiting step in the biosynthesis of the bile acids. This transformation requires



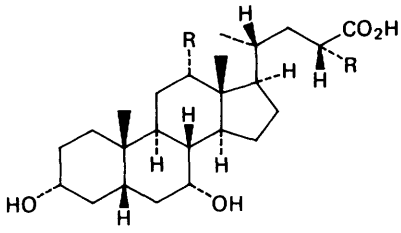
(59) a; R^1 = OH, R^2 = CH_2CHMe_2
b; R^1 = H, R^2 = CO_2H



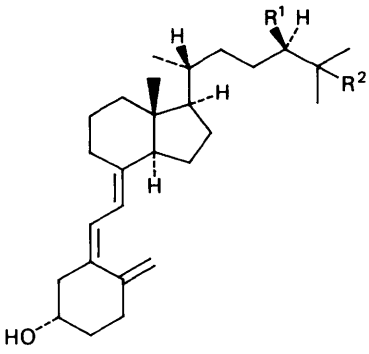
(60) a; R = H
b; R = OH



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



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



(63) a; R^1 = R^2 = H
b; R^1 = H, R^2 = OH
c; R^1 = R^2 = OH

NADPH and oxygen, and is catalysed by the enzyme cholesterol 7α-mono-oxygenase [E.C. 1.14.13.7]. The enzyme was inactivated by treatment with disulphides, in a liver microsomal preparation from rats that had been fed with cholestyramine. This inactivation was reversed by treatment with mercaptoethanol, which suggests that the enzyme contains a highly reactive, essential cysteine residue.[111] The cholestyramine-induced cytochrome P-450 component (mol. wt. 52000) has been isolated and purified; cholesterol 7α-mono-oxygenase activity was reconstituted by addition of NADPH-cytochrome P-450 reductase.[112] The reconstituted activity was highly specific for the 7α-hydroxylation of cholesterol and of 5α-cholestan-3β-ol. The cytochrome P-450 component of this enzyme has also been isolated and purified from the liver of untreated rabbits.[113] The addition of cytochrome b_5, as well as NADPH-cytochrome P-450 reductase, was necessary in the latter case for maximal reconstitution of the enzyme activity.

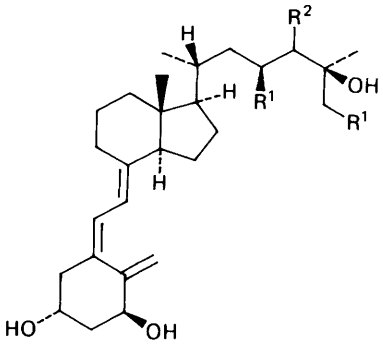
It was suggested earlier that the activity of cholesterol mono-oxygenase is modulated by reversible phosphorylation and that, in contrast to the case of HMG-CoA reductase, the active form of the enzyme is phosphorylated.[47] Much circumstantial evidence may be cited in favour of this postulate.[111,114] Furthermore, it has been shown that the activity of a reconstituted 7α-hydroxylase was inhibited by treatment with a phosphatase, and that the activity was partially restored by treatment with a protein kinase.[115] However, other workers have failed to confirm that the activity of the enzyme is modulated by reversible phosphorylation.[116]

Microsomes of cholestyramine-treated rats converted the 5α,6α-methanocholestanol (60a) into its 7α-hydroxy-derivative (60b).[117] The observation that compound (60a) did not inhibit the 7α-hydroxylase in a time-dependent manner was interpreted to mean that the hydroxylation does not proceed *via* a long-lived C-7 radical intermediate. Cholesterol-7α-mono-oxygenase has been comprehensively reviewed.[118]

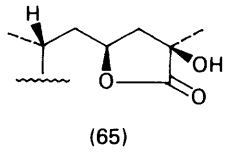
Further studies have been reported on the metabolism of steroids by clinical patients who were suffering from cerebrotendinous xanthomatosis, which is a rare disorder of cholesterol metabolism. Such patients displayed a reduced ability to hydroxylate 7α-hydroxycholestenone (61a) at C-26,[119] and excreted excessive amounts of 23-hydroxylated bile alcohols and bile acids, such as (23*R*)-23-hydroxycholic acid (62a).[120] Inhibition studies have been reported on the 12α-hydroxylase of rabbit liver microsomes.[121] The role of 'sterol carrier protein-2' (SCP-2) in the biosynthesis of bile acids has been investigated.[122] It was reported that SCP-2 caused a 2- to 3-fold stimulation of the 7α-hydroxylation of cholesterol, the 12α-hydroxylation of 5β-cholestane-3α,7α-diol, and the oxidation of cholest-5-ene-3β,7α-diol (59a) to the conjugated ketone (61a) in microsomes, but that the stimulatory effect was greatly reduced, or non-existent when the purified enzyme systems were assayed.[122] Assay procedures for the 7α- and 12α-hydroxylases of bile acid biosynthesis have been reviewed.[123]

It has been reported that rabbits, humans, and hamsters can metabolize 3β-hydroxychol-5-enoic acid (59b) to form chenodeoxycholic acid (62b).[124] An NADPH-dependent 7α-hydroxysteroid dehydrogenase has been isolated from rat liver microsomes and purified 160-fold.[125]

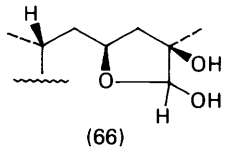
Critical steps in the metabolism of vitamin D_3 [calciol] (63a) are the hydroxylations at carbons 1 and 25 to furnish 1α,25-



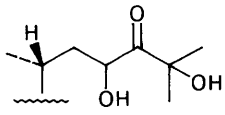
(64) a; $R^1 = R^2 = H$
b; $R^1 = OH$, $R^2 = H$
c; $R^1 = H$, $R^2 = OH$



(65)



(66)



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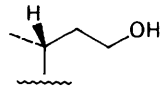
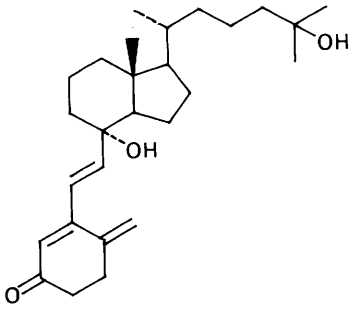
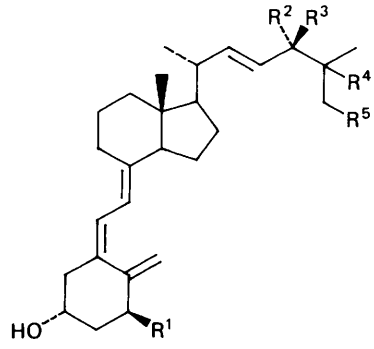


(68)



(69)



(70) a; $R^1 = R^3 = R^4 = OH$, $R^2 = Me$, $R^5 = H$
b; $R^1 = R^4 = OH$, $R^2 = Me$, $R^3 = R^5 = H$
c; $R^1 = R^5 = H$, $R^2 = Me$, $R^3 = R^4 = OH$
d; $R^1 = R^2 = R^5 = H$, $R^3 = Me$, $R^4 = OH$
e; $R^1 = R^4 = OH$, $R^2 = R^5 = H$, $R^3 = Me$
f; $R^1 = R^5 = H$, $R^2 = R^4 = OH$, $R^3 = Me$
g; $R^1 = R^4 = OH$, $R^2 = R^3 = H$, $R^5 = Me$

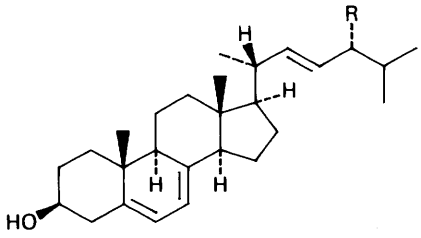
dihydroxyvitamin D_3 (64a), which is the active form of the vitamin. Details of the isolation and purification of the cytochrome P-450 component of the 25-hydroxylase have now been published.[126] The subcellular location and properties of the rat kidney 25-hydroxyvitamin D_3 1α-hydroxylase have been investigated.[127] On the basis of metabolic studies, that were conducted in chick kidney homogenates, it has been suggested that the biosynthesis of the γ-lactone (65) involves the stereospecific hydroxylation of (64a) at carbons 23 and 26, in either order, to give the tetrahydroxyvitamin D_3 (64b), which is further oxidized to the lactol (66) and thence to the lactone (65).[128] Other metabolites of 1α,25-dihydroxyvitamin D_3 (64a), in rat kidney, include the known tetrol (64c), the known ketone (67) and the new tetranor-vitamin D_3 derivative (68). It was also shown that the latter compound was formed during the metabolism of ketone (67).[129] The highly conjugated ketone (69) has been identified as a metabolite of 25-hydroxyvitamin D_3 in phagocytic mouse cells.[130] A 25-hydroxyvitamin D_3 24-hydroxylase, has been isolated from chick kidneys; enzymic activity was reconstituted by addition of ferredoxin and ferredoxin reductase in the presence of NADPH and oxygen.[131] The reconstituted hydroxylase converted 25-hydroxyvitamin D_3 (63b) into its (24*R*)-24-hydroxy-derivative (63c).

1,24,25-Trihydroxyvitamin D_2 (70a) has been isolated from bovine kidney homogenates that were incubated with 1,25-dihydroxyvitamin D_2 (70b), and from chick kidney homogenates that were incubated with 24,25-dihydroxyvitamin D_2 (70c).[132] Curious results have been reported in the 24-*epi* series.[133] Thus the diol (70d), upon incubation with a chick kidney homogenate, furnished the unexceptional triols (70e) and (70f), together with a metabolite that was tentatively identified as the *homo*vitamin D_3-derivative (70g). Further evidence for the latter structure, and for the implied 1,3-migration of a methyl group, is clearly desirable.[133]

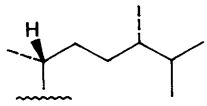
6 Steroids and Triterpenoids in Higher Plants, Algae, and Fungi

6.1 The Biosynthesis of Sterols in Fungi

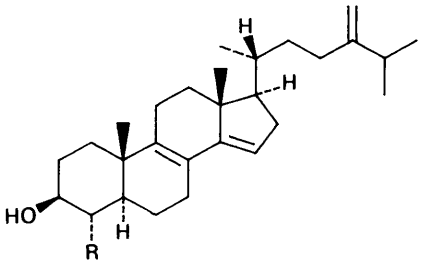
The first degradative step in the biosynthesis of ergosterol (71a) in the yeast *Saccharomyces cerevisiae* is the oxidative removal of the 14α-methyl group of lanosterol (19a) catalysed by a microsomal mono-oxygenase. The isolation and purification of the cytochrome P-450 component (cytochrome $P\text{-}450_{14DM}$) from yeast, and the reconstitution of 14α-demethylase activity by addition of NADPH-cytochrome P-450 reductase, was reported previously.[1] The reconstituted enzyme system catalysed the aerobic conversion of 32-hydroxydihydrolanosterol (37a) into the conjugated diene (39a) in a reaction that required two moles of NADPH per mole of the primary alcohol.[134] This observation provides further evidence that all



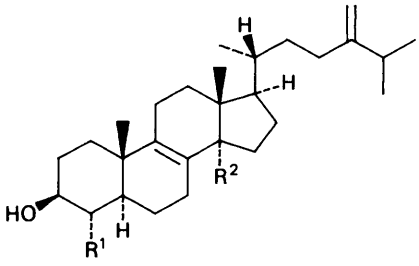
(71) a; R = Me
b; R = Et



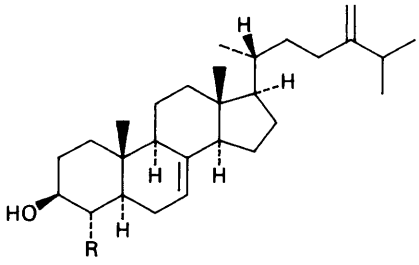
(72)



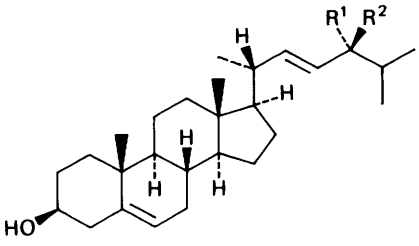
(73) a; R = H
b; R = Me



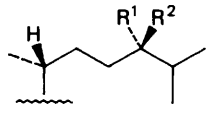
(74) a; $R^1 = R^2 = H$
b; $R^1 = R^2 = Me$
c; $R^1 = Me$, $R^2 = H$



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



(76) a; $R^1 = Me$, $R^2 = H$
b; $R^1 = H$, $R^2 = Et$



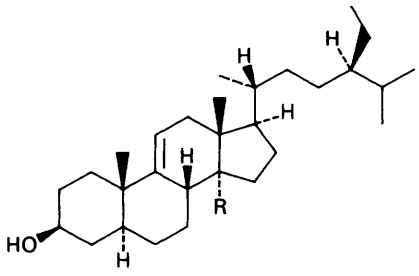
(77) a; $R^1 = Me$, $R^2 = H$
b; $R^1 = H$, $R^2 = Et$
c; $R^1 = H$, $R^2 = Me$

three steps in the conversion of the unnatural substrate dihydrolanosterol (36) into the diene (39a)—and by implication, all three steps in the conversion of the natural substrate lanosterol (19a) to the triene (39c)—are catalysed by the same mono-oxygenase. The conversions of both dihydrolanosterol (36) and its 32-hydroxy derivative (37a) into the conjugated diene (39a) were inhibited by low concentrations of ketoconazole.[134] It was shown that inactivation of the demethylase by azole antifungal agents, such as ketoconazole, occurs as a consequence of their interaction with the prosthetic group of cytochrome $P\text{-}450_{14DM}$.[135] The reconstituted demethylase was inhibited also by 7-oxo-24,25-dihydrolanosterol.[136]

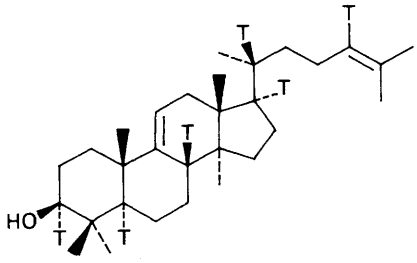
A modified version of cytochrome $P\text{-}450_{14DM}$, whose 14α-demethylase activity was *not* restored by addition of NADPH-cytochrome P-450 reductase, has been isolated from a mutant of *Saccharomyces cerevisiae* that was defective in the demethylation of lanosterol.[137] It was suggested that the imidazole group of a histidine residue in the defective protein occupied the axial coordination site, *trans* to the thiolate ligand, of the iron atom in the heme unit.[137] The base-sequence of the gene coding for cytochrome $P\text{-}450_{14DM}$, in the wild-type *Saccharomyces cerevisiae*, has been determined.[138] The gene for the same demethylase has been isolated from the yeast *Candida tropicalis*.[139]

Lanosterol (19a) was converted into 4,4-dimethylzymosterol (40d) when it was incubated with microsomes from *Saccharomyces cerevisiae* in the presence of potassium cyanide. It was observed that the trienol (39c) accumulated in place of 4,4-dimethylzymosterol (40d) when the drug AY-9944 was added to the reaction system. These observations confirm that the 14α-demethylation of lanosterol in yeast, as in mammals, requires the sequential operation of two enzymes namely the 14α-demethylase (cytochrome $P\text{-}450_{14DM}$), and a Δ^{14}-reductase which is sensitive to AY-9944.[140] Additional evidence has been published to support the earlier contention that the biosynthetic conversion of ergosta-5,7-dien-3b-ol (72) to ergosterol, by a microsomal enzyme system from the same yeast, involves a cytochrome P-450-based mono-oxygenase.[141] It was shown that ergosterol acquired the expected labelling pattern after incorporation of isotopic label from [2-$^{13}C_1$, 2-$^{2}H_3$]acetate in *S. cerevisiae*. In particular, the three 1,2-hydride shifts that occur during the biosynthesis of ergosterol, (from carbon 13 to 17, from carbon 17 to 20, and from carbon 24 to 25), were verified by observation of the associated β-deuterium isotope effects in the ^{13}C-NMR spectrum.[142]

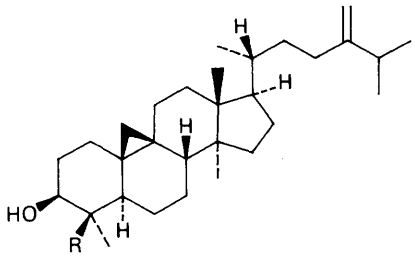
Further studies have been reported on the inhibition of sterol biosynthesis by fenpropimorph, fenpropidine, and tridemorph, in a cell-free enzyme system from *S. cerevisiae*. The former two fungicides were equally effective as inhibitors of the reduction of [^{14}C]ergosta-8,14,24(28)-trienol (73a) to the $\Delta^{8,24(28)}$-diene fecosterol (74a), while fenpropimorph was the best inhibitor of the isomerization of fecosterol to its Δ^{7}-isomer (75a).[143] It has been reported that the biosynthesis of ergosterol in yeast is regulated by feed-back inhibition of the enzymes acetoacetyl coenzyme A thiolase, and HMG-CoA synthase.[144] The biosynthesis of sterols from [U-^{14}C]acetate, [2-^{14}C]mevalonate, and [*methyl*-^{14}C]methionine was blocked by the antifungal agents tolnaftate and tolciclate in *Trichophyton mentagrophytes*, *Candida parapsilosis*, and *Candida albicans*. It was shown that the site of inhibition was the enzyme squalene epoxidase.[145] [2-^{14}C]Acetate was incorporated into ergosterol (71a), brassicasterol (76a), and 22,23-dihydrobrassicasterol (77a) by *Gibberella fujikuroi*.[146] It was shown that an external supply of each of these sterols was necessary for normal growth of the fungus, when the biosynthesis of sterols had been blocked by the inhibitor 2,3-iminosqualene. Accordingly, it was suggested that these sterols are end-products of biosynthesis in this



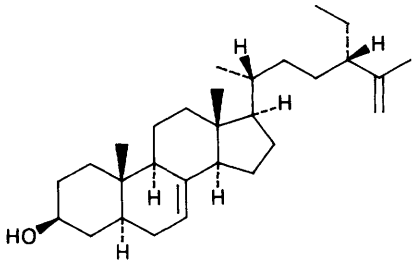
(78) a; R = H
b; R = Me



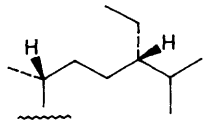
(79)



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



(81)



(82)

fungus, and are not interconvertible.[146] The biosynthesis of ergosterol has been reviewed, with particular reference to fungicides that inhibit the biosynthesis of sterols.[147]

It is generally believed that cycloartenol (22a) is the tetracyclic triterpenoid from which sterols are synthesized in photosynthetic plants and algae, whilst lanosterol serves the equivalent function in vertebrate animals and fungi. Cold-trap experiments have demonstrated that the Oomycete *Saprolegnia ferax* can incorporate radioactivity from [2-^{14}C]acetate into lanosterol, but not into cycloartenol. However, [2-^{3}H]cycloartenol was incorporated into 24,25-didehydropollinastanol (22b) by the same species.[148]

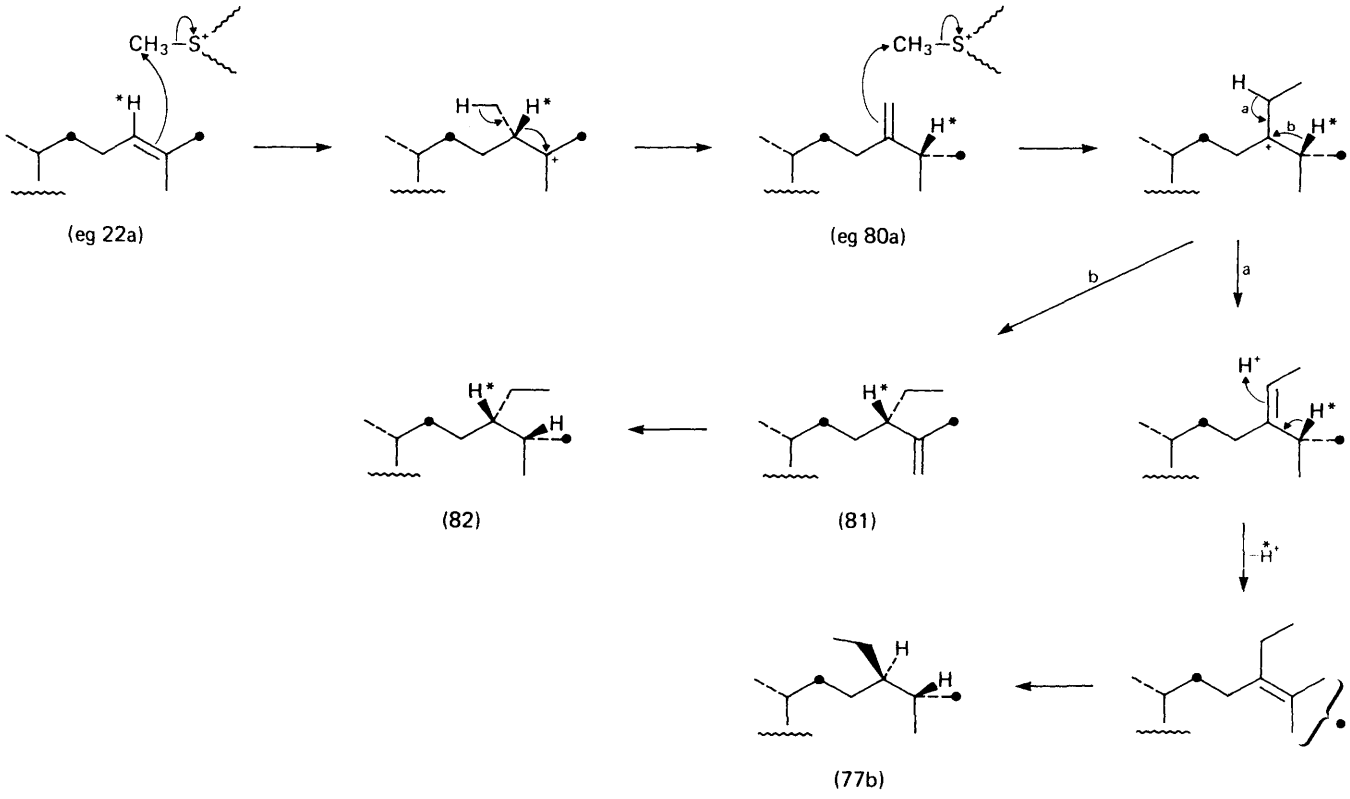
6.2 The Biosynthesis of Phytosterols

The biosynthesis of 5α-stigmast-9(11)-en-3β-ol (78a) in *Costus speciosus*, from [2-^{14}C, 4*R*-^{3}H]mevalonate of nominal ^{3}H/^{14}C ratio 1:1, has been reported.[149] The $\Delta^{9(11)}$-phytosterol had a normalized ^{3}H/^{14}C ratio of 0.996:1, which is consistent with the retention of five of the six tritium labels that must have been present in the intermediate squalene. Appropriate degradations established that there was no tritium attached to C-3, as expected, but that 1/5th of the tritium was located at C-8 (or C-7) of (78a), and must have migrated from C-9 of an intermediate tetracyclic triterpenoid carbocation. This observation was taken to mean that the presumed intermediate, lanosta-9(11),24-dienol (79), was formed directly from squalene epoxide (rather than *via* lanosterol), although the data do not appear to preclude initial formation of cycloartenol and subsequent opening of the cyclopropane ring to furnish a $\Delta^{9(11)}$-sterol.[149] However, the retention of tritium at C-8 of phytosterol (78a) is somewhat surprising, since a Δ^{8}- or Δ^{7}-double bond is normally required for the oxidative removal of the 14α-methyl group (see below). Indeed the isolation of a 14α-methyl-$\Delta^{9(11)}$-phytosterol (78b), from aerial parts of *Cucumis sativus*, underlines this point.[150]

The early stages in the normal route for the biosynthesis of phytosterols from squalene epoxide involves the sequence cycloartenol (22a), 24-methylenecycloartanol (80a), cycloeucalenol[151] (80b), obtusifoliol (74b), and thence to the $\Delta^{8,14,24(28)}$-trienol (73b), the 4α-methyl-$\Delta^{8,24(28)}$-dienol (74c), and 24-methylenelophenol (75b). Further evidence for this sequence has been gleaned from biosynthetic studies with a microsomal enzyme system from embryos of corn (*Zea mays*) which demethylated [28-^{3}H]obtusifoliol (74b) to furnish the $\Delta^{8,24(28)}$- and $\Delta^{7,24(28)}$-sterols (74c) and (75b) respectively, when supplemented with NADPH in the presence of air.[152] When the microsomal sterol-Δ^{14}-reductase was specifically inhibited by the *N*-oxide (28b) obtusifoliol was converted only to the $\Delta^{8,14,24(28)}$-triene (73b); it was shown that this demethylation reaction was brought about by a cytochrome P-450 dependent mono-oxygenase. Alternatively, if the amine (28a) was used to inhibit the sterol Δ^{8}-Δ^{7} isomerase, the $\Delta^{8,24(28)}$-sterol (74c) alone accumulated.[152] It is significant that no $\Delta^{8(14),24(28)}$-sterol intermediate was observed in these studies (*cf.* Section 5.1).

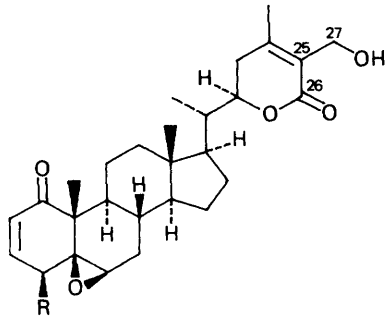
The bicyclic amine (28a), which was an efficient inhibitor of cycloartenol synthase (see Section 4) and of the sterol Δ^{8}-Δ^{7} isomerase (see above), also inhibited the cycloeucalenol–obtusifoliol isomerase of higher plants.[153] In particular, a suspension culture of bramble cells (*Rubus fruticosus*) or roots of maize seedlings (*Zea mays*) each accumulated 9,19-cyclosterols such as cycloeucalenol (both species) and 24-methylenecycloartanol (bramble cells) or 24-methylpollinastanol (maize) when grown in the presence of low concentrations of (28a).[153] The rationale behind the design of such inhibitors, which mimic sterically and electronically the transient carbocation-like intermediates of sterol biosynthesis, has been discussed in detail.[56,154] Structure-activity relationships in the inhibition of phytosterol biosynthesis by *N*-substituted morpholine derivatives have been investigated.[155] The growth of a cell suspension culture of celery (*Apium gravidens*), and the incorporation of [2-^{14}C]acetate into phytosterols, were both inhibited by the plant growth regulator paclobutrazol, owing to blocking of the enzyme obtusifoliol 14α-demethylase.[156] The incorporation of [2-^{14}C]acetate into phytosterols by a bramble cell culture was inhibited by treatment with 2-aza-2,3-dihydrosqualene (27a) or with the derived *N*-oxide (27b), leading to the accumulation of squalene epoxide; these aza-squalene derivatives also inhibited the sterol Δ^{8}-Δ^{7} isomerase of bramble cells.[157]

The sterol composition of genera of the family Cucurbitaceae is unusual in several respects. For example, the mature plants contain predominantly or exclusively sterols with the Δ^{7}-nucleus, whilst seeds of these plants contain also the 'normal' Δ^{5}-sterols.[158] In the case of *Cucurbita maxima*, it has been shown that the Δ^{5}-sterols disappear during germination, at a time when Δ^{7}-sterols are being actively synthesized.[159] Moreover, these genera furnish sterols which display a wide variety of side-chain structure; in particular, individual species contained substantial amounts of sterols of both the 24α-ethyl type (especially in the mature plants) and the epimeric 24β-ethyl series (particularly in the seeds).[160] The biosynthesis of the 24β-ethyl-sterols 22,23-dihydro-25,26-didehydrochondrillasterol (81) and 22,23-dihydrochondrillasterol (82), from [1,2-$^{13}C_2$]acetate and from [2-^{13}C, 2-$^{2}H_3$]acetate, has been studied in tissue cultures of *Trichosanthes kirilowii* var. *japonica* (Cucurbitaceae).[161] In the case of the latter precursor, retention or 1,2-migration of deuterium in the biosynthesized sterols was recognized by the appearance of appropriate α- or β-deuterium isotope shifts in the ^{13}C-NMR spectra that were recorded with



^{14}C-label from C-2 of mevalonate is shown as ●, and ^{2}H-label from H-24 of (22a) is shown as H*. All other ^{2}H-labels are ignored, for the sake of clarity

Scheme 10



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

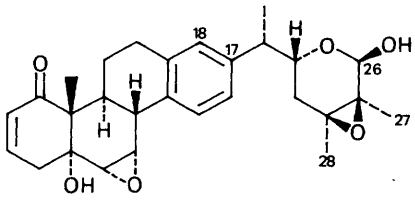
simultaneous decoupling of hydrogen and deuterium. The following observations were made, *inter alia*, for both sterols (81) and (82): (i) ^{2}H was retained at C-5, which precludes the formation of these Δ^{7}-sterols from their Δ^{5}-isomers; (ii) two deuterium labels only were retained at C-19, which is consistent with the role of cycloartenol as a precursor of these sterols; (iii) two deuterium labels were retained at C-22, which precludes the intermediacy of a Δ^{22}-sterol. Furthermore, ^{2}H was retained at C-24 of both sterols, but had migrated to C-25 in 24-methylenecycloartanol (80a) that was isolated also.[161] In a parallel experiment, [2-^{13}C, 2-$^{2}H_{3}$]acetate was incorporated into sitosterol (77b), by a tissue culture of *Rabdosia japonica*, with loss of the deuterium label that must have been present at C-24 of the cycloartenol precursor.[142] These observations may be accommodated mechanistically as summarized in Scheme 10, in which the biosynthetic origins recorded for the C-25 methyls of dihydrochondrillasterol (82)[162,163] and of sitosterol (77b)[162] are based on reassignments in the ^{13}C-NMR spectra that have been made outside of the period covered by this report.

The earlier claim[1] that [4-^{14}C]sitosterol (77b) was incorporated into stigmasterol (76b) in seedlings of barley (*Hordeum vulgare*) could not be confirmed by another group.[164] However, it has now been reported that [4-^{14}C]sitosterol is incorporated into stigmasterol, albeit in only 0.1% radiochemical yield, by leaves of *sorghum* (*Sorghum bicolor*).[165] The time-course of the incorporation of isotopic label from [2-^{14}C]mevalonate and [*methyl*-^{14}C]methionine, into sitosterol (77b) and into the epimeric 24-methylcholesterols (77a) and (77c), has been studied in shoots of *Zea mays*.[166] The results showed that the C_{29}-sterol was synthesized in maize at about 3 to 4 times the rate of synthesis of the epimeric C_{28}-sterols.

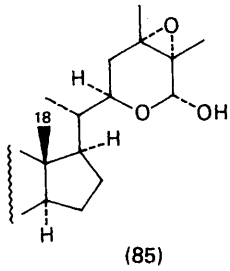
It has been shown that mevalonic acid can moderate the effect of inhibitors of sterol biosynthesis in plants *in vivo*.[167] For example, treatment of seedlings of *Medicago sativa* with a 30 μM solution of L-ethionine (which blocks transmethylation at C-24) led to the accumulation of cholesterol in the hypocotyls, which resulted in a reduced sterol concentration in the roots. However, when mevalonic acid (2 mM) was supplied together with the inhibitor, cycloartenol accumulated in the roots.[167] The biosynthesis of phytosterols has been reviewed.[168]

6.3 Further Metabolism of Steroids and Triterpenoids

Further degradations have been reported on withaferin A (83a) and jaborosalactone A (83b) that had been labelled biosynthetically following the feeding of [2-^{14}C]mevalonolactone to leaves of *Acnistus breviflorus*.[169, *cf.* 1] It was shown that only 1% of the ^{14}C-label in jaborosalactone A resided at C-26, whilst 6% of the isotopic label in withaferin A was found at C-26 and 17% at C-1. It was concluded that a major route to the biosynthesis of withanolides in this plant involves cleavage of the side-chain of the sterol precursor,[169] though other explanations appear to be possible.



(84)



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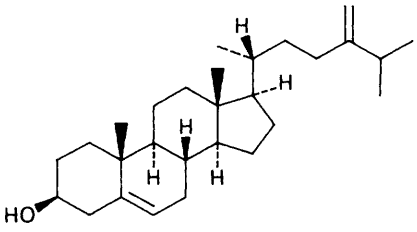
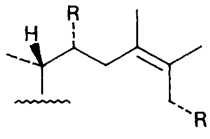
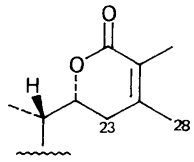


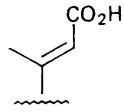
(86)



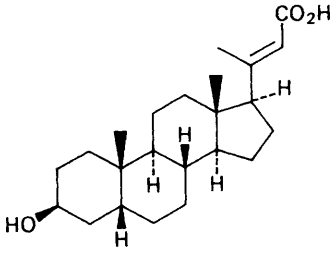
(87) a; R = H
b; R = OH



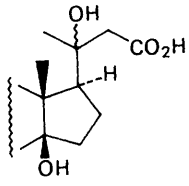
(88)



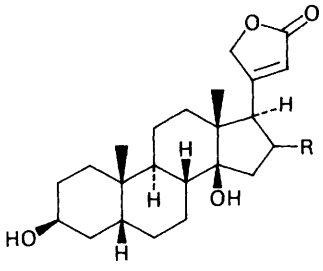
(89)



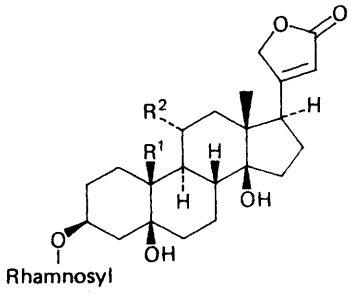
(90)



(91)



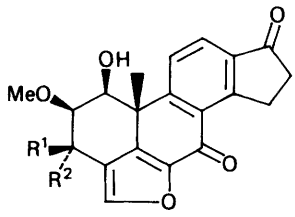
(92) a; R = H
b; R = OH



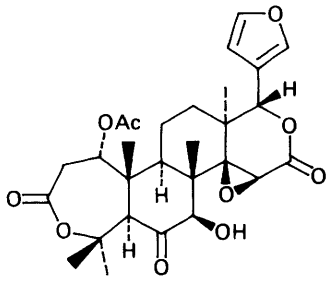
(93) a; R^1 = Me, R^2 = OH
b; R^1 = CH_2OH, R^2 = H
c; R^1 = CH_2OH, R^2 = OH
d; R^1 = CHO, R^2 = OH
e; R^1 = CHO, R^2 = H

The curious D-homo aromatic steroid Nic-1 (84) presumably arises from the more conventional withanolide Nic-3 (85) with which it co-occurs in the South American plant *Nicandra physaloides*. [*methyl*-^{14}C]Mevalonolactone showed a 0.1% incorporation into Nic-1, oxidative degradation of which furnished benzene-1,2,4-tricarboxylic acid, which contained 26% of the isotopic label. The proposition that the 13β-methyl group of a sterol precursor becomes the aromatic C-18 of Nic-1 was established by ^{2}H-NMR spectroscopy, on the same degradation product, following the incorporation of [*methyl*-$^2H_3$]mevalonate.[170] The steroids [28-^{14}C]24-methylenecholesterol (86), [28-^{14}C]24-methyldesmosterol (87a), the [23,28-^{3}H]diol (87b), and [23,28-^{3}H]lactone (88), were each incorporated into Nic-1, in low yield, by leaves of *Nicandra physaloides*.[171] However, it was noted that the lactone (88) could not be an obligatory intermediate in the biosynthesis of Nic-1 since the ^{2}H-NMR spectrum of Nic-1, that had incorporated [*methyl*-$^2H_3$]mevalonate, showed the presence of deuterium attached to carbons 18, 19, 21, and significantly 26.[171]

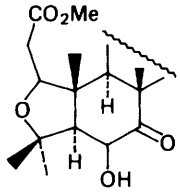
It is normally accepted that a late step in the biosynthesis of the cardenolides is the condensation of acetate with a highly functionalized 20-ketopregnane. It has now been shown that a range of norcholanic acid derivatives can serve as precursors of the glycosides digitoxin and gitoxin in leaves of *Digitalis purpurea*.[172] For example, the [23-^{14}C]norcholanic acid derivatives (89), (90), and (91) were each incorporated efficiently (0.5—3.0%) into both digitoxin and gitoxin. In each case the specificity of the incorporation was established by hydrolysis of the glycoside to furnish the aglycon [digitoxigenin (92a) and gitoxigenin (92b) respectively] which contained all of the isotopic label.[172] It has been reported that [21-^{14}C]3β-hydroxy-5β-pregn-8(14)-en-20-one was not incorporated into digitoxin, in leaves of *D. purpurea*, whilst in parallel experiments the unexceptional precursor [21-^{14}C]3β-hydroxy-5β-pregnan-20-one gave typical incorporations of 1%.[173] This negative result leaves open the question of the mechanism of introduction of the 14β-hydroxy group, of digitoxin, with apparent inversion of configuration. A number of biosynthetic interconversions have been demonstrated amongst the complex cardiac glycosides of *Convallaria majalis*. For example, the [U-^{14}C]glycosides lokundjoside (93a) and convallatoxol (93b) each furnished sarhamnoloside (93c) in leaves of the plant, whilst the latter was further oxidized to tholloside (93d).[174] [23-^{14}C]Digitoxigenin (92a) was incorporated by rhizomes of *C. majalis* into convallatoxol (93b) and convallatoxin (93e), amongst other products.[175]



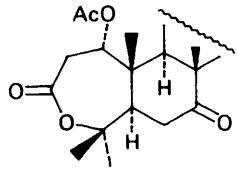
(94) a; $R^1, R^2 = O$
b; $R^1 = OH$, $R^2 = H$



(95)



(96)



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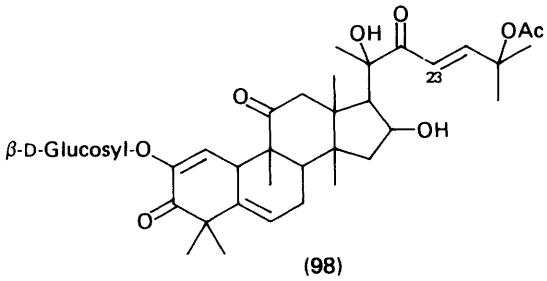

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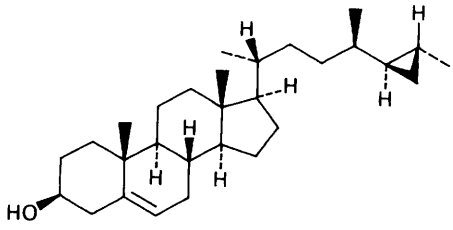
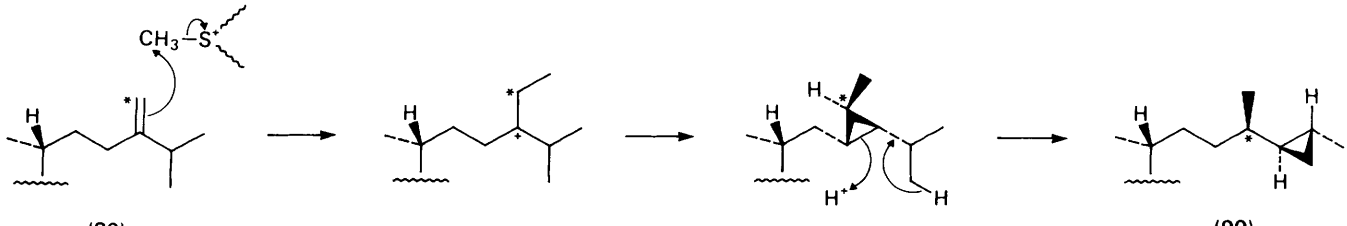
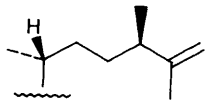


(99)

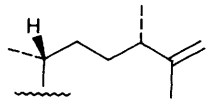


(86) (99)

Scheme 11



(100)



(101)

The highly oxidized fungal metabolite viridin (94a), that had been labelled biosynthetically by incorporation of [*methyl*-^{14}C]methionine, was reduced to viridiol (94b) by *Gliocladium virens*; since the reverse transformation did not occur, it was concluded that viridiol and not viridin is the end-product in this mould.[176]

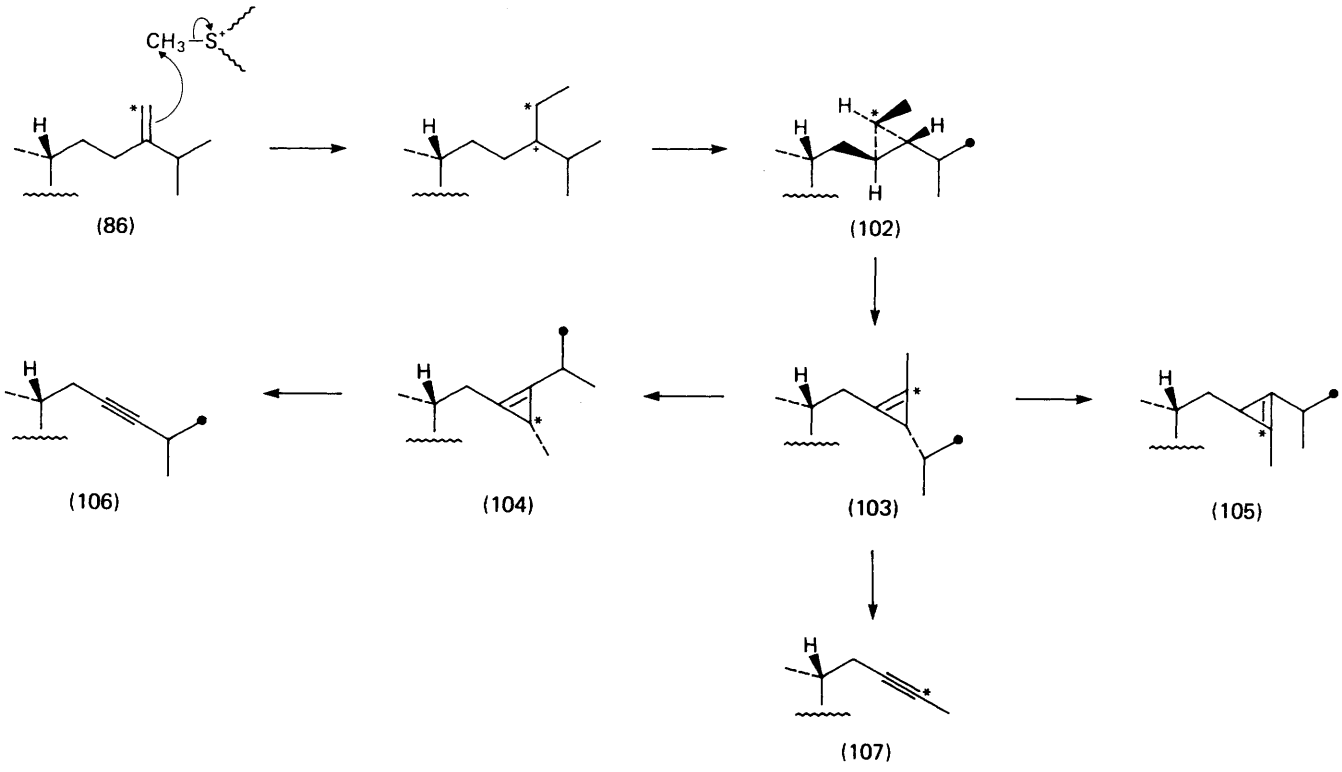
Studies have continued on the metabolism of limonoids in *Citrus limon*[177] and in the *Citrus* hybrid 'calamondin' (*i.e. Citrus reticulata* cv. Austera × Fortunella sp.).[178] 6-Keto-7β-nomilol (95) served as a precursor of isocyclocalamin (96) in the latter species.[179] The sites of biosynthesis of nomilin (97) have been identified in *Citrus limon*;[180] auxins were potent inhibitors of the biosynthesis of nomilin in seedlings of the same species.[181]

Further studies have been reported on the enzyme cucurbitacin Δ^{23}-reductase [E.C. 1.3.1.5], which catalyses the NADH- or NADPH-dependent reduction of cucurbitacin derivatives.[182] For example, elaterinide (which is the β-D-glucoside of cucurbitacin E) (98) is reduced to its 23,24-dihydro-derivative.

7 Steroids and Triterpenoids in Invertebrates

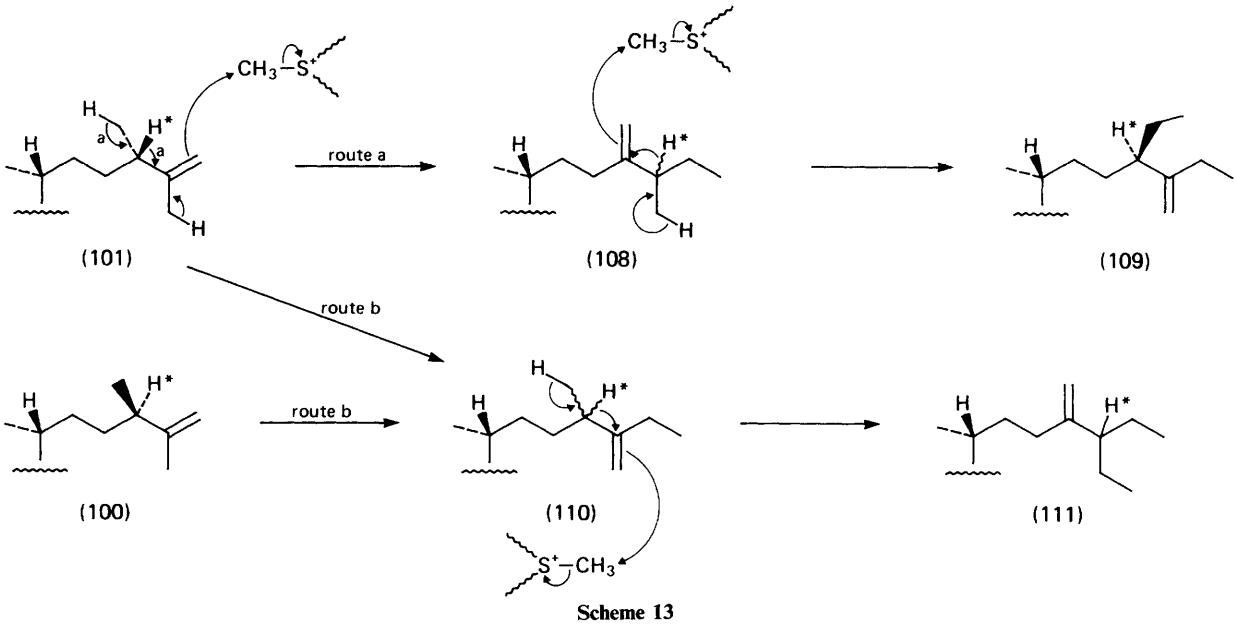
7.1 Marine Sponges

For many years marine invertebrates, particularly sponges, have furnished a plentiful supply of structurally interesting new steroids, particularly those with unusual, highly alkylated side-chains or with novel types of nucleus. Important and timely progress has been made in the elucidation of the details of the biosynthesis of some of these steroids, as summarized below. It was predicted that petrosterol (99), which is the major sterol of the sponge *Petrosia ficiformis*, was derived biosynthetically from epicodisterol (100). In the event, neither [26-^{14}C]epicodisterol (100) nor [26-^{14}C]codisterol (101) was incorporated into petrosterol in the same sponge; however, [28-



Labels * and ● represent the fates of C-28 of (86) and C-26 of (102) respectively. The steroid nucleus is that of cholesterol throughout

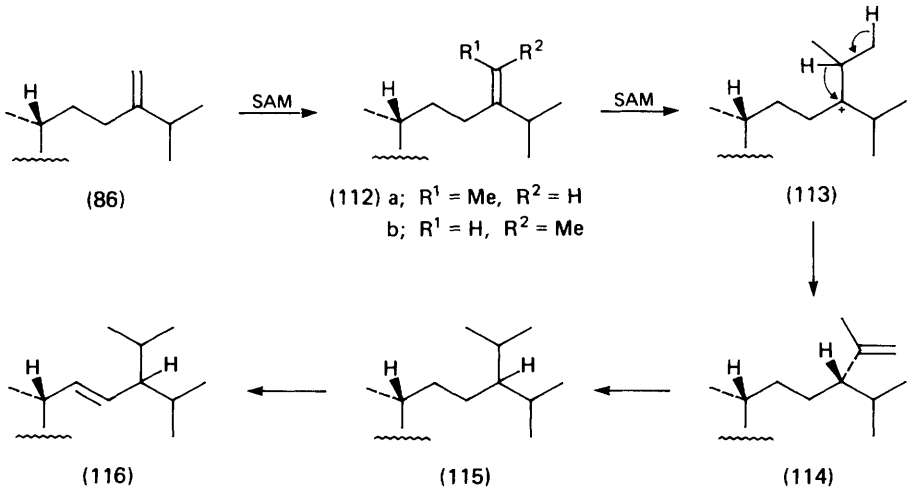
Scheme 12



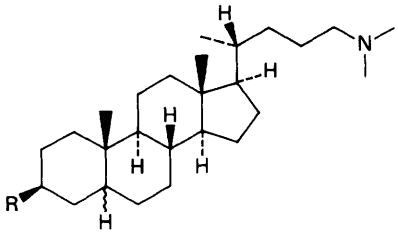
Scheme 13

^{14}C]24-methylenecholesterol (86) gave a 19% incorporation into the latter steroid.[183] Chemical degradation revealed that the isotopic label of the precursor was located at C-24 of petrosterol, which is consistent with the biosynthetic route summarized in Scheme 11.

The sponge *Calyx niceaensis* produces a wealth of fascinating sterols, which include the cyclopropane (23*S*, 24*S*, 28*R*)-dihydrocalysterol (102), the cyclopropenes 24*H*-isocalysterol (103), calysterol (104), and 23*H*-isocalysterol (105) (which together constitute up to 75% of the total steroids of this sponge), and the unique steroidal acetylenes (106) and (107). Each of these compounds, *except the acetylene* (*106*), became isotopically labelled when [28-^{14}C]24-methylenecholesterol was tested as a precursor in the same sponge.[184] Furthermore, each of the cyclopropene-containing sterols, and the acetylene (106) *but not the acetylene* (*107*), incorporated isotopic label when [26-^{3}H]-(23*S*,24*S*,28*R*)-dihydrocalysterol (102) was supplied to the sponge. These data are best accommodated by the biosynthetic route depicted in Scheme 12, which was supported also by the relative specific activities of the metabolites of each labelled precursor.[184] The differential retention of isotopic label in the two acetylenes provides evidence for the specificity of the incorporations. The formation of the original cyclopropane [*e.g.* (102)] by loss of a proton from a carbocation, and the acid-catalysed rearrangement of cyclopropanes in biosynthesis, were discussed in terms of



Scheme 14



(117) a; R = OH, Δ^5
b; R = H, 5αH
c; R = H, 5βH

protonated cyclopropanes as intermediates.[183,184] The latter description has been used to predict and correlate the biosynthesis of a number of marine sterols.[185]

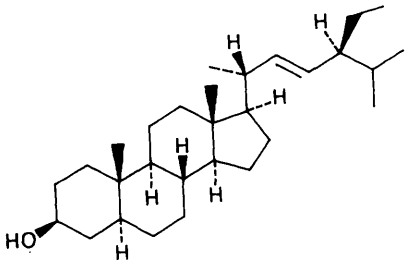
The biosynthesis of the triply alkylated sterols strongylosterol (109) and xestosterol (111) have been studied in the sponges *Strongylophora durissima*[186] and *Xestospongia testudinaria*[187] respectively. [26-^{14}C]Codisterol (101) [but not epicodisterol (100)] and the two 25-epimeric [6-^{3}H]-sterols (108) were efficiently incorporated into strongylosterol. When a mixture of [24-^{3}H]codisterol and [24-^{3}H]epicodisterol was supplied to *Strongylophora durissima*, the strongylosterol that was formed retained tritium at C-24, which is consistent with the double migration of the 24-hydrogen of codisterol that is depicted in Scheme 13a.[186] [26-^{14}C]Codisterol (101), [26-^{14}C]epicodisterol (100), and the mixed [27-^{14}C]-24-epimers (110) were each incorporated efficiently into xestosterol (111) in *Xestospongia testudinaria*. The incorporation of the mixture of [24-^{3}H]codisterol and [24-^{3}H]epicodisterol into xestosterol, and subsequent degradation of the latter, established that the predicted hydride migration from C-24 of (101) to C-25 of xestosterol does indeed occur (Scheme 13b).[187]

The sterols of the sponge *Pseudaxinyssa* sp. (Axinellida), which is found on the Australian Great Barrier Reef, consist virtually entirely of the 24-isopropyl-sterols (115) and (116), together with traces of the (24*S*)-24-isopropenyl-sterol (114).[188] In the course of biosynthetic studies it was shown that [28-^{14}C]-24-methylenecholesterol (86), [22-^{3}H]fucosterol (112a), and [22-^{3}H]isofucosterol (112b) were incorporated efficiently into the above three sterols in this sponge. The specificity of the incorporations of the first two precursors was established, in each case, by partial degradation of the biosynthetically-derived Δ^{22}-sterol (116). It was also reported that the natural [29-^{14}C]isopropenyl-sterol (114) gave higher incorporations into the 24-isopropyl-sterols (115) and (116) than did its 24-epimer. These data are consistent with the biosynthesis of sterols (114), (115), and (116) by the route that is summarized in Scheme 14, but without commitment as to whether fucosterol (112a) or isofucosterol (112b) is the true precursor.[188] It was further shown that the 24-isopropenyl-steroid (114) incorporated isotopic label when [26-^{3}H]-24-methylenecholesterol was supplied to the sponge, but that none of the label was found in the isopropenyl group of (114), and must therefore be located at C-26.[189] The reader will appreciate that the two isopropyl groups of the putative intermediate carbocation (113) are *equivalent*. Consequently, their different fates must reflect conformational constraints imposed by the enzyme on the carbocation.

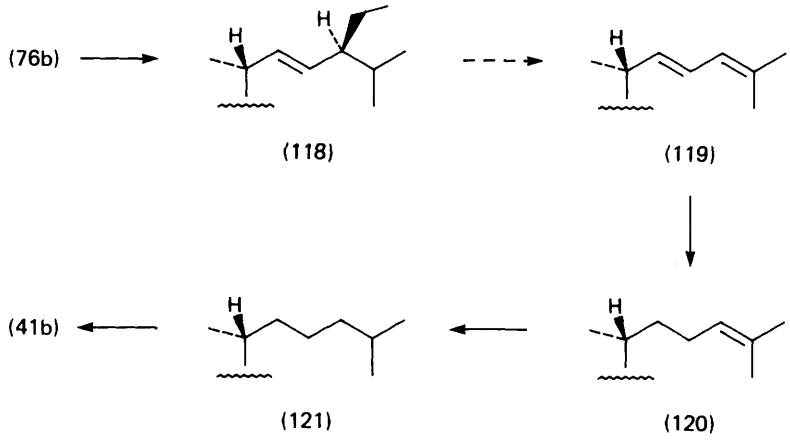
A second *Pseudaxinyssa* species, distinct from that discussed above, furnished the $\Delta^{5,7}$-sterols ergosterol (71a) and its 24β-ethyl homologue (71b) as the major sterols, together with smaller amounts of ergosta-5,7-diene-3β-ol (72). It was reported that [28-^{14}C]24-methylenecholesterol (86) and its [3-^{3}H]-$\Delta^{7,24(28)}$-isomer (75a) were both incorporated efficiently into (71b) and (72), and that [26-^{14}C]codisterol (101) was an excellent precursor of both ergosterol and ergosta-5,7-diene-3β-ol in this sponge.[190] [24-^{14}C]Desmosterol (3d) was incorporated into all three $\Delta^{5,7}$-sterols. It is clear from the foregoing that this *Pseudaxinyssa* species is capable not only of modifying the side-chain of appropriate precursor sterols, but also of converting a Δ^{5}- or Δ^{7}-steroid nucleus into the $\Delta^{5,7}$-diene system.[190] However, the ultimate source of the endogenous sterols that are modified by this sponge, and by those other sponges that are discussed in the paragraphs above, remains uncertain. The relatively low incorporations of [2-^{14}C]mevalonate into the mixed sterols of these sponges[186,188,190] suggest that these organisms are incapable of the efficient biosynthesis of sterols *de novo*. It is unclear at present whether symbiotic organisms provide the sponges with appropriate sterol precursors, or whether the latter are of dietary origin.

7.2 Insects

It is generally believed that insects are incapable of the biosynthesis of sterols *de novo*. The cholesterol requirement of several phytophagous insect species, both for essential membrane functions and for the biosynthesis of moulting hormones (the ecdysteroids), is satisfied by the dealkylation (at C-24) of dietary phytosterols. The penultimate step in the dealkylation sequence is the reduction of desmosterol (3d) to cholesterol (3a).[1,47] Typically, the tobacco army worm *Spodoptera litura*, which acts as a serious pest towards a wide range of crops, is able to degrade sitosterol (77b) and campesterol (77c) to cholesterol. The inclusion in the diet of 25-azacholesterol (117a) or 25-azacholestane (117b) led to a reduction in growth of the organism, accompanied by the accumulation of desmosterol and of undegraded phytosterols.[191]

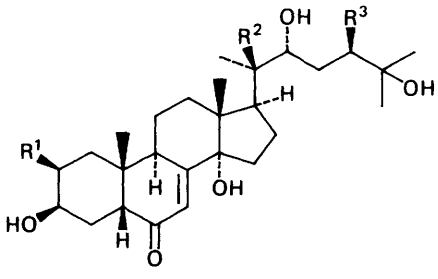


(118)

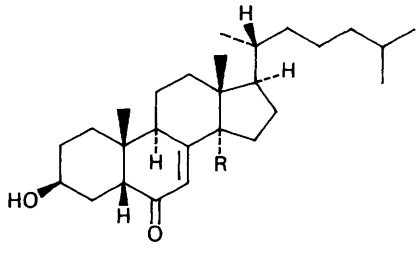


(118) (119) (120) (121) (41b) (76b)

Scheme 15



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



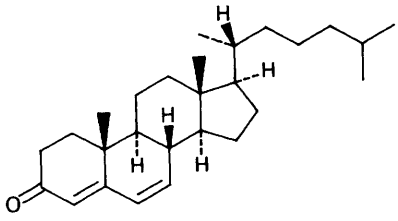
(123) a; R = OH
b; R = H

The Mexican bean beetle, *Ephilachna varivestis*, metabolizes dietary stigmasterol (76b) to 5α-cholestanol (121) [rather than to cholesterol] and to lesser quantities of Δ^7-cholestenol (41b). It was shown that inclusion of 25-azacholesterol or 25-azacoprostane (117c) in the diet reduced the conversion of [2,4-^{3}H]stigmasterol to cholestanol, and led to the accumulation of tritium-labelled Δ^{22}-stigmastenol (118), $\Delta^{22,24}$-cholestadienol (119), and Δ^{24}-cholestenol (120).[192] It was suggested that this organism degrades stigmasterol by the route summarized in Scheme 15.

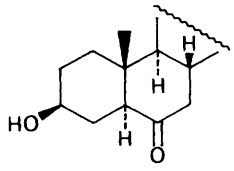
The honey bee *Apis mellifera*, and other hymenoptera,[193] is unable to convert phytosterols to cholesterol and the former species selectively accumulates 24-methylenecholesterol (86) from amongst dietary phytosterols.[194] Consequently, 25-azacoprostane and 25-azacholestane had no long-term effect on this species.[195] The C_{28}-ecdysteroid makisterone A (122a) has been isolated from ovaries of *Apis mellifera*.[196] [2,4-^{3}H]Campesterol (77c) was incorporated into makisterone A in a radiochemical yield of 0.045%; neither sitosterol (77b) nor 24-methylenecholesterol (86) acted as a precursor of ecdysteroids.[197] [4-^{14}C]Cholesterol was incorporated (0.02%) into a metabolite which was tentatively identified as 20-hydroxyecdysone (122b).

Wheat seedlings (*Triticum sativum*) that had germinated in the presence of fenpropimorph (see Section 6.1) accumulated 9β,19-cyclo-sterols in place of the normal Δ^5-sterols; adult females of the locust *Locusta migratoria*, that had been reared on these seedlings, showed a dramatic decrease in cholesterol content and their eggs contained only 20% of the normal concentration of ecdysteroids.[198] It was concluded that 9β,19-cyclo-steroids cannot act as precursors for the biosynthesis of ecdysteroids.

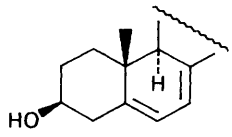
Despite intensive study, the early stages in the biosynthesis of ecdysone (122c) from cholesterol remain poorly understood, and the first undisputed intermediate is the 5β-ketodiol (123a).[199] None of the potential precursors cholest-4-en-3-one



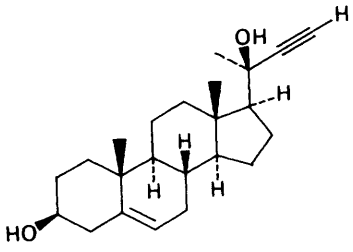
(124)



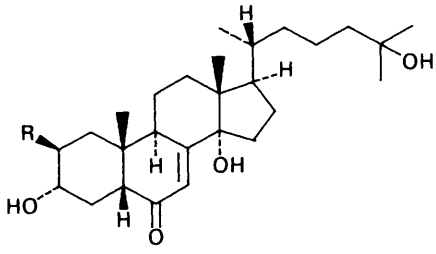
(125)



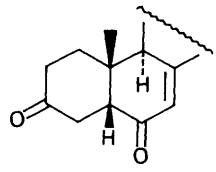
(126)



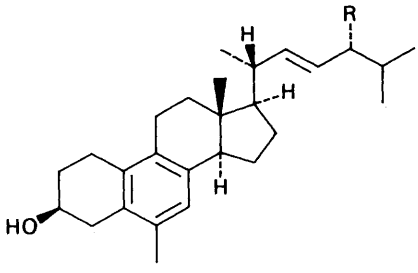
(127)



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



(129)



(130) a; R = Me
b; R = Et

(61b), cholest-4,6-dien-3-one (124), and 5α-cholestan-6-one-3β-ol (125), each labelled with ^{14}C at C-4, was incorporated into ecdysteroids by the desert locust *Schistocerca gregaria*.[200] However, [4-^{14}C, 7-^{3}H]cholesta-5,7-dienol (126) was incorporated into ecdysone and 20-hydroxyecdysone, by pupae of *Spodoptera littoralis*, without change in the $^{3}H/^{14}C$ atomic ratio. The observed retention of tritium was taken to mean that the conjugated diene was a genuine intermediate between cholesterol and ecdysteroids. It was claimed that if the diene had first been reduced to cholesterol, which had then been incorporated into these ecdysteroids, complete loss of tritium would have occurred. This prediction was based on the knowledge that the 7β-proton of cholesterol is lost during the biosynthesis of ecdysone, and on the *assumption* that the reduction of the diene would have occurred, in this insect, with the same stereochemistry as that which had been established for the biosynthesis of cholesterol in vertebrates. Loss of about half of the tritium label occurred during the biosynthesis of ecdysone and 2-deoxyecdysone from the same labelled diene in *Schistocerca gregaria*.[200]

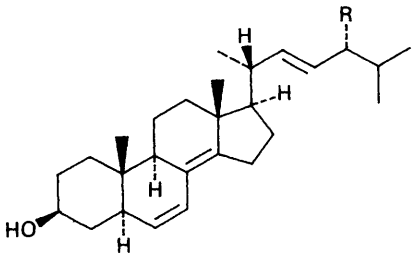
The ketol 3β-hydroxy-5β-cholest-7-en-6-one (123b) had been isolated earlier from ovaries and eggs of locusts. However, tissues of *Locusta migratoria* were unable to incorporate the [22,23,24,25-^{3}H]-ketol into ecdysteroids; the 14α-hydroxylation that sometimes occurred was shown to be a non-enzymatic reaction.[201] A series of analogues of cholesterol, containing a 22-yne moiety, have been tested as suicide inhibitors of the 22-hydroxylation of precursors of ecdysone.[202] The best inhibitor tested was the acetylene (127) which inhibited the incorporation of tritium from [22,23-^{3}H]-2,22-dideoxyecdysone into ecdysone, by prothoracic glands of *Locusta migratoria*, while not affecting the 2-hydroxylation of 2-deoxyecdysone.

The capacity of various tissues of larvae and adults of *Locusta migratoria* to incorporate [23,24-^{3}H]-2-deoxyecdysone (122d) into ecdysone has been studied.[203] The 2-hydroxylase has been solubilized and studied *in vitro*. Although the hydroxylase appeared to be a cytochrome P-450-based mono-oxygenase, its ability to hydroxyate 2-deoxyecdysone was not inhibited by carbon monoxide.[203] Eggs of the locust were able to incorporate the same precursor into ecdysone and 3-*epi*-2-deoxyecdysone (128a).[204] It has been shown that the 3-ketone (129) is an intermediate in the epimerization of ecdysone in a cell-free enzyme system from the cotton leaf worm *Spodoptera littoralis*.[205] The results of these studies suggested that there are three enzymes involved in the interconversions between ecdysone and 3-*epi*-ecdysone (128b), namely the oxygen-dependent ecdysone oxidase [E.C. 1.1.3.16], an NADPH-dependent 3-dehydroecdysone reductase (3α-hydroxyl-forming), and an NADPH- or NADH-requiring 3-deoxyecdysone reductase (3β-hydroxyl-forming).[205] It is normally considered that 3-*epi*-ecdysone is hormonally inactive, and that 20-hydroxyecdysone is the true, active moulting hormone. The enzyme ecdysone 20-mono-oxygenase [E.C. 1.14.99.22] has been studied in larvae of *Drosophila melanogaster*[206] and in *Pieris brassicae*.[207]

[4-^{14}C]Cholesterol was incorporated into 26-hydroxyecdysone 26-phosphate by female pupae of the tobacco hornworm *Manduca sexta*.[208] The concentration of the 26-phosphate falls during embryogenesis in eggs of the latter species and a new metabolite, namely 26-hydroxyecdysone 22-glucoside, appears.[209] 26-Hydroxyecdysone 2-phosphate has also been identified as a metabolite of ecdysone in eggs and ovaries of *M. sexta*.[210] The metabolism of ecdysone and of 20-hydroxyecdysone has been studied also in larvae of *Pieris brassicae*.[211]

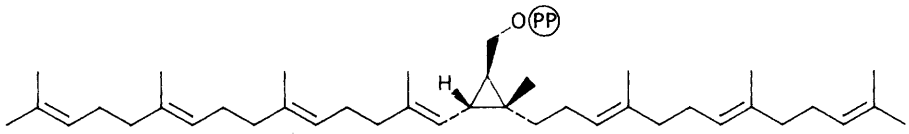
7.3 Other Invertebrates

The main sterols of the soil amoeba *Acanthamoeba polyphaga* are ergosterol (71a) and the homologue (71b), which co-occur with traces of poriferasterol (3c) and the unusual aromatic sterols (130a) and (130b).[212] It was shown earlier that these sterols are biosynthesized *via* cycloartenol (22a), despite the fact that this protozoon is not a photosynthetic organism.[1] The amoeba could be adapted to growth in the presence of high concentrations of the fungicide tridemorph. Under these

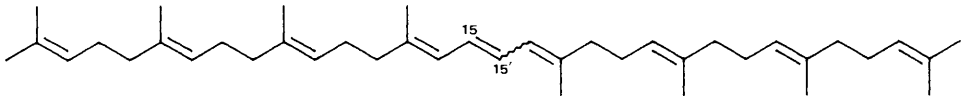


(131) a; R = Me
b; R = Et

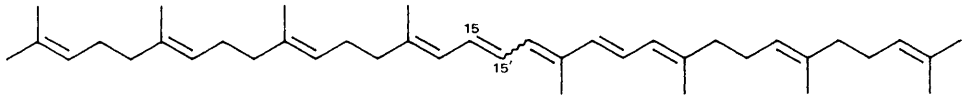
conditions the isomerization of cycloeucalenol (80b) to obtusifoliol (74b) was partially inhibited. Furthermore, the conversion of Δ^8-sterols to their Δ^7-isomers was completely blocked, as judged by the accumulation of Δ^8-sterols, particularly the unusual $\Delta^{6,8,22}$-trienols (131a) and (131b).[213] The related soil-borne amoebae *Naegleria lovaniensis* and *Naegleria gruberi* also contained cycloartenol, accompanied in the former species by lanosterol (19a) and parkeol (79).[214] Ergosterol was the main sterol but was accompanied by unusually high concentrations of 4α-methyl-sterols in both species. *De novo* biosynthesis of sterols from [1-^{14}C]acetate was demonstrated in *N. lovaniensis*, and a cell-free enzyme system from the latter amoeba incorporated [3-^{3}H]squalene epoxide into cycloartenol.[214] In contrast to the results described above, the protozoa *Crithidia fasciculata*[215] and *Trypanosoma cruzi*[216]



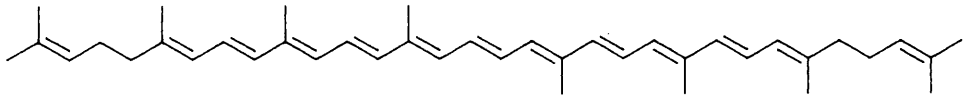
(132)



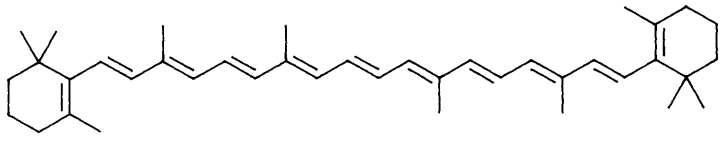
(133) a; 15,15′-*cis*
b; 15,15′-*trans*



(134) a; 15,15′-*cis*
b; 15,15′-*trans*

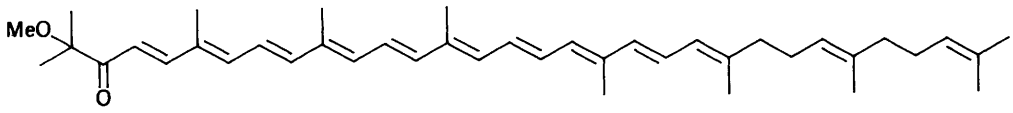


(135)

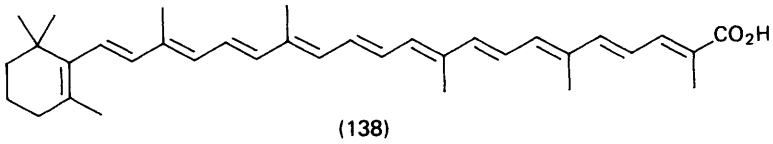


(136)

Scheme 16



(137)



(138)

both contained lanosterol rather than cycloartenol. It was reported that [1-^{14}C]acetate was incorporated into ergosterol *via* lanosterol in the former species,[215] and that ketoconazole blocked the incorporation of [2-^{14}C]mevalonate into sterols, in the latter species, leading to the accumulation of lanosterol and 24-methylenedihydrolanosterol.[216]

Further studies have been reported on the metabolism of sterols in the free-living nematodes *Caenorhabditis elegans*,[217,218] *Panagrellus redivivus*,[218] and *Turbatrix aceti*.[218] Ecdysone (122c) was metabolized to its 25-*O*-β-D-glucoside by the parasitic nematode *Parascaris equorum*.[219]

8 The Biosynthesis of Carotenoids

The biosynthesis of carotenoids in higher plants proceeds *via* prephytoene diphosphate (132) and *cis*-phytoene (133a). A series of dehydrogenations follow, with notable intermediates being *cis*-phytofluene (134a), *trans*-phytofluene (134b), and lycopene [ψ,ψ-carotene] (135), which may be cyclized to furnish β-carotene [β,β-carotene] (136) (Scheme 16). The conversion of isopentenyl diphosphate and prephytoene diphosphate into *cis*-phytoene has been demonstrated in the stromal fractions of chloroplasts, etioplasts, and amyloplasts of higher plants.[220] The biosynthesis of carotenoids in several species was inhibited by the herbicide FMC 57020,[221] and the formation of *cis*-phytoene in an enzyme system from chromoplasts of *Capsicum annuum* was inhibited by phenethyl diphosphate derivatives.[222] In both studies the sites of inhibition were identified as the enzymes isopentenyl diphosphate Δ-isomerase and prenyl transferase. The effects of other inhibitors on the biosynthesis of carotenoids have been studied in fruits of *Capsicum annuum*.[223]

The membrane-bound enzyme system that catalyses the biosynthesis of β-carotene (136) from *cis*-phytoene (133a) in chromoplasts of the daffodil *Narcissus pseudonarcissus* has been solubilized with the aid of a zwitterionic detergent.[224] The ability of this enzyme system to convert *cis*-phytoene into β-carotene was regained upon reconstitution of the soluble proteins into liposomes. *cis*-Phytofluene (134a) was the only intermediate that was detected between phytoene and β-carotene. Furthermore, any *cis*-phytofluene that was released was only poorly incorporated into β-carotene. It was suggested that the reconstituted enzyme system acted as an 'assembly line' with only limited access for exogenous precursors.[224] The efficient incorporation of [15,15′-^{3}H]lycopene (135) into β-carotene, by a soluble enzyme system prepared from chloroplast membranes of *Capsicum* fruits, has been reported.[225] The crude cyclase contained an essential thiol group and was inhibited by longchain tertiary amines. The time-course of the biosynthesis of β-carotene and its C_{40}-precursors from isopentenyl diphosphate has been studied in cotyledons of mustard seedlings (*Sinapsis alba*).[226] The biosynthetic relationships between the oxygenated carotenoids of the green alga *Chlorococcales* have been discussed.[227] The biosynthesis of carotenoids in higher plants has been reviewed.[228]

A photosynthetic membrane preparation of the cyanobacterium *Aphanocapsa* is one of the most efficient systems for the study of carotenoid biosynthesis *in vitro*. A soluble enzyme system, that catalyses the incorporation of [^{14}C]phytoene and [^{14}C]lycopene into β-carotene, has now been prepared from these membranes.[229] Under aerobic growth conditions the photosynthetic bacterium *Rhodopseudomonas capsulata* furnishes predominantly the acyclic carotenoid spheroidenone (137), which is formed *via trans*-phytoene (133b) and *trans*-phytofluene (134b). The role of several genes in the biosynthesis of carotenoids in this bacterium was reported earlier.[230] Further work with mutants that do not synthesize coloured carotenoids have shown that the gene 'crt I' controls the dehydrogenation of phytoene to furnish phytofluene.[231]

[2-^{14}C]Mevalonate was incorporated into phytoene, lycopene, and β-carotene by a cell-free enzyme system from *Aspergillus giganteus* mut. *alba* that had been grown in the light.[232] The cytosolic fraction contained phytoene synthetase activity whilst the enzymes that catalysed the conversion of phytoene into β-carotene were located in the microsomal fraction. The orange mutant SG22 of *Gibberella fujikuroi* produces large amounts of carotenoids such as neurosporaxanthin (138). The biosynthesis of carotenoids was inhibited by β-ionone and diphenylamine, but many other compounds that stimulated or inhibited the formation of carotenoids in other species had no effect on this mutant.[233] A green strain (S442) of the fungus *Phycomyces blakesleeanus* furnished phytoene, and smaller amounts of phytofluene. It was suggested that this strain represents a new mutation at the carB gene, which controls the dehydrogenation of phytoene to give lycopene in this species.[234]

The metabolism of oxygenated carotenoids in the yolk of hen's eggs has been discussed.[235]

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