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Biochemistry of Plant Secondary Metabolism: Second Edition Edited by Michael Wink
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Biochemistry of Plant Secondary Metabolism

Second Edition

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Chapter 5

BIOCHEMISTRY OF TERPENOIDS: MONOTERPENES, SESQUITERPENES AND DITERPENES*

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Abstract: Terpenoids represent the largest class of secondary metabolites and usually do not contain nitrogen or sulfur in their structures. Many terpenoids serve as defence compounds against microbes and herbivores and/or are signal molecules to attract pollinating insects, fruit-dispersing animals or predators which can destroy insect herbivores. As a consequence, many terpenoids have pronounced pharmacological activities and are therefore interesting for medicine and biotechnology. The first part of the biosynthesis is the generation of a C5 unit, such as isopentenyl diphosphate (IPP) or dimethylallyl diphosphate (DMAPP). Two independent pathways have been discovered that can produce the C5 unit: the mevalonate and the methylerythritol phosphate (MEP) pathway. Depending on the number of C5 units, we distinguish hemiterpenes C5, monoterpenes including iridoids (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (including steroids) (C30), tetraterpenes (C40) and polyterpenes (>C40). The biosynthesis (including enzymes, genes and their regulation) of mevalonate and the methylerythritol phosphate pathway and the consecutive pathways leading to mono-, sesqui- and diterpenes are discussed in this chapter in detail.

Keywords: biosynthesis; genes; monoterpenes; sesquiterpenes; diterpenes; mevalonate pathway; methylerythritol phosphate pathway

* This chapter is an update from an earlier version from J. Gershenzon and W. Kreis printed in the first edition in 1999.

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5.1 Introduction

The largest class of plant secondary metabolites is undoubtedly that of the terpenoids or isoprenoids. Over 36 000 individual members of this class have been reported (Buckingham, 2007) and new structures are currently being added at the rate of about 1000 every year. Compilations of newly described terpenoids appear periodically in *Natural Product Reports* (e.g. Grayson, 2000; Hanson, 2005; Connolly and Hill, 2008; Fraga, 2008). Terpenoids are not only numerous, but also extremely variable in structure, exhibiting hundreds of different carbon skeletons and a large assortment of functional groups. In spite of such diversity, all terpenoids are unified by a common mode of biosynthesis: the fusion of C_5 units with an isopentenoid structure.

Since the origins of organic chemistry, terpenoids have been a source of fascination for many practitioners of this discipline. However, the basic structural unity of terpenoids has only been appreciated since the end of the past century, when pioneers, such as the German Otto Wallach, discovered that some members of this class could be pyrolized to give isoprene, a C_5 diene with an isopentenoid skeleton (Fig. 5.1). These studies gave rise to the so-called isoprene rule, which states that all terpenoids are derived from the ordered, head-to-tail joining of isoprene units. More recent workers have refined the original concept, recognizing that non-head-to-tail condensations of isoprene units also occur in a few secondary metabolites (pyrethrins) which seem to be limited almost exclusively to members of family Asteraceae. Substantial structural rearrangements or loss of carbons during biosynthesis have been observed (Dewick, 2002). Nevertheless, the original isoprene rule was a very useful concept in determining the structures of many unknown substances and assessing their biogenetic origin. In this context, terpenoids have frequently been referred to as isoprenoids, and the terms isoprenoids, terpenoids and terpenes are now used interchangeably.

The classification of terpenoids is based on the number of isoprenoid units present in their structure. The largest categories are those made up of compounds with two isoprenoid units (monoterpenes), three isoprenoid units (sesquiterpenes), four isoprenoid units (diterpenes), five isoprenoid units (sesterterpenes), six isoprenoid units (triterpenes) and eight isoprenoid units (tetraterpenes) (Table 5.1). Although the biosynthesis is based on a unit of five-carbon atoms terpenoid nomenclature is based on a unit of ten carbon atoms since the C_{10} terpenoids were once thought to be the smallest naturally occurring representatives of this class. Designation of the C_{10} terpenoids as mono-('one')-terpenes made it necessary to name the subsequently described C_5 terpenes as hemi-('half')-terpenes, the C_{15} terpenes as sesqui-('one-and-a-half')-terpenes and so on. In this section, the biosynthesis and functional significance of the lower (C_5–C_{20}) terpenes are surveyed, with emphasis on the major advances in the past five years. Triterpenes (C_{30}), cardiac glycosides and steroid saponins are treated in Chapter 6. Relevant monographs of outstanding coverage and quality (Cane, 1998; Leeper

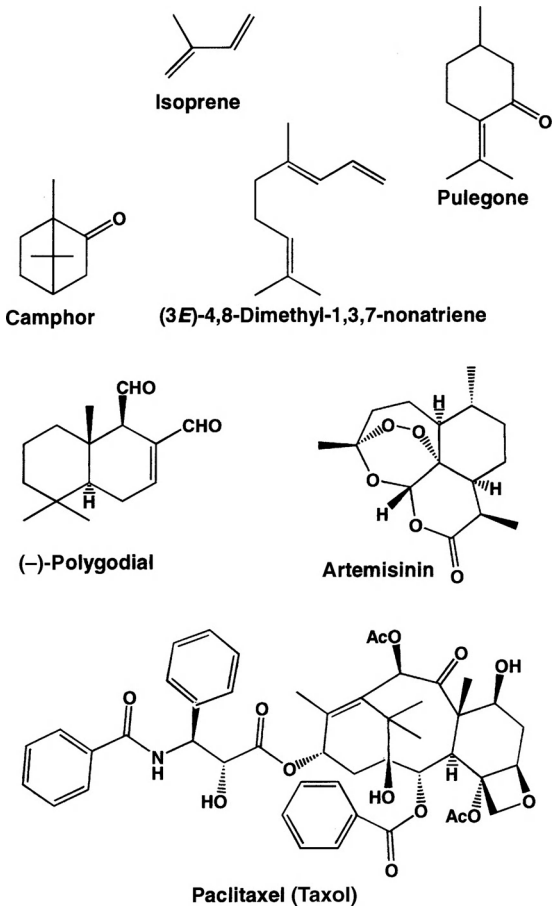

Figure 5.1 Examples of terpenoids that are of commercial importance or whose functional role in plants has recently been investigated. Isoprene may stabilize membranes at high temperatures. Camphor, artemisinin and paclitaxel (taxol) are valuable pharmaceuticals. The other three compounds appear to be involved in plant defence: pulegone is toxic to herbivores; polygodial is a herbivore feeding deterrent; and (3*E*)-4,8-dimethyl-1,3,7-nonatriene, a C_{11} homoterpene, functions to attract herbivore enemies to herbivore-damaged plants.

and Vederas 2000) and several excellent individual reviews (Chappell, 1995; McCaskill and Croteau, 1997) have covered many aspects of this subject.

5.2 Function

The enormous structural diversity of the terpenoids is almost matched by their functional variability. Terpenoids have well-established roles in almost all basic plant processes, including growth, development, reproduction and

Table 5.1 The classification of terpenoids is based on the number of C_5 isoprenoid units in their structures

Isoprene units *n*	Carbon atoms *n*	Name	Example
1	5	Hemiterpenes	Isoprene
2	10	Monoterpenes	Pulegone
3	15	Sesquiterpenes	Polygodial
4	20	Diterpenes	Paclitaxel
5	25	Sesterterpenes	
6	30	Triterpenes	β-Amyrin
8	40	Tetraterpenes	β-Carotene
9—30 000	> 40	Polyterpenes	Rubber

defence (Wink and van Wyk, 2008). Among the best-known lower (C_5–C_{20}) terpenes are the gibberellins, a large group of diterpene plant hormones involved in the control of seed germination, stem elongation and flower induction (Thomas *et al.*, 2005). Another terpenoid hormone, the C_{15} compound, abscisic acid (ABA), is not properly considered a lower terpenoid, since it is formed from the oxidative cleavage of a C_{40} carotenoid precursor (Schwartz *et al.*, 1997).

Several important groups of plant compounds, including cytokinins, chlorophylls and the quinone-based electron carriers (the plastoquinones and ubiquinones), have terpenoid side chains attached to a non-terpenoid nucleus. These side chains facilitate anchoring to or movement within membranes. In the past decade, proteins have also been found to have terpenoid side chains attached. In fact, all eukaryotic cells appear to contain proteins that have been post-translationally modified by the attachment of C_{15} and C_{20} terpenoid side chains via a thioether linkage.

Prenylation substantially increases protein hydrophobicity and serves to target proteins to membranes or direct protein–protein interactions (Zhang and Casey, 1996). In plants, prenylated proteins may be involved in the control of the cell cycle (Qian *et al.*, 1996; Crowell, 2000), nutrient allocation (Zhou *et al.*, 1997) and ABA signal transduction (Clark *et al.*, 2001).

The most abundant hydrocarbon emitted by plants is the hemiterpene (C_5) isoprene, 2-methyl-1,3-butadiene (Fig. 5.1). Emitted from many taxa, especially woody species, isoprene has a major impact on the redox balance of the atmosphere, affecting levels of ozone, carbon monoxide and methane (Lerdau *et al.*, 1997). The release of isoprene from plants is strongly influenced by light and temperature, with the greatest release rates typically occurring under conditions of high light and high temperature (Lichtenthaler, 2007). Although the direct function of isoprene in plants themselves has been a mystery for many years, there are now indications that it may serve to prevent cellular damage at high temperatures, perhaps by reacting with free radicals to stabilize membrane components (Sasaki *et al.*, 2007). Instead of isoprene,

some plant species emit large amounts of monoterpene (C_{10}) hydrocarbons, which may function in a similar fashion (Loreto *et al.*, 1998).

Most of the thousands of terpenoids produced by plants have no discernible role in growth and development and are, therefore, often classified as 'secondary' metabolites. Although comparatively few of these substances have been investigated in depth, they are thought to serve primarily in ecological roles, providing defence against herbivores or pathogens (Wittstock and Gershenzon, 2002; Wink, 2007) and acting as attractants for animals that disperse pollen or seeds or as inhibitors of germination and growth of neighbouring plants (Harborne and Tomas-Barberan, 1991; Langenheim, 1994; Wink, 2010). One of the best-known examples of a lower terpene involved in plant defence is polygodial, a drimane-type sesquiterpene dialdehyde found in *Polygonum hydropiper* (Fig. 5.1). Among the most potent deterrents to insect feeding known, polygodial has been shown to inhibit the feeding of a diverse assortment of herbivorous insects (Moreno-Osorio *et al.*, 2008). The deterrent effect appears to be a direct result of the action of polygodial on taste receptors. In lepidopteran larvae, polygodial and other drimane dialdehydes block the stimulatory effects of glucose and sucrose on chemosensory receptor cells found on the mouthparts (Frazier, 1986; Jansen and de Groot, 2004). The aldehyde groups can covalently bond with the free amino group of proteins under physiological conditions; such modification can change the three-dimensional structure of proteins and thus alter their bioactivity (Wink, 2008).

Although a few lower terpenes have been studied in as much detail as polygodial, many other members of this group serve as toxins, feeding deterrents or oviposition deterrents to herbivores, and so are also thought to function in plant defence. As toxins or deterrents, these substances possess many diverse modes of action on herbivores. For example, the monoterpenoid ketone, pulegone (Fig. 5.1), is a liver toxin in mammals (Chen *et al.*, 2001); the pyrethrins, monoterpene esters, function as insect nerve poisons by interacting with the voltage-gated sodium channel proteins found in insect nerve cell membranes, leading to paralysis and eventual death (Davies *et al.*, 2007); and the diterpene, atractyloside, inhibits ADP/ATP translocation in the mitochondria (Stewart and Steenkamp, 2000).

In the past few years, a new role for lower terpenes in plant defence has emerged. Certain plant species respond to herbivore attack by emitting volatile terpenes that attract the enemies of herbivores. For example, lima bean (*Phaseolus lunatus*) plants damaged by the spider mite, *Tetranychus urticae*, emit a mixture of monoterpenes, C_{11} and C_{16} homoterpenes (Fig. 5.1) and methyl salicylate, which attracts a carnivorous mite, *Phytoseiulus persimilis*, that preys on spider mites (Dicke *et al.*, 1990; Dicke, 1994). When maize or cotton is fed upon by lepidopteran larvae, a blend of monoterpenes, sesquiterpenes, homoterpenes and other compounds is released, which attracts parasitic wasps that oviposit on the larvae (Turlings *et al.*, 1990, 1995). The majority of these volatiles are emitted only by arthropod-damaged plants

and not by unattacked or artificially damaged plants. The terpenoids released are largely synthesized de novo following an initial herbivore attack (Pare and Tumlinson, 1997) and are released systemically throughout the plant (Dicke *et al.*, 1993; Rose *et al.*, 1996). The use of volatile terpenoids to attract the enemies of herbivores may be a valuable complement to the more direct modes of antiherbivore defence.

The functions of the lower terpenes are not limited to the natural world. Many play important roles in human society, such as the myriad of monoterpene and sesquiterpene flavour and fragrance agents that are added to foods, beverages, perfumes, soaps, toothpaste, tobacco and other products (Berger, 2007). Some lower terpenes find use in industry as raw materials in the manufacture of adhesives, coatings, emulsifiers and speciality chemicals, whilst others, such as limonene and the pyrethrins, are of increasing commercial importance as insecticides because of their low toxicity to mammals and lack of persistence in the environment. The pharmaceutical importance of plant lower terpenes has steadily increased in the past decade. In addition to the well-known roles of camphor (Fig. 5.1) and cineole in preparations to relieve the pain of burns, strains and other inflammations, the past few years have seen the acceptance of artemisinin, a sesquiterpene endoperoxide derived from the traditional Chinese medicinal plant, *Artemisia annua* (Fig. 5.1), as a valuable antimalarial compound (Balint, 2001), and the development of paclitaxel (Fig. 5.1), a highly functionalized diterpene from yew (*Taxus* spp.), as a new drug for the treatment of ovarian and breast cancer (Kingston and Newman, 2007). Recently, and after the thorough research carried by Thomas Efferth and co-workers, it was proven that artemisinin and its semisynthestic artemether derivatives have not only antimalarial activity, but also antiviral and cytotoxic activities against different cancer cells (Efferth *et al.*, 2007, 2008; Youns *et al.*, 2009). These sesquiterpene lactone drugs with the highly active endoperoxide bridge can bind covalently to DNA and protein leading to permanent inactivation of many molecular targets (Wink, 2008). Paclitaxel, also known as taxol, enhances the polymerization of tubulin, a protein component of the microtubules of the mitotic spindle, resulting in stabilized, non-functional tubules and blocking the cell cycle. The potential of other lower terpenes in the therapy and prevention of cancer is currently under active investigation (Gould, 1995). Several sesquiterpene lactones have exocyclic methylene groups that are highly reactive. They can easily make covalent bonds with SH-groups of proteins or glutathione and thus alter their bioactivity (Wink, 2008). These interactions can explain the activity of several sesquiterpene lactones against inflammation and as antiinfectants.

5.3 Biosynthesis

The biosynthetic pathway to terpenoids (Fig. 5.2) is conveniently treated as comprising four stages, the first of which involves the formation of IPP,

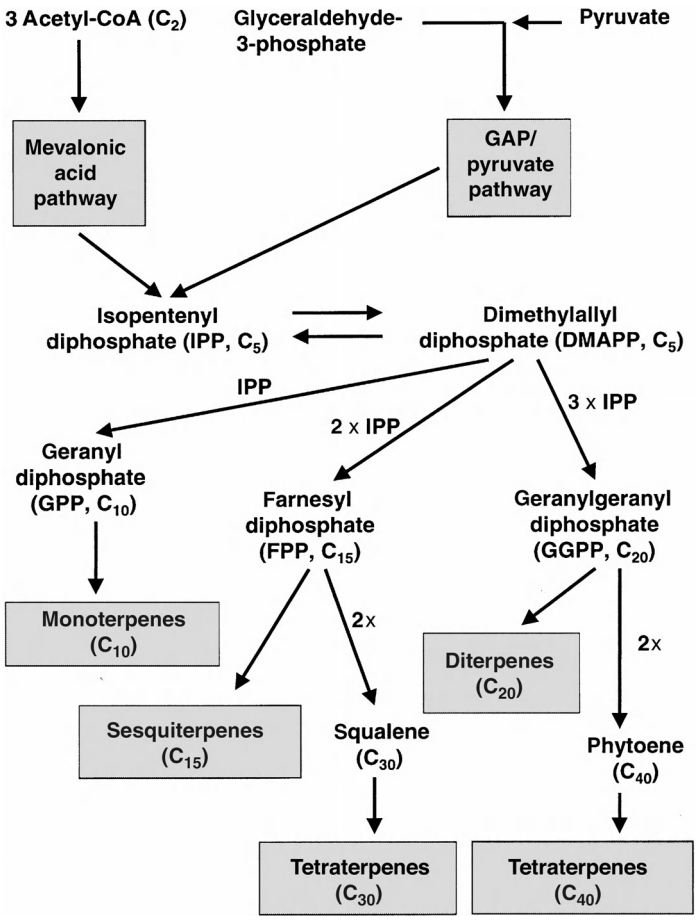

Figure 5.2 Overview of terpenoid biosynthesis in plants, showing the basic stages of this process and major groups of end products. CoA, coenzyme A; GAP, glyceraldehyde-3-phosphate.

the biological C_5 isoprene unit. Plants synthesize IPP and its allylic isomer, DMAPP, by one of two routes: the well-known mevalonic acid pathway, or the newly discovered methylerythritol phosphate (MEP) pathway. In the second stage, the basic C_5 units condense to generate three larger prenyl diphosphates, geranyl diphosphate (GPP, C_{10}), farnesyl diphosphate (FPP, C_{15}) and geranylgeranyl diphosphate (GGPP, C_{20}). In the third stage, the C_{10}–C_{20} diphosphates undergo a wide range of cyclizations and rearrangements to produce the parent carbon skeletons of each terpene class. GPP is converted to the monoterpenes, FPP is converted to the sesquiterpenes and GGPP is converted to the diterpenes. FPP and GGPP can also dimerize in a head-to-head fashion to form the precursors of the C_{30} and the C_{40} terpenoids,

respectively. The fourth and final stage encompasses a variety of oxidations, reductions isomerizations, conjugations and other transformations by which the parent skeletons of each terpene class are converted to thousands of distinct terpene metabolites. This section discusses the latest findings concerning each of the four stages of terpenoid biosynthesis in plants. The portions of the third and fourth stages that are not involved in the formation of the lower (C_5–C_{20}) terpenes are dealt with in Section 5.2.

5.3.1 Formation of the basic C_5-unit: the mevalonate pathway

The classic route for the formation of the C_5 building blocks of terpenoid biosynthesis in plants is via the reactions of the mevalonate pathway, first demonstrated in yeast and mammals. This well-characterized sequence (Fig. 5.3) involves the stepwise condensation of three molecules of acetyl coenzyme A (AcCoA) to form the branched C6 compound, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). Following the reduction of HMG-CoA to mevalonic acid, two successive phosphorylations and a decarboxylationelimination yield the C_5 compound, IPP.

Among the most recent developments in mevalonate pathway research is the successful cloning of the plant genes encoding all the enzymes (acetyl-CoA acetyltransferase genes, HMG-CoA synthase genes and HMG-CoA reductase) that catalyse the intial steps of the meralonic acid (MVA) pathway in rubber (Sando *et al*., 2008). Along with the work done earlier on acetoacetyl-CoA thiolase (Vollack and Bach, 1996) and HMG-CoA synthase (Montamant *et al*., 1995), it was obvious that the two sequences of acetyl-CoA acetyltransferase, HMG-CoA synthase are separated and distinct from each other, in contrast to an earlier report suggesting that in plants, in contrast to animals and microorganisms, both reactions are catalysed by a single protein (Weber and Bach, 1994). Each sequence is highly homologous to that of corresponding genes in the mevalonate pathways of mammals and microbes.

The third step of the mevalonic acid pathway is the conversion of HMG-CoA to mevalonic acid, a two-step, nicotinamide adenine diphosphate (reduced form) (NADPH)-requiring reduction catalysed by HMG-CoA reductase (HMGR) (Fig. 5.3). Researchers have lavished considerable attention on HMGR, since it catalyses a critical, rate-determining step in the biosynthesis of sterols in animals, and has been assumed to play a role of similar importance in the formation of plant terpenoids. Plant HMGR is a membrane-bound enzyme, a feature that has greatly hindered efforts to purify and characterize it. However, now that HMGR genes from more than ten species have been cloned and analysed (Table 5.2), our knowledge of this important catalyst has increased substantially. All plant genes isolated so far encode polypeptides of 60–65 kDa each, with three distinct regions: a very divergent NH_2-terminal domain, a more conserved membrane-binding region with two membrane-spanning sequences and a highly conserved COOH-terminal domain containing the catalytic site.

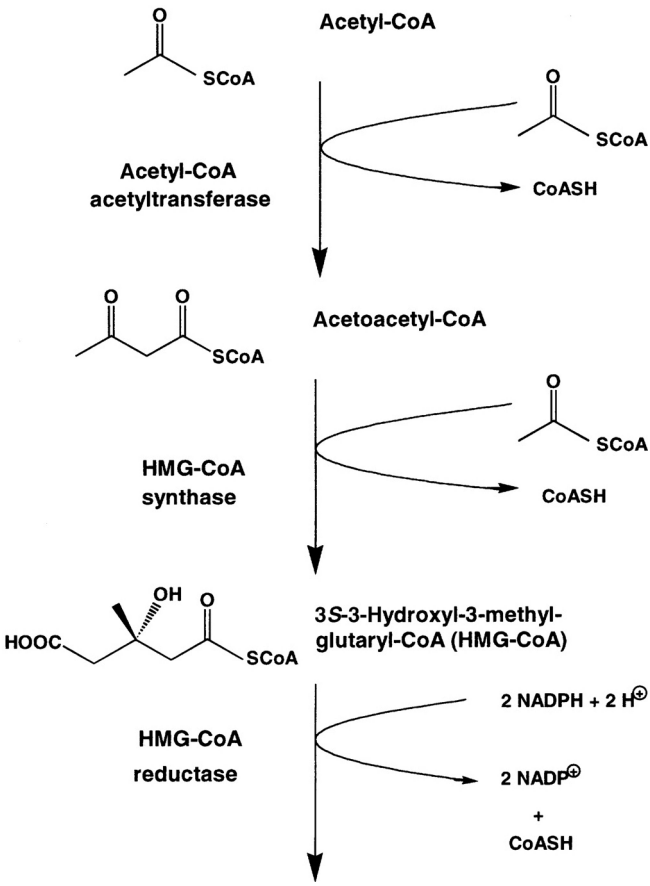

Figure 5.3 Outline of the mevalonate pathway for the formation of C_5 isoprenoid units. Most research has focused on HMG-CoA reductase (HMGR), the rate-determining step in terpenoid biosynthesis in mammals. P indicates a phosphate moiety. HMG-CoA, 3-hydroxy-3 methylglutaryl coenzyme A; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); SCoA, S-Coenzyme A (to which acetate is attached); CoASH, free coenzyme A.

Experiments with cloned genes have contributed to the resolution of a long-standing controversy concerning the subcellular location of HMGR in plants. Over the past 25 years, it has been claimed that HMGR is present in the endoplasmic reticulum (ER), the plastids and the mitochondria (Bach *et al.*, 1991). However, HMGR gene products from both *Arabidopsis thaliana* (Enjuto *et al.*, 1994; Campos and Boronat, 1995) and tomato (Denbow *et al.*, 1996) have recently been demonstrated to be co-translationally inserted into ER-derived microsomal membranes in vitro. Since the insertion is mediated by the two transmembrane regions (Enjuto *et al.*, 1994; Denbow *et al.*, 1996; Re *et al.*, 1997) whose sequences are conserved among all plant HMGR genes

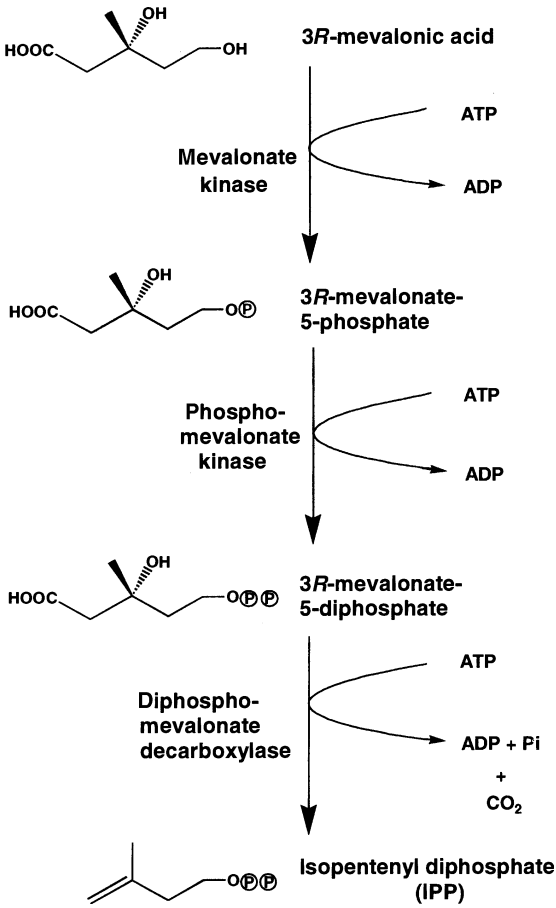

Figure 5.3 (*Continued*)

so far isolated, it seems probable that all known plant HMGRs are targeted to the ER (Campos and Boronat, 1995). Nevertheless, claims regarding the plastidial localization of HMGR have continued to appear (Nakagawara *et al.*, 1993; Bestwick *et al.*, 1995; Kim *et al.*, 1996). While an as yet uncharacterized HMGR may be present in the plastids, reports of plastidial localization are more likely to be due to contamination of plastid fractions with microsomes (Gray, 1987). Marker enzymes or electron microscopy have seldom been used to verify the purity of subcellular fractions in such studies.

Evidence for the regulatory role of HMGR in the formation of plant terpenoids comes from numerous studies that have demonstrated a close correlation between changes in HMGR activity and alterations in the rate of terpenoid biosynthesis. For example, Heide and co-workers (Gaisser and Heide, 1996; Lange *et al.*, 1998a) have been studying the formation of shikonin,

Table 5.2 Isolated genes encoding several major classes of enzymes in terpene biosynthesis

Enzyme	Species	Reference
A) HMG-CoA reductase	*Arabidopsis thaliana*	Caelles *et al.* (1989) D'Auria and Gershenzon (2005) Enjuto *et al.* (1994) Learned and Fink (1989)
	Camptotheca acuminata	Burnett *et al.* (1993)
	Catharanthus roseus	Maldonado-Mendoza *et al.* (1992)
	Corylus avellana	Wang *et al*. (2007)
	Eucommia ulmoides	Jiang *et al.* (2006)
	Euphorbia Pekinensis	Cao *et al.* (2009)
	Ginkgo biloba	Shen *et al*. (2006)
	Gossypium harbadense	Joost *et al.* (1995)
	Gossypium hirsutum	
	Hevea brasiliensis	Sando *et al.* (2008)
	Lycopersicon esculentum	Narita and Gruissem (1989) Park *et al.* (1992)
	Nicotiana sylvestris	Genschik *et al.* (1992)
	Oryza sativa	Nelson *et al.* (1994)
	Pisum sativum	Monfar *et al.* (1990)
	Raphanus sativus	Wettstein *et al.* (1989) Vollack *et al.* (1994)
	Solanum tuberosum	Bhattacharyya *et al.* (1995) Choi *et al.* (1992) Korth *et al.* (1997) Oosterhaven *et al.* (1993) Yang *et al.* (1991)
	Triticum aestivum	Aoyagi *et al.* (1993)
B) Prenyltransferases		
FPP synthase	*Arabidopsis thaliana*	Delourme *et al.* (1994)
	Artemisia annua	Matsushita *et al.* (1996)
	Capsicum annuum	Hugueney *et al.* (1996)
	Centella asiatica	Kim *et al.* (2005)
	Ginkgo biloba	Wang *et al.* (2004)
	Hevea brasiliensis	Adiwilaga and Kush (1996)
	Lupinus albus	Attucci *et al.* (1995)
	Oryza sativa	Sanmiya *et al.* (1997)
	Parthenium argentatum	Pan *et al.* (1996)
	Picea abies	Schmidt and Gershenzon (2007)
	Zea mays	Li and Larkins (1996)
GGPP synthase	*Arabidopsis thaliana*	Scolnick and Bartley (1996) Scolnick and Bartley (1994)
	Brassica campestris	Lim *et al.* (1996)
	Capsicum annuum	Badillo *et al.* (1995) Kuntz *et al.* (1992)
	Catharanthus roseus	Bantignies *et al.* (1996)
	Ginkgo biloba	Liao *et al.* (2004)
	Lupinus albus	Aitken *et al.* (1995)
	Picea abies	Schmidt and Gershenzon (2007)

Table 5.2 (*Continued*)

Enzyme	Species	Reference
C) Terpene synthases	*Abies grandis*	Bohlmann *et al.* (1997) Bohlmann *et al.* (1998a) Steele *et al.* (1998a) Vogel *et al.* (1996)
	Arabidopsis thaliana	Corey *et al.* (1993) Sun and Kamiya (1994) Yamaguchi *et al.* (1998)
	Artemisia annua	Bertea *et al.* (2006)
	Cichorium intybus	Lu *et al.* (2002)
	Citrus junos	Mercke *et al.* (2000)
	Clarkia brewerii	Bouwmeester *et al.* (2002) Maruyama *et al.* (2001) Dudareva *et al.* (1996)
	Cucurbita maxima	Yamaguchi *et al.* (1996)
	Gossypium arboreum	Chen *et al.* (1995) Chen *et al.* (1996)
	Hyoscyamus muticus	Back and Chappell (1995)
	Luffa cylindrical	Hayashi *et al.* (2001)
	Lycopersicon esculentum	Colby *et al.* (1998)
	Mentha x piperita	Crock *et al.* (1997)
	Mentha spicata	Colby *et al.* (1993)
	Nicotiana tabacum	Facchini and Chappell (1992)
	Perilla frutescens	Yuba *et al.* (1996)
	Picea sitchensis	Mckay *et al.* (2003)
	Pinus taeda	Phillips *et al.* (2003)
	Pisum sativum	Ait-Ali *et al.* (1997)
	Ricinus communis	Mau and West (1994)
	Salvia officinalis	Wise *et al.* (1998)
	Taxus brevifolia	Wildung and Croteau (1996)
	Vitis vinifera	Lucker *et al.* (2004)
	Zea mays	Bensen *et al.* (1995)
	Zingiber zerumbet	Yu *et al.* (2008)

HMG-CoA, 3-hydroxy-3 methylglutaryl coenzyme A; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate.

a napthoquinone pigment constructed from a benzenoid ring and a molecule of GPP. In *Lithospermum erythrorhizon* cultures, they showed that increases in the level of HMGR enzyme activity under various light and inhibitor treatments were associated with greater accumulation of shikonin and its derivatives. Other recent examples include correlations between the level of HMGR and the formation of: sesquiterpenes in lettuce (Bestwick *et al.*, 1995), sesquiterpenes in cotton (Joost *et al.*, 1995), triterpenes in *Tabernaemontana divaricata* (Fulton *et al.*, 1994) and rubber in guayule (Ji *et al.*, 1993).

To obtain a more rigorous proof of the regulatory role of plant HMGR, researchers have used constitutive promoters to overexpress HMGR in various

species. For example, tobacco transformed with a constitutively expressed HMGR construct showed a three–eightfold increase in HMGR enzyme activity and a three–tenfold increase in total sterols (Chappell *et al.*, 1995; Schaller *et al.*, 1995). However, there was no change in the level of other terpenoid end products, including sesquiterpenes, phytol (the C_{20} side chain of chlorophyll) and carotenoids. Curiously, the sterol composition of these HMGR-overexpressing plants differed from that of untransformed tobacco in having a much higher proportion of biosynthetic intermediates, such as cycloartenol (often conjugated as esters), rather than end products, such as sitosterol or stigmasterol. A mutant tobacco cell line resistant to a sterol inhibitor showed a very similar phenotype (Gondet *et al.*, 1992, 1994). Taken together, these results make a strong case for HMGR being a rate-determining step, at least in the formation of sterols, although later enzymes in the pathway also have a significant influence on the rate of sterol biosynthesis. However, this conclusion may not be applicable to all plant species, since the overexpression of HMGR in *A. thaliana* had no effect on the accumulation of sterols and other terpenoids (Re *et al.*, 1997).

If HMGR activity limits the rate of terpenoid formation, it is important to understand the mechanism of this control. In mammals, HMGR activity is subject to feedback inhibition by sterols that regulates the rates of transcription and translation, and post-translational controls involving allosteric effects and reversible phosphorylation (Panda and Devi, 2004). HMGR activity in plants appears to be modulated in similar ways, although we are only just beginning to understand the mechanisms of control. The close correlation of HMGR activity with the abundance of HMGR mRNA in *L. erythrorhizon* (Lange *et al.*, 1998a), tomato (Yang *et al.*, 1991) and other species (Stermer *et al.*, 1994) is good evidence for transcriptional control. At the post-translational level, HMGR from *Brassica oleracea* was shown to be inactivated by reversible phosphorylation, mediated by a specific kinase (MacKintosh *et al.*, 1992; Dale *et al.*, 1995). Since plants produce a much wider assortment of terpenoid end products than mammals do, they might be expected to regulate HMGR in unique ways not found in mammals. While only a single HMGR gene is known from each of the mammal species studied so far, all plants examined possess a small gene family with as many as nine members (Bhattacharyya *et al.*, 1995; Joost *et al.*, 1995). Detailed studies in tomato and potato reveal that different HMGR genes may be expressed in different organs or under different environmental conditions (Choi *et al.*, 1994; Enjuto *et al.*, 1995; Daraselia *et al.*, 1996), raising the possibility that a differential expression of HMGR genes could serve as a major mechanism for the control of HMGR activity.

Mevalonic acid, the product of HMGR, is converted to IPP by the sequential action of three enzymes: mevalonate kinase, phosphomevalonate kinase and diphosphomevalonate decarboxylase (Fig. 5.3). These three catalysts have not previously been considered to be important control points in plant terpenoid biosynthesis, and little new information has appeared to alter this view. The

activities of all the three enzymes were shown to be higher than that of HMGR (Bianchini *et al.*, 1996), similar to each other (Sandmann and Albrecht, 1994) and unrelated to fluctuations in the rate of terpenoid formation (Ji *et al.*, 1993; Bianchini *et al.*, 1996). A cDNA encoding mevalonate kinase was recently isolated from *A. thaliana* by genetic complementation in yeast (Riou *et al.*, 1994). The lack of a transit peptide and the presence of only a single gene, as deduced from Southern blotting, make it appear that plant mevalonate kinase, like HMGR, is a cytosolic enzyme.

5.3.2 Formation of the basic C_5 unit: the methylerythritol phosphate pathway

The most exciting advance in the field of plant terpenoid biosynthesis is the discovery of a second route for making the basic C_5 building block of terpenes, completely distinct from the mevalonate pathway (Lichtenthaler, 2000). This new route, which starts from glyceraldehyde phosphate and pyruvate (Fig. 5.4), has also been detected in bacteria and other microorganisms. With the advantage of hindsight, one can list many observations made during the past 30 years that, taken together, should have persuaded researchers of the existence of a non-mevalonate pathway to terpenoids in higher plants. For example, it was demonstrated numerous times that mevalonate itself is a very poor precursor for many classes of terpenoids (Croteau and Loomis, 1972; Charlwood and Banthorpe, 1978). However, there was no reasonable alternative to the mevalonate pathway prior to the pioneering investigations of terpenoid biosynthesis in eubacteria, carried out by Michel Rohmer, Hermann Sahm and co-workers. These investigators discovered that the incorporation of ^{13}C-labelled precursors, such as glucose, acetate and pyruvate, into bacterial terpenoids (hopanoids and ubiquinones) was not consistent with the operation of the mevalonate pathway (Flesch and Rohmer, 1988; Rohmer, 2008). In addition, when intermediates of the mevalonate pathway were fed to species such as *Escherichia coli*, they were not incorporated (Horbach *et al.*, 1993). Analysis of the ^{13}C incorporation patterns from labelled glucose and acetate allowed the deduction that a C_3-unit from glycolysis and a C_2-unit from pyruvate combined in some manner to form the basic C_5 isopentenoid unit (Rohmer, 1999). Subsequent experiments with *E. coli* mutants, blocked in specific steps of triose phosphate metabolism, pointed to glyceraldehyde phosphate and pyruvate as the actual precursors of this new pathway (Rohmer *et al.*, 1996).

The existence of a similar non-mevalonate route to terpenoids in plants was first reported in 1994. When Duilio Arigoni and co-workers fed different ^{13}C-labelled forms of glucose to *Ginkgo biloba* embryos, the ^{13}C-nuclear magnetic resonance (NMR) spectra of the resulting diterpenes were not what would have been expected from the normal operation of the mevalonate pathway (Cartayrade *et al.*, 1994), but showed an incorporation pattern identical to that seen with the *E. coli* terpenoids. Subsequent studies employing

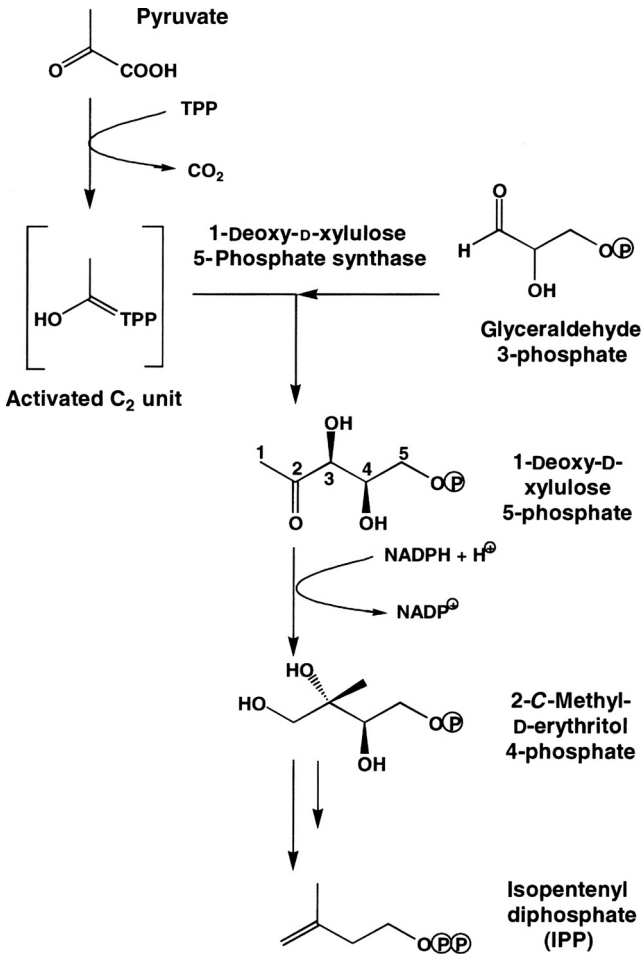

Figure 5.4 Outline of the newly discovered glyceraldehyde phosphate/pyruvate pathway for the formation of C_5 isoprenoid units. None of the intermediates after 2-C-methyl-D-erythritol 4-phosphate is known. P indicates a phosphate moiety. TPP, thiamine pyrophosphate; NADP, nicotinamide adenine dinucleotide phosphate.

a similar methodology have demonstrated that an assortment of terpenoids from angiosperms, gymnosperms and bryophytes, including monoterpenes (Eisenreich *et al.*, 1997; Adam *et al.*, 1998), diterpenes (Knoss *et al.*, 1997; Jennewein and Croteau, 2001), carotenoids (Lichtenthaler *et al.*, 1997) and the side chains of chlorophyll (phytol) and quinones (Lichtenthaler *et al.*, 1997; Adam *et al.*, 1998) are formed in a non-mevalonate fashion, while the labelling of sesquiterpenes and sterols was consistent with their origin from the mevalonate pathway (Schwarz, 1994; Lichtenthaler *et al.*, 1997; Adam *et al.*, 1998).

Several research groups are now actively involved in elucidating the sequence of the new pathway. In 1996, Rohmer and co-workers refined their concept of the first step, proposing that hydroxyethylthiamine diphosphate, a C_2-unit derived from pyruvate, condenses with glyceraldehyde-3-phosphate to form 1-deoxy-D-xylulose 5-phosphate (Rohmer *et al.*, 1996) (Fig. 5.4). This hypothesis was based on the pattern of labelling in terpenoids formed from [^{13}C]-pyruvate, [^{13}C]-glycerol and various [^{13}C]-glucoses, and the natural occurrence of 1-deoxy-D-xylulose, a precursor of the enzyme cofactors thiamine (vitamin B_1) diphosphate and pyridoxal (vitamin B_6) 5′-phosphate. Additional support comes from the high rate of 1-deoxy-D-xylulose incorporation into terpenoids measured in *E. coli* (Broers, 1994) and several plant species (Zeidler *et al.*, 1997; Sagner *et al.*, 1998b; Eisenreich *et al.*, 2001). A more rigorous proof of the nature of the first step of the non-mevalonate pathway has become available in the past years, with the isolation of cDNAs for enzymes that catalyse the conversion of glyceraldehyde phosphate and pyruvate to 1-deoxy-D-xylulose 5-phosphate from *E. coli* (Sprenger *et al.*, 1997; Lois *et al.*, 1998), *Capsicum annuum* (Bouvier *et al.*, 1998) and *Mentha x piperita* (Lange *et al.*, 1998b). The encoded enzymes are novel transketolases that are distinct from other members of this enzyme family, such as the well-characterized transketolases of the pentose phosphate pathway.

After 1-deoxy-D-xylulose 5-phosphate, subsequent reactions of the new pathway must transform the linear five-carbon backbone of this sugar phosphate to a branched, isopentenoid carbon skeleton. Just recently, 1-deoxy-D-xylulose 5-phosphate has been shown to be converted to 2-C-methyl-D-erythritol 4-phosphate in *E. coli* (Duvold *et al.*, 1997; Wanke *et al.*, 2001; Kuzuyama, 2002) (Fig. 5.4), and the same reaction was demonstrated to occur in several species of plants (Sagner *et al.*, 1998a). This intramolecular rearrangement involves the cleavage of the C3–C4 bond of the deoxyxylulose backbone and the establishment of a new bond between C2 and C4. Similar skeletal rearrangements are involved in both riboflavin and valine biosynthesis. While nothing is yet known of any additional intermediates in the pathway, several dehydration steps, reductions and at least one phosphorylation seem to be required to transform 2-C-methyl-D-erythritol 4-phosphate to IPP. Given the high level of interest in this work and the participation of several excellent research groups, it would be surprising if the remaining steps of this novel pathway were not rapidly elucidated.

The non-mevalonate route to terpenoids appears to be localized in the plastids. In plant cells, terpenoids are manufactured both in the plastids and the cytosol (Gray, 1987; Kleinig, 1989). As a general rule, the plastids produce monoterpenes, diterpenes, phytol, carotenoids and the side chains of plastoquinone and α-tocopherol, while the cytosol/ER compartment produces sesquiterpenes, sterols and dolichols. In the studies discussed above, nearly all of the terpenoids labelled by deoxyxylulose (Sagner *et al.*, 1998b; Eisenreich *et al.*, 2001) and 2-C-methyl erythritol feeding (Duvold *et al.*, 1997) or

showing ^{13}C-patterns indicative of a non-mevalonate origin (Cartayrade *et al.*, 1994; Eisenreich *et al.*, 1997, 2001; Lichtenthaler *et al.*, 1997; Adam *et al.*, 1998) are thought to be plastid derived. Consistent with this generalization is the fact that the genes of the non-mevalonate pathway that have been isolated so far all encode plastid-targeting sequences (Bouvier *et al.*, 1998; Lange *et al.*, 1998b). In contrast, the mevalonate pathway appears to reside solely in the cytosol/ER compartment based on the sequence analysis and expression of genes encoding pathway enzymes, including acetoacetyl-CoA thiolase (Vollack and Bach, 1996), HMG-CoA synthase (Montamant *et al.*, 1995), HMGR (discussed in Section 5.3.1) and mevalonate kinase (Riou *et al.*, 1994). A third subcellular compartment, the mitochondrion, also participates in terpenoid biosynthesis, making the prenyl side chain of ubiquinone, an electron transport system component found in this organelle, using IPP derived from the cytosol/ER pathway (Hemmerlin *et al.*, 2004).

It was once difficult to reconcile the terpenoid-manufacturing capabilities of the plastids with the usual absence of detectable HMGR activity in these organelles. Models proposed that the basic reactions of terpenoid biosynthesis are confined to the cytosol, with the preformed C_5-units being transferred to other subcellular compartments (Gray, 1987; Luetke-Brinkhaus and Kleinig, 1987). However, current knowledge suggests a more accurate generalization: the plastids biosynthesize terpenoids primarily via the methylerythritol phosphate pathway, while in the cytosol/ER terpenoid formation occurs largely via the mevalonate pathway. Reviewing the older literature with this paradigm in mind, it is not surprising that mevalonate was found to be so poorly incorporated into many plastid-formed terpenoids (Croteau and Loomis, 1972; Charlwood and Banthorpe, 1978; Keene and Wagner, 1985; Lunn, 2007), that levels of HMGR activity were often noted to be poorly correlated with the formation of plastidial terpenoids (Chappell *et al.*, 1989; Narita and Gruissem, 1989) and that the HMGR inhibitor, mevinolin, was shown to have a negligible effect on the production of plastidial terpenoids (Bach and Lichtenthaler, 1983; Bach *et al.*, 1999).

The existence of a non-mevalonate route to terpenoids also helps account for other puzzling observations, such as the complete failure of green algae to incorporate mevalonate into terpenoids (Lichtenthaler, 2000). Feeding experiments with ^{13}C-labelled glucose and acetate have now shown that all terpenoids in *Scenedesmus obliquus* (Rohmer, 1999) and other green algae (Disch *et al.*, 1998) are formed by the glyceraldehyde–pyruvate pathway. Among other photosynthetic microorganisms surveyed, the red alga, *Cyanidium caldarium*, and the chrysophyte, *Ochromonas danica* (Disch *et al.*, 1998), use both pathways, *Euglena gracilis* (Disch *et al.*, 1998) and the eubacterium, *Chloroflexus aurantiacus* (Rieder *et al.*, 1998), use only the mevalonate pathway, while the cyanobacterium, *Synechocystis* PCC 6714 (Disch *et al.*, 1998), employs only the glyceraldehyde–pyruvate pathway, like the plastids of higher plants. These results are in accord with the endosymbiotic origin of higher plant plastids from a cyanobacterium-like symbiont.

A strict division between the mevalonate and non-mevalonate pathways may not always exist for a given end product. The biosynthesis of certain terpenoids appears to involve the participation of both routes (Schwarz, 1994; Nabeta *et al.*, 1995; Adam and Zapp, 1998; Piel *et al.*, 1998). For example, the first two C_5 units of the sesquiterpenes of chamomile (*Matricaria recutita*) are formed via the methylerythritol phosphate pathway, while the third unit is derived from both the mevalonate pathway and the glyceraldehyde–pyruvate pathway (Adam and Zapp, 1998). Joint participation of the two pathways may be a result of the transport of prenyl diphosphate intermediates between the different sites of terpenoid biosynthesis (Heintze *et al.*, 1990; Soler *et al.*, 1993; McCaskill and Croteau, 1997), or the actual presence of both pathways in the same compartment. While the preponderance of evidence argues for the localization of the mevalonate pathway in the cytosol and the glyceraldehyde–pyruvate pathway in the plastids, as discussed above, there are some indications that the mevalonate pathway may also be found in the plastids, at least in certain species (Kim *et al.*, 1996) at certain developmental stages (Heintze *et al.*, 1990, 1994).

The occurrence of both terpenoid pathways at the same subcellular site, or the exchange of prenyl diphosphates between sites, may also help explain other curious phenomena noted in previous biosynthetic studies, such as the unequal labelling of different C_5 units. Administration of mevalonate has frequently been shown to result in the IPP-derived portion of the molecule being much more heavily labelled than the portion derived from DMAPP (Croteau and Loomis, 1972; Charlwood and Banthorpe, 1978). Such asymmetry has been attributed to the existence of a large pool of DMAPP that dilutes any DMAPP formed from an exogenous, labelled precursor. However, asymmetric labelling could also be a consequence of having separate pathways to each of the two basic C_5 units. The actual C_5 product of the alternative pathway is not known, and might be DMAPP rather than IPP. If DMAPP arising from the non-mevalonate pathway condensed with mevalonate-derived IPP (produced in situ or transported from another compartment), this could result in the unequal labelling of C_5 units. More research is needed not only to identify the remaining intermediates in the methylerythritol phosphate pathway, but also to determine in which species, tissues and compartments it operates, as well as to understand its regulation.

5.3.3 Assembly of C_5 units into C_{10}, C_{15} and C_{20} prenyl diphosphates

The second stage of terpene biosynthesis involves the fusion of the basic C_5 building blocks to yield larger metabolic intermediates (Fig. 5.2). IPP and its more reactive allylic isomer, DMAPP, condense in a head-to-tail orientation to form C_{10}, C_{15} and C_{20} prenyl diphosphates (Fig. 5.5). The requisite DMAPP is derived directly from IPP by the action of IPP isomerase, which is also capable of catalysing the reverse reaction. In the past years, genes encoding this

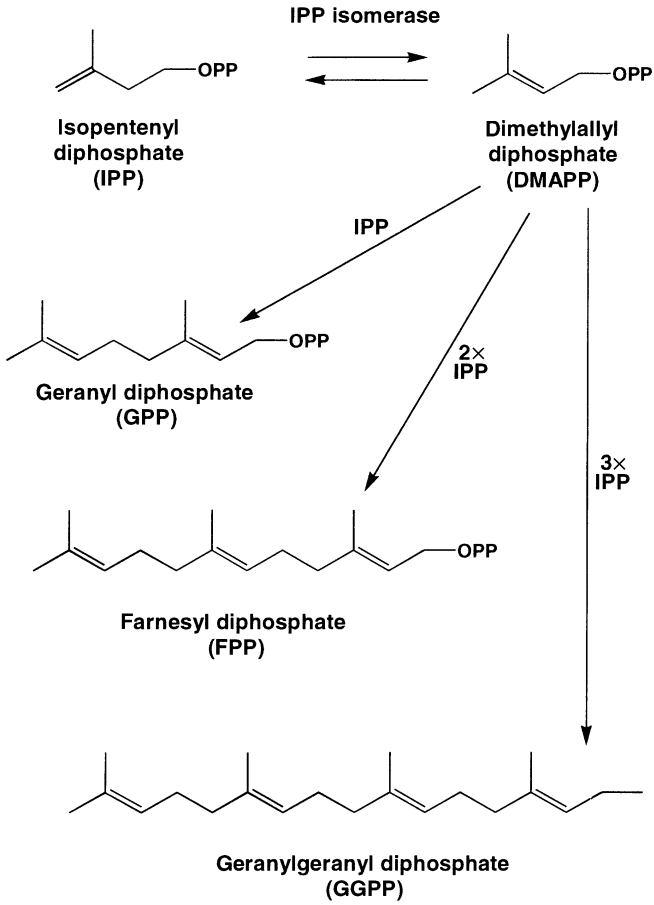

Figure 5.5 The formation of C_{10}, C_{15} and C_{20} prenyl diphosphates from the fusion of C_5 isoprenoid units. PP indicates a diphosphate moiety.

enzyme have been isolated from *A. thaliana* (Okada *et al.*, 2008) and *Clarkia breweri* (Blanc and Pichersky, 1995). The sequences reported exhibit high homology to the IPP isomerase gene sequences of other organisms, except at their *N*-termini, which seem to encode transit peptides for plastid localization. *Arabidopsis thaliana* possesses an IPP isomerase gene family consisting of at least two members (Phillips *et al.*, 2008), a finding consistent with the detection of multiple forms of this enzyme in cell cultures of several plant species (Ramosvaldivia *et al.*, 1998). In *Cinchona robusta*, e.g., the two isoforms of IPP isomerase had different kinetic parameters, different preferences for divalent metal ion cofactors and different patterns of occurrence; one form was present only after induction by a fungal elicitor (Ramosvaldivia *et al.*, 1997c). Although there is no strong evidence that IPP isomerase has any control of

flux through the terpenoid pathway (Ramosvaldivia *et al.*, 1997b), the activity of this enzyme in maize increases significantly after stimulation of carotenoid biosynthesis by light (Sandmann, 2001), and activity in cell cultures of several species increases after induction of phytoalexin formation by treatment with fungal elicitors (Hanley *et al.*, 1992; Fulton *et al.*, 1994; Ramosvaldivia *et al.*, 1997a).

The substrate (IPP) and the product (DMAPP) of IPP isomerase are both involved in the fundamental reactions by which C_5 isopentenoid units are joined together. Enzymes known as prenyltransferases add varying numbers of IPP units to a DMAPP primer in sequential chain elongation steps. The initial head-to-tail (1′-4) condensation of IPP and DMAPP yields the C_{10} allylic diphosphate, GPP. Further 1′-4 condensations of IPP with the enlarging allylic diphosphate chain give the C_{15} allylic diphosphate, farnesyl diphosphate (FPP) and the C_{20} allylic diphosphate, GGPP. In plants, FPP and GGPP are produced by well-characterized, product-specific enzymes that catalyse two- or three-step elongation sequences starting with IPP and DMAPP (Fig. 5.5). For example, GGPP synthases convert DMAPP and IPP directly to GGPP (Spurgeon *et al.*, 1984; Dogbo and Camara, 1987; Laskaris *et al.*, 2000). The reaction proceeds through the intermediacy of GPP and FPP, but under normal conditions GGPP is the first product to leave the active site. In contrast to FPP and GGPP synthases, much less attention has been devoted to GPP synthases. In fact, the very existence of this class of prenyltransferases in plants was once doubted, in the belief that amounts of GPP sufficient to sustain monoterpene biosynthesis were released during the formation of the larger allylic diphosphates (Tello *et al.*, 2008). However, prenyltransferases that synthesize GPP exclusively have now been discovered in several plant species that produce monoterpenes or natural products incorporating a monoterpene unit (Croteau and Purkett, 1989; Clastre *et al.*, 1993; Tello *et al.*, 2008).

In the past six years, cDNAs encoding FPP and GGPP synthases have been isolated from a diverse assortment of plant species (Table 5.2). The amino acid sequences deduced have a high degree of similarity to the FPP and GGPP synthases of other organisms (Chen *et al.*, 1994), which means that the recent determination of the crystal structure of an avian FPP synthase has a considerable value for the study of plant prenyltransferases as well. The structure of FPP synthase from avian liver consists of a novel arrangement of ten parallel α-helices positioned around a large central cavity (Tarshis *et al.*, 1994). Two aspartate-rich sequences (DDxxD) that are highly conserved among other prenyltransferases (Chen *et al.*, 1994) and essential for catalysis (Joly and Edwards, 1993; Song and Poulter, 1994; Harris and Poulter, 2000) are found on opposite sides of the cavity, with their aspartate carboxyl side chains pointing towards the cavity centre. These aspartate residues had previously been suggested to bind the diphosphate moieties of the substrates via Mg^{2+} bridges (Harris and Poulter, 2000). Structural analysis of a samarium-containing heavy atom derivative of an avian FPP synthase (samarium commonly adheres to Mg^{2+}-binding sites in enzymes) showed

samarium atoms bound to each of the two aspartate-rich regions, supporting the role of the aspartate residues in binding Mg^{2+} (Tarshis *et al.*, 1994). Work has now begun to identify other amino acid residues involved in the reaction mechanism. Prenyltransferases are one of the few groups of enzymes in which carbon–carbon bond formation results from electrophilic attack of a carbocationic species on a pre-existing double bond (Poulter and Rilling, 1981). The initial carbocation is formed by the ionization of the allylic substrate through hydrolysis of the diphosphate ester. Subsequently, addition to the double bond of IPP forms a new carbocation, which is then stabilized by proton elimination.

A long-standing goal in the study of prenyltransferases is to understand how these catalysts control the length of the growing chain during the reaction sequence. The availability of cloned prenyltransferase sequences and a three-dimensional structure for this enzyme class has provided new tools to approach this problem. Random and site-directed mutagenesis of bacterial FPP and GGPP synthases has demonstrated that several amino acid residues near the conserved aspartate-rich domains were most critical in determining chain length (Tarshis *et al.*, 1996; Wang and Ohnuma, 2000). For example, when an avian FPP synthase was altered so that two phenylalanine residues, located just on the *N*-terminal side of the first aspartate-rich domain, were changed to serine and alanine, the mutant enzyme produced products up to Coo, with an average size of C_{35}–C_{40} (Tarshis *et al.*, 1996). Structural analysis carried out in parallel with the mutagenesis revealed that the mutant FPP synthase had a larger binding pocket for allylic diphosphate substrates than native FPP synthase. Other amino acid residues involved in the substrate and product specificity of prenyltransferases are being actively sought.

The prenyltransferases that catalyse the syntheses of GPP, FPP and GGPP may be important regulatory enzymes in plant terpenoid biosynthesis since they are situated at the primary branch points of the pathway, directing flux among the various major classes of terpenoids. The level of prenyltransferase activity is, in fact, closely correlated with the rate of terpenoid formation in many experimental systems (Dudley *et al.*, 1986; Hanley *et al.*, 1992; Hugueney *et al.*, 1996) consistent with the regulatory importance of these catalysts. The localization of specific prenyltransferases in particular types of tissue or subcellular compartments may control the flux and direction of terpenoid synthesis at these sites. For example, the GPP synthase in *Salvia officinalis* is restricted to the secretory cells of the glandular trichomes, which are the sole site of monoterpene biosynthesis in this species (Croteau and Purkett, 1989).

5.3.4 Formation of parent carbon skeletons

The prenyl diphosphates, GPP, FPP and GGPP, are the central intermediates of terpenoid biosynthesis. Under the catalysis of monoterpene, sesquiterpene and diterpene synthases, respectively, these substances are transformed into

the primary representatives of each terpene skeletal type. Recent progress in the area of terpene synthases has been remarkable. In the past years, many novel activities have been described (Guo *et al.*, 1994; Dekraker *et al.*, 1998; Lu *et al.*, 2002; Dudareva *et al.*, 2004), over 30 terpene synthase cDNAs have been isolated from plants (Table 5.2) and the first crystal structures of terpene synthases have been obtained (Starks *et al.*, 1997; Christianson, 2006; Abe, 2007). These achievements have permitted new insights into the evolutionary origin and genetic regulation of terpene synthases and have provided unprecedented opportunities for exploring the reaction mechanisms of these catalysts.

A sequence comparison of the isolated terpene synthase cDNAs suggests that all appear to be derived from a single ancestral stock (Bohlmann *et al.*, 1998b). Overall, the amino acid sequences deduced share a high degree of similarity, and the positions of many residues thought to be involved in catalysis are conserved. When genomic sequences are compared (Facchini and Chappell, 1992; Mau and West, 1994; Back and Chappell, 1995), a common pattern of intron–exon organization is evident. Within the terpene synthases, phylogenetic reconstruction divides the known sequences into six subfamilies, each of which has a minimum of 40% identity among its members (Bohlmann *et al.*, 1998b). The pattern of sequence relationships is influenced by the taxonomic affinities of plant species, as well as by the chemical similarities among enzyme products and the reaction mechanism employed. For example, the limonene synthases of *Abies grandis*, a gymnosperm, are more closely related to other gymnosperm monoterpene and sesquiterpene synthases than they are to the limonene synthases from angiosperms.

Terpene synthases, also known as terpene cyclases because most of their products are cyclic, utilize a carbocationic reaction mechanism very similar to that employed by the prenyltransferases. Numerous experiments with inhibitors, substrate analogues and chemical model systems (Croteau, 1987; Cane, 1990, 1998) have revealed that the reaction usually begins with the divalent metal ion-assisted cleavage of the diphosphate moiety (Fig. 5.6). The resulting allylic carbocation may then cyclize by addition of the resonance-stabilized cationic centre to one of the other carbon–carbon double bonds in the substrate. The cyclization is followed by a series of rearrangements that may include hydride shifts, alkyl shifts, deprotonation, reprotonation and additional cyclizations, all mediated through enzyme-bound carbocationic intermediates. The reaction cascade terminates by deprotonation of the cation to an olefin or capture by a nucleophile, such as water. Since the native substrates of terpene synthases are all configured with *trans* (*E*) double bonds, they are unable to cyclize directly to many of the carbon skeletons found in nature. In such cases, the cyclization process is preceded by isomerization of the initial carbocation to an intermediate capable of cyclization.

The recently published crystal structure of tobacco epi-aristolochene synthase (a sesquiterpene synthase) has provided the first look at the three-dimensional configuration of a plant terpene synthase (Starks *et al.*, 1997).

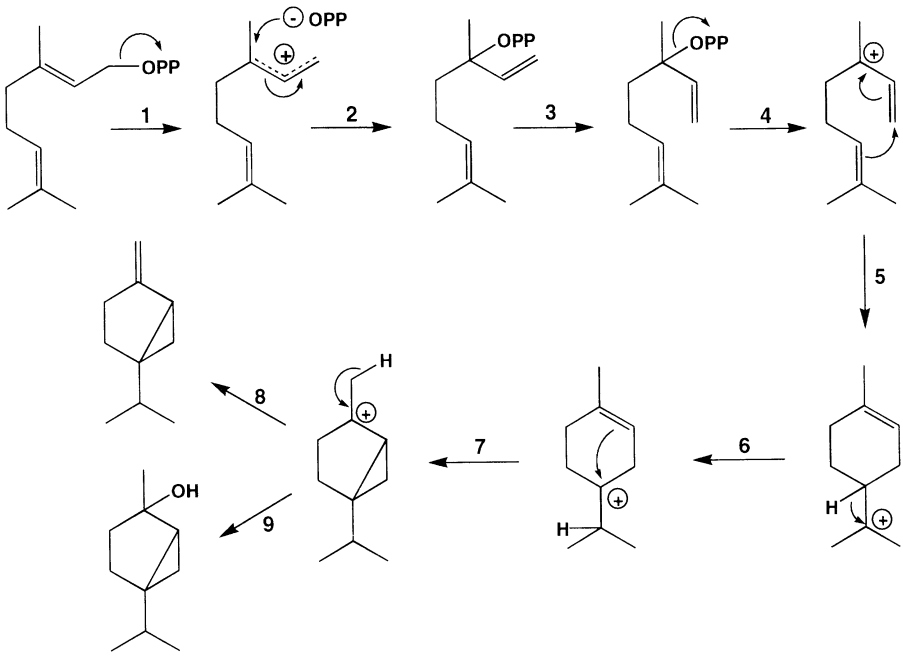

Figure 5.6 Proposed mechanism for the cyclization of geranyl diphosphate to sabinene and sabinene hydrate under catalysis by monoterpene synthases: the reaction begins with the hydrolysis of the diphosphate moiety to generate a resonance-stabilized carbocation (1); the carbocation then isomerizes to an intermediate capable of cyclization by return of the diphosphate (2); and rotation around a single bond (3); after a second diphosphate hydrolysis (4); the resulting carbocation undergoes a cyclization (5); a hydride shift (6); and a second cyclization (7); before the reaction terminates by deprotonation (8); or capture of the cation by water (9). Cyclizations, hydride shifts and a variety of other rearrangements of carbocationic intermediates are a characteristic of the mechanisms of terpene synthases. No known terpene synthase actually produces both sabinene and sabinene hydrate; these are shown to indicate the possibilities for reaction termination. PP indicates a diphosphate moiety.

The structure provides a physical basis for some of the proposed mechanistic features and reveals several elements responsible for controlling the course of reaction. The arrangement of the protein backbone, consisting of eight antiparallel α-helices that form a large cavity, is very similar to that reported for two other terpene synthases, a fungal sesquiterpene synthase (Lesburg *et al.*, 1997) and a bacterial triterpene synthase (Wendt *et al.*, 1997). It is also strongly reminiscent of the structure of avian liver FPP synthase (discussed in Section 5.3.3) despite only a low level of sequence similarity, reflecting the parallels in the reaction mechanism between terpene synthases and prenyltransferases. Among the notable features of the epi-aristolochene synthase structure is the presence of an aspartate-rich cluster, DDxxD, in the active site (just like those found in prenyltransferases) that serves to bind the diphosphate moiety of

the substrate via a Mg^{2+} bridge. Prenyltransferases, which simultaneously bind two different diphosphate-containing substrates, have two such clusters, while epi-aristolochene synthase and other terpene synthases, which bind only one diphosphate-containing substrate, have only one. The active site of epi-aristolochene synthase also contains a variety of aromatic amino acid residues that may serve to stabilize the enzyme-bound carbocationic intermediates by π–cation interactions (Wise and Croteau, 1998). Other amino acid residues were identified that direct the released diphosphate moiety away from the active site, that complex two additional Mg^{2+} ions and that participate in protonation and deprotonation.

Terpene synthases employ two other modes of generating the initial carbocationic intermediate in addition to hydrolysis of the diphosphate ester. The reaction may be initiated by protonation of an epoxide, as in the cyclization of oxidosqualene to sterols and triterpenes (Abe, 2007), or by protonation of the carbon–carbon double bond at the opposite end of the molecule from the diphosphate moiety. Mechanisms initiated by double-bond protonation are a characteristic of the formation of many diterpenes, such as copalyl diphosphate (West, 1981) (Fig. 5.7). Isolated cDNA sequences encoding copalyl diphosphate synthase have some homology to the sequences of terpene synthases in which the reaction is initiated by diphosphate hydrolysis, but lack the characteristic DDxxD motif, possessing instead an alternate aspartate-rich motif, DxDDTA, at a very different position in the sequence (Sun and Kamiya, 1994; Bensen *et al.*, 1995; Ait-Ali *et al.*, 1997). A second category of diterpene synthases has more in common with the majority of terpene synthases discussed above, catalysing diphosphate hydrolysis-initiated cyclizations while possessing typical DDxxD motifs (Yamaguchi *et al.*, 1996, 1998). Notable members of this group include the ent-kaurene synthases involved in gibberellin biosynthesis, which use copalyl diphosphate as a substrate rather than a product. There is also a third type of diterpene synthase that seems to combine the properties of the other two classes. For example, abietadiene synthase from *A. grandis* catalyses two sequential cyclization steps: first cyclizing GGPP to copalyl diphosphate via a double-bond protonation-initiated cyclization and then converting copalyl diphosphate to the olefin, abietadiene, via a diphosphate hydrolysis-initiated process (Keeling and Bohlmann, 2006). Appropriately, the *A. grandis* abietadiene synthase cDNA has regions of sequence homologous to both other types of diterpene synthases and contains both DDxxD and DxDDTA elements (Vogel *et al.*, 1996).

Not all terpene synthases catalyse complex reactions. Isoprene synthase converts DMAPP to the hemiterpene (C_5), isoprene (Fig. 5.1), a comparatively simple process involving the ionization of the diphosphate group, followed by double-bond migration and proton elimination (Silver and Fall, 1991). Present in chloroplasts in both stromal and thylakoid-bound forms, isoprene synthase is a homodimer that differs from other terpene synthases in many properties, such as subunit architecture, optimum pH and kinetic parameters

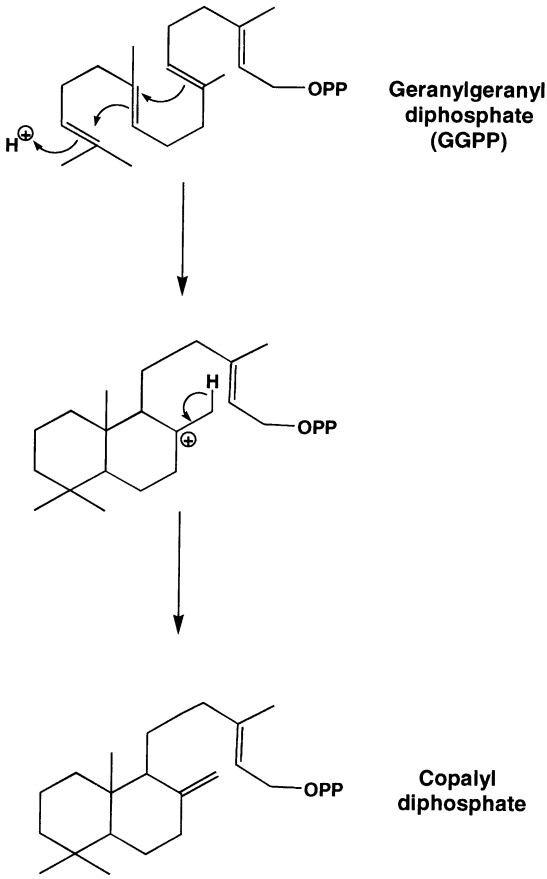

Figure 5.7 Proposed mechanism for the cyclization of geranylgeranyl diphosphate (GGPP) to the diterpene copalyl diphosphate, an example of terpene synthase-catalysed cyclization initiated by double-bond protonation, rather than by hydrolysis of the diphosphate ester. PP indicates a diphosphate moiety.

(Silver and Fall, 1995; Wildermuth and Fall, 1998). Its key role in the formation of isoprene, an abundant plant volatile with a major influence on atmospheric chemistry, has made it a popular target for cloning efforts.

An unusual feature of terpene synthases is the ability of a single enzyme to catalyse the formation of more than one product species. First suggested by the copurification of separate activities and differential inactivation studies, and later demonstrated by isotopically sensitive branching experiments (Wagschal *et al.*, 1991; Rajaonarivony *et al.*, 1992), this property has been unequivocally proved by cDNA cloning. Heterologous expression of many cloned terpene synthases, such as 1,8-cineole synthase from *S. officinalis*, leads to a mixture of products (Wise *et al.*, 1998). In a spectacular, recently published example, two sesquiterpene synthases from *A. grandis*, δ-selinene synthase

and γ-humulene synthase, were shown to synthesize 34 and 52 different sesquiterpenes, respectively (Steele *et al.*, 1998a). The tendency of terpene synthases to form multiple products is probably a consequence of their reaction mechanisms, which involve highly reactive carbocationic intermediates that may have more than one chemical fate. Interestingly, exon-swapping experiments on epi-aristolochene synthase converted this single product sesquiterpene synthase to one making multiple products (Back and Chappell, 1996). Further correlations between elements of protein structure and features of the reaction mechanism using three-dimensional structures will increase our understanding of how terpene synthases are able to make multiple products.

Terpene synthases are likely to serve as important agents of flux control in terpene biosynthesis because they operate at metabolic branch points where pathways diverge to different terpene types. However, there is still insufficient information available to assess the regulatory significance of these catalysts. Direct relationships between terpene synthase activity and changes in the rate of terpene formation have been noted on several occasions (Dudley *et al.*, 1986; Gijzen *et al.*, 1991; Zook *et al.*, 1992; Bohlmann and Croteau, 1999; Sharkey and Yeh, 2001), but terpene synthase activity is not always well correlated with the accumulation of end products of the pathway (Keller *et al.*, 1998a; Jennewein and Croteau, 2001; Pichersky *et al.*, 2006). In evaluating the regulatory importance of terpene synthases, it is necessary to consider not only the level of activity, but also its subcellular location. As we have noted above, monoterpenes and diterpenes are generally formed in the plastids, while sesquiterpene and triterpene biosynthesis is restricted to the cytosol (Mettal *et al.*, 1988; Kleinig, 1989; Turner *et al.*, 1999). Based on subcellular fractionation studies and the presence or absence of plastid transit peptides, the distribution of most terpene synthases follows this pattern. Most monoterpene and diterpene synthases are localized in the plastids (Mau and West, 1994; Aach *et al.*, 1995, 1997; Vogel *et al.*, 1996; Wise *et al.*, 1998; Yamaguchi *et al.*, 1998), while sesquiterpene and triterpene synthases are cytosolic (Belingheri *et al.*, 1988; Kleinig, 1989; Bohlmann *et al.*, 1998a; Steele *et al.*, 1998a). Terpene synthase activity itself seems to be regulated by the level of the corresponding mRNA (Facchini and Chappell, 1992; Chen *et al.*, 1995; Dudareva *et al.*, 1996; Keller *et al.*, 1998a; Steele *et al.*, 1998b). Reports of multi-gene families (Facchini and Chappell, 1992; Colby *et al.*, 1993; Back and Chappell, 1995) may imply complex developmental and tissue-specific patterns of regulation or may just indicate the existence of different synthases with closely related sequences.

In addition to terpene synthases, the construction of terpenoid carbon skeletons in plants also involves a number of prenyltransferases distinct from those that make the C_{10}, C_{15} and C_{20} diphosphates. One class of prenyltransferases catalyses 1′-4 condensations of IPP with an FPP or GGPP starter unit to make long-chain polyterpenes, such as rubber, a linear hydrocarbon with *cis* (*Z*) double bonds and as many as 30 000 isoprene units. The

cis-polyprenyltransferase participating in rubber biosynthesis has been characterized in several species of plants (Mooibroek and Cornish, 2000; Cornish, 2001; Takahashi and Koyama, 2006), but efforts to purify this protein or clone the corresponding gene have not yet been successful. Another class of prenyltransferases mediates condensations between allylic diphosphates and non-isoprenoid substrates, in which dimethylallyl, geranyl, farnesyl or geranylgeranyl moieties are transferred to a nucleophilic acceptor. These are key reactions in the formation of many different prenylated compounds, including prenylated proteins, prenylated flavonoids, furanocoumarins, cytokinins, ubiquinone, plastoquinone and the tocopherols. Several of the enzymes responsible have been well studied and are similar in gross properties to other prenyltransferases (Laflamme *et al.*, 1993; Cutler *et al.*, 1996; Qian *et al.*, 1996; Fellermeier and Zenk, 1998; Muhlenweg *et al.*, 1998; Yamamoto *et al.*, 2000).

5.3.5 Secondary transformations

The cyclic terpenes formed initially are subject to an assortment of further enzymatic modifications, including oxidations, reductions, isomerizations and conjugations, to produce the wide array of terpenoid end products found in plants. Unfortunately, few of these conversions have been well studied, and there is little evidence from most of the biosynthetic routes proposed, except in the case of the gibberellin (Yamaguchi, 2008) pathway. Many of the secondary transformations belong to a series of well-known reaction types that are not restricted to terpenoid biosynthesis. For example, the hydroxylation of terpenes by cytochrome P450-dependent oxygenases has been the subject of much investigation (Mihaliak *et al.*, 1993) (Fig. 5.8a). This large family of membrane-bound enzymes catalyses the position-specific hydroxylation of many terpenoids, using molecular oxygen and NADPH (Hallahan *et al.*, 1992; Hoshino *et al.*, 1995; Kato *et al.*, 1995; Winkler and Helentjaris, 1995; Helliwell *et al.*, 1998; Ro and Bohlmann, 2006). The first cDNA encoding a cytochrome P450-dependent terpene hydroxylase has recently been isolated (Lupien *et al.*, 1995).

A second group of oxidative enzymes, the 2-oxoglutarate-dependent dioxygenases, are soluble, nonhaeme iron-containing catalysts (Prescott and John, 1996) that participate in several reactions in terpene biosynthesis (Lange *et al.*, 1994; Phillips *et al.*, 1995; Xu *et al.*, 1995; Yamaguchi, 2008) (Fig. 5.8b). Several other types of secondary transformation that have been characterized include the oxidation of acyclic monoterpene alcohols to their corresponding aldehydes during iridoid biosynthesis in *Nepeta racemosa* (Hallahan *et al.*, 1995), the reduction of the geranylgeranyl moiety of chlorophylls, tocopherols and phylloquinone in *A. thaliana* (Keller *et al.*, 1998b) and the glucosylation of diterpene alcohols by glucosyltransferases in *Stevia rebaudiana* (Shibata *et al.*, 1995).

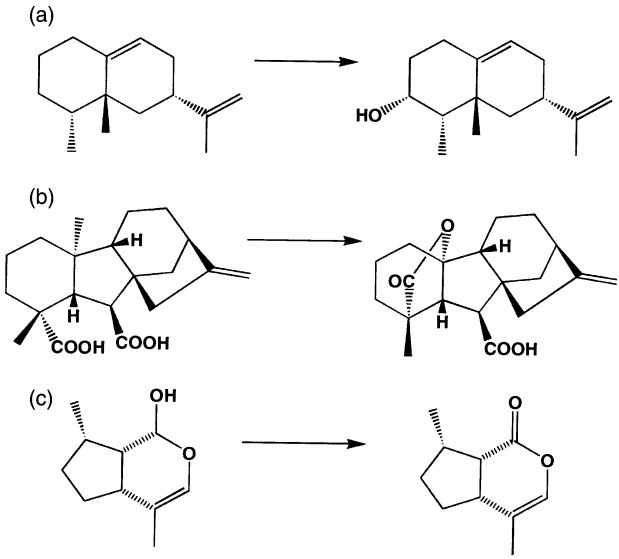

Figure 5.8 Examples of oxidative secondary transformations in terpenoid biosynthesis. (a) Hydroxylation of epi-aristolochene at the 3-position by a cytochrome P450-dependent terpene hydroxylase in *Capsicum annuum* (Hoshino *et al.*, 1995). (b) Conversion of GA_{12} to GA_9 by a 2-oxoglutarate-dependent dioxygenase involved in gibberellin biosynthesis. A single enzyme catalyses three successive oxidations leading to the loss of a methyl group and lactone formation (Lange *et al.*, 1994; Phillips *et al.*, 1995; Xu *et al.*, 1995). (c) Oxidation of *cis*, *cis*-nepetalactol to *cis*, *cis*-nepetalactone by a nicotinamide adenine dinucleotide (NAD^+)-dependent soluble oxidoreductase in *Nepeta racemosa* (Hallahan *et al.*, 1998).

5.4 Conclusions

Research on the formation and function of plant terpenoids has flourished in the past decades. The greatest achievement has been the discovery of a new, non-mevalonate route for the synthesis of the C_5 building blocks of terpenoids. While many of the intermediates of the new glyceraldehyde phosphate/pyruvate pathway are still unidentified and most of the enzymes are completely unknown, such details should be rapidly eludicated, setting the stage for studies on the distribution of the new pathway in plants and its relationship to the 'classical' mevalonate pathway. At present, the glyceraldehyde phosphate/pyruvate route appears to be found in the plastids of all higher plant species and is the likely source of substrate for the plastid-associated terpenoids, including monoterpenes, diterpenes, phytol, plastoquinones and carotenoids. In contrast, the mevalonate pathway appears to be restricted to the cytosol/ER based on the finding that all known pathway genes are targeted to this compartment. The mevalonate route may be the chief source of substrate for cytosolic (sesquiterpenes, triterpenes, dolichols) and mitochondrial (ubiquinone) terpenoids. Further

research is urgently needed to confirm these generalizations concerning the subcellular compartmentation of terpenoid biosynthesis. The extent to which the two pathways interact must also be clarified and the existence of a cryptic mevalonate pathway in the plastids, at least in certain taxa or specific developmental stages, must be investigated. With the basic features of the new, non-mevalonate pathway coming into focus, it is also time to re-evaluate the regulation of terpenoid formation in general, especially the role of HMGR, to determine which steps are the main modulators of flux.

As in most other branches of plant science, the application of molecular biology to terpenoid biosynthesis has led to enormous progress. The cloning and heterologous expression of biosynthetic enzymes have permitted new inferences about the evolution of these catalysts and have opened the door to site-specific mutagenesis and X-ray structure determination, which in turn have revealed much new information on enzyme structure and mechanism. For prenyltransferases and terpene synthases, two major groups of terpenoid-synthesizing enzymes that catalyse complex reactions involving carbocationic intermediates, we will soon achieve a detailed understanding of not only how the enzyme directs the outcome of the reaction, but also how redesign of the protein can give altered product distributions.

As terpenoids constitute the largest class of plant secondary compounds, it is fitting that terpenoid metabolites play a wide assortment of roles in nearly all basic plant processes. Recent research has added to this list, suggesting new functions for terpenoids, such as isoprene (stabilizing membranes at high temperatures), prenylated proteins (control of the cell cycle, allocation of nutrients) and certain mono- and sesquiterpenes (attraction of the enemies of herbivores). Nevertheless, the roles of most terpenoids are completely unstudied. Many compounds are thought to be involved in protecting plants from herbivores and pathogens, but supporting data are often fragmentary and unconvincing. In the coming years, the use of molecular techniques to make precise alterations to the levels of individual compounds should facilitate more rigorous investigation of the functional significance of terpenoids and give us a greater appreciation of their roles in plants.

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Chapter 6

BIOCHEMISTRY OF STEROLS, CARDIAC GLYCOSIDES, BRASSINOSTEROIDS, PHYTOECDYSTEROIDS AND STEROID SAPONINS

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Abstract: Phytosterols are synthesized via the mevalonate pathway of terpenoid formation and arise from the initial cyclization of 3*S*-squalene-2,3-epoxide. Plant steroids are derived from sterols and comprise steroid saponins, steroid alkaloids, pregnanes, androstanes, estranes, ecdysteroids, withanolides and cardiac glycosides. The typical route of sterol and steroid biosynthesis follows the cycloartenol pathway, whereas the lanosterol route seems to be operative mainly in fungi and animals. It was demonstrated, however, that both sterol pathways can be operative in higher plants. Crucial steps in the conversion of cycloartenol/lanosterol to sterols are the events leading to the removal of the methyl groups at C-4 and C-14. Meanwhile, all steps in the sterol pathway have been elucidated and the respective enzymes/genes characterized. The biosynthetic pathway leading from phytosterol precursors to the cardiac glycosides – important compounds in the treatment of cardiac insufficiency in humans – was basically deduced from studies using radiolabelled precursors. The more recent identification and characterization of several enzymes/genes involved in pregnane and cardenolide metabolism, such as 3β-hydroxysteroid dehydrogenase and progesterone 5β-reductase, have further clarified the pathway. Brassinosteroids (BRs) are hydroxylated derivatives of cholestane and they are specific plant steroid hormones that are essential for normal plant development. The biosynthesis of BRs has mainly been studied in *Arabidopsis thaliana*. Many of the genes encoding biosynthetic enzymes have been cloned using mutants of *Arabidopsis thaliana*, pea, tomato and rice which revert to a wild-type phenotype following treatment with exogenous BRs. Phytoecdysteroids are related in structure to the invertebrate steroid hormones. Their biological significance in plants is still under discussion. The understanding of the biosynthetic

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pathway(s) for phytoecdysteroids is very limited. Steroid saponins constitute a vast group of glycosides present almost exclusively in the monocotyledonous angiosperms, and occurring in only a few dicotyledonous families. As far as enzymatic and genetic aspects are concerned, the biosynthesis of steroid saponins (including the steroid alkaloids) has not been studied extensively. The withanolides are C_{28}-steroids and biogenetically related to the steroid saponins in that they are derived from ergostane-type sterols. These compounds appear to be specific for the Solanaceae and their biosynthesis has not yet been studied at the enzyme/gene level.

Keywords: biosynthesis; cardiac glycosides; ecdysteroids; metabolic pathways; phylogeny; pregnanes; saponins; secondary metabolites; withanolides

6.1 Introduction

Sterols, cardiac glycosides, BRs, phytoecdysteroids and steroid saponins are plant metabolites that may be considered to be triterpenes which have lost a minimum of three methyl groups during their biogenesis and are thus supposed to be derived from mevalonic acid via the triterpenoid pathway. All triterpenes originate from squalene, and the cyclic representatives, including the steroids, are composed of cyclohexane and cyclopropane units annelated *trans* or *cis*, the annelation being specific for the different groups of otherwise structurally closely related compounds (Table 6.1).

Separating triterpenes from steroids is not always easy, especially with regard to the close structural relationship between some tetracyclic structures, such as the ginsenosides, the cucurbitacins and cycloartenol (Fig. 6.1); only by considering the biosynthetic routes it is possible to separate the two groups. Members of both groups generally arise from the initial cyclization of 3*S*-squalene-2,3-epoxide (2,3-oxidosqualene). The opening of the epoxide initiates the cyclization and it is the initial conformation of 2,3-oxidosqualene which determines the biosynthetic route to follow. Therefore, different 2,3-oxidosqualene cyclases must be involved in the formation of the more than 4000 triterpenes (including steroids) isolated from plants so far (Abe, 2007; Vincken *et al.*, 2007).

Cardiac glycoside and steroid saponin biosynthesis in vascular plants cannot be separated from sterol biosynthesis, which will therefore also be discussed in this chapter. BRs and phytoecdysteroids may be synthesized on routes in part similar to sterol and/or cardenolide biosynthesis and therefore will also considered here. In higher plants, triterpenoids most often occur as 3-*O*-glycosides, 3-*O*-acyl esters and/or glucose esters; the hydroxyl group in position C-3 arising from the opening of the 2,3-epoxide of oxidosqualene. It is assumed that 2,3-oxidosqualene cyclases are regulatory key enzymes in the isoprenoid pathway with a high degree of specificity, thus orienting the biosynthetic flux towards either tetracyclic or pentacyclic structures (e.g. Henry *et al.*, 1992). More recent findings concerning the formation of ginseng

Table 6.1 Ring annelation in different steroids

Group	Individual substance	Rings A/B	Substituents in position 5/10	Rings B/C	Substituents in position 8/9	Rings C/D	Substituents in position 13/14
Sterols	Lanosterol	*trans*	α/β	—	—	*trans*	α/β
	Cycloartenol	*trans*	α/β	*cis*	β/β	*trans*	α/β
	Euphol	*trans*	α/β	—	—	*trans*	α/β
	Cholesterol	—	—	*trans*	α/β	*trans*	α/β
Saponins	Smilagenin	*cis*	β/β	*trans*	α/β	*trans*	α/β
	Tigogenin	*trans*	α/β	*trans*	α/β	*trans*	α/β
	Diosgenin	—	—	*trans*	α/β	*trans*	α/β
C_{27}-Steroid alkaloids	α-Tomatine	*trans*	α/β	*trans*	α/β	*trans*	α/β
	Solasodine	—	—	*trans*	α/β	*trans*	α/β
Bile alcohols and bile acids	Allocholic acid	*trans*	α/β	*trans*	β/α	*trans*	β/α
	Cholic acid	*cis*	β/β	*trans*	β/α	*trans*	β/α
Pregnanes and allopregnanes	Urocortisol	*cis*	β/β	*trans*	β/α	*trans*	β/α
	Alloconolone	*trans*	α/β	*trans*	α/β	*trans*	α/β
	Progesterone	—	—	*trans*	α/β	*trans*	α/β
	Digipurpurogenin	—	—	*trans*	α/β	*cis*	β/β
	Androstanes	—	—	*trans*	α/β	*trans*	α/β
	Testosterone						
	5α-Androstane-17β-ol-3-one	*trans*	α/β	*trans*	α/β	*trans*	α/β
Estranes	Estradiol	—	—	*trans*	α/β	*trans*	α/β
Cardiac glycosides	Digitoxigenin	*cis*	β/β	*trans*	α/β	*cis*	β/β
	Uzarigenin	*trans*	α/β	*trans*	α/β	*cis*	β/β
	Scillarenin	—	—	*trans*	β/a	*cis*	R/P

Source: After Luckner (1990).

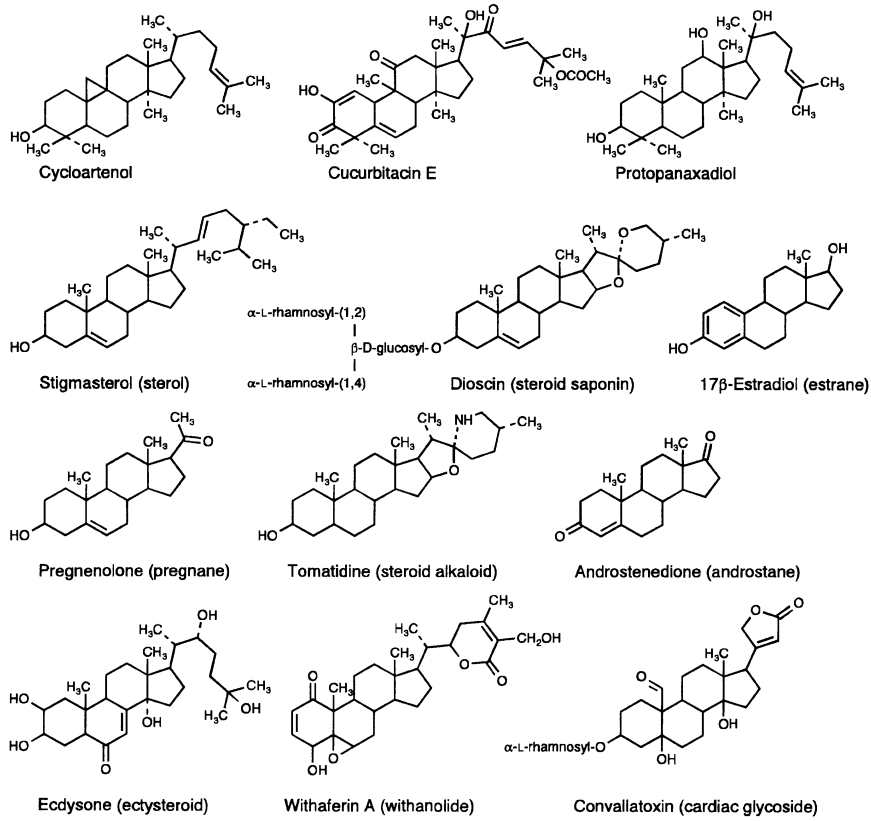


Figure 6.1 Chemical structures of plant metabolites synthesized from squalene-2,3-epoxide.

saponins support this assumption. (*RS*)-(3-3H)-2,3-oxidosqualene was converted into (20*S*)-dammarenediol (= protopanaxadiol, Fig. 6.1), but not to (20*R*)-dammarenediol by a microsomal fraction prepared from hairy roots of *Panax ginseng*. The properties of the cyclase differed significantly from those of other 2,3-oxidosqualene cyclases reported from higher plants (Kushiro *et al.*, 1997; Abe, 2007). The enzymatic cyclizations of squalene and oxidosqualene are important steps in the biosynthesis of sterols and triterpenes. The polyenes are converted to various polycyclic triterpenes by different enzyme systems employing only small modification of the active site. Abe (2007) recently reviewed crystallographic and structure-based mutagenesis studies which revealed structural details of the different cyclases.

Although bearing a sterane nucleus, the biosyntheses of cucurbitacins and dammaran-type saponins will not be reviewed here. Both groups belong to the triterpenoids, the chemistry, biosynthesis and biological activities of which have been described elsewhere (Charlwood and Banthorpe, 1991;

Mahato *et al.*, 1992; Haralampidis *et al.*, 2002; Vincken *et al.*, 2007). In this chapter, emphasis will be laid on the formation of sterols and steroids in plants; the pathways leading to the tetra- and pentacyclic triterpenes will not be considered further.

Steroids are widely used as drugs and constitute anti-inflammatory, contraceptive and anti-cancer agents. Most are obtained by semi-synthesis using natural substances, such as sterols (from plants or animals), saponins, including steroid alkaloids (from plants) and bile acids (from animals) as precursors. Plant steroids comprise sterols, steroid saponins, steroid alkaloids, pregnanes, androstanes, estranes, ectysteroids, withanolides and cardiac glycosides (Fig. 6.1), which all share the same basic skeleton. Some of them are widespread (sterols, pregnanes) in the plant kingdom, whereas the occurrence of others (androstanes, estranes, withanolides) is limited. Estranes, e.g., have been found in seeds of *Punica granatum* (Dean *et al.*, 1971) and androstenes accumulate in pollen of *Pinus sylvestris* (Saden-Krehula *et al.*, 1976).

6.2 Sterols

Sterols (Fig. 6.1) are primary metabolites and have essential functions in all eukaryotes. For example, free sterols are integral components of the membrane lipid bilayer where they play an important role in the regulation of membrane fluidity and permeability. While cholesterol is the major sterol in animals, a mixture of various sterols is present in higher plants, with sitosterol usually predominating. Higher plants, algae, most fungi and vertebrates are capable of synthesizing sterols. In sterol biosynthesis, squalene 2,3-epoxide can cyclize in two ways, to form lanosterol and cycloartenol, respectively. The cycloartenol pathway of steroid biosynthesis appears to be specific for photosynthetic eukaryotes, whereas the lanosterol route seems to be operative mainly in fungi and animals. An *Arabidopsis thaliana* gene encoding cycloartenol synthase was expressed in a yeast mutant lacking lanosterol synthase (LSS). Several of the transformants were able to cyclize squalene 2,3-epoxide to cycloartenol (Corey *et al.*, 1993). Although most plant steroids are derived from cycloartenol, it has to be mentioned that lanosterol and lanosterol oligosaccharides have been detected in various plants, e.g. in the latex of different *Euphorbia* species. Since the conversion of cycloartenol to lanosterol could not be demonstrated, it was proposed that both sterol pathways are operative in these plants (Giner and Djerassi, 1995). Only recently, genome-mining experiments revealed that *Arabidopsis thaliana* encodes, in addition to cycloartenol synthase, an LSS. The co-existence of cycloartenol synthase and LSS implies specific roles for both cyclopropyl and conventional sterols in plants. Phylogenetic reconstructions revealed that LSSs are broadly distributed in eudicots, but evolved independently from those in animals and fungi. Novel catalytic motifs establish that plant LSSs comprise a third catalytically distinct class of LSS (Kolesnikova *et al.*, 2005).

6.2.1 Biosynthesis

The biosynthesis of plant sterols was comprehensively reviewed by Benveniste (1986, 2004). A matrix of alternative routes along a main road was proposed (Oehlschlager *et al.*, 1984). As in cardenolide formation (see Section 6.3) and gibberellin formation (e.g. Hedden and Kamiya, 1997), we have to consider multi-dimensional grids instead of linear biosynthetic pathways (Figs 6.2 and 6.3). Recently, various genes and enzymes that are involved in the formation of plant sterols have been identified and characterized. The mechanisms of enzyme action were elucidated in studies using analogues of the high-energy carbocationic intermediates supposed to be involved in the various biosynthetic steps. Additional information was provided by

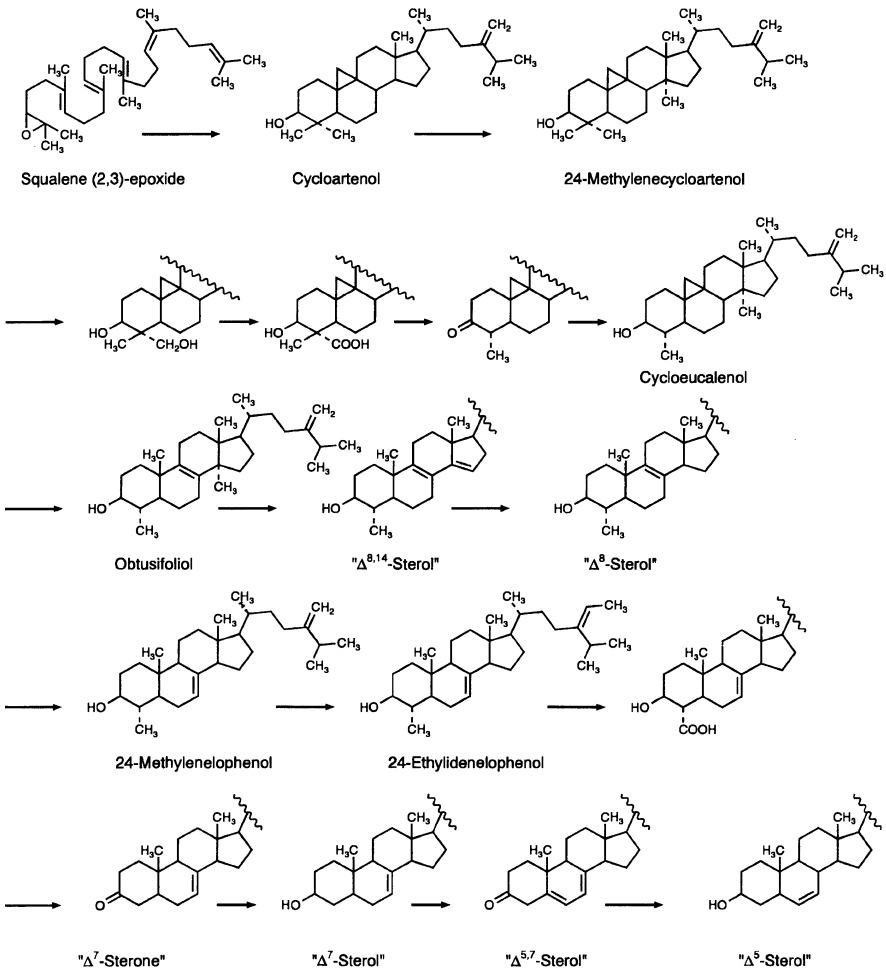


Figure 6.2 Proposed pathway for sterol biosynthesis in higher plants.

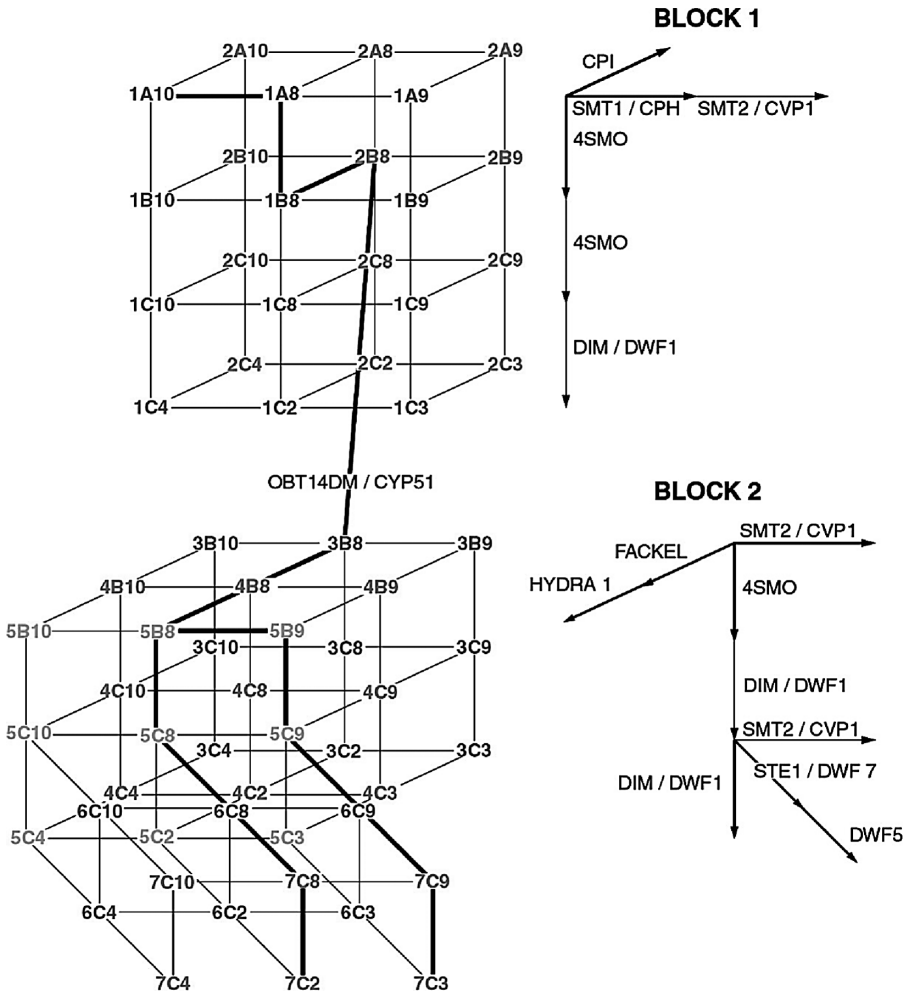


Figure 6.3 Biosynthesis of (24ξ)-24-methyl cholesterol (7C2) and (24R)-24-ethyl cholesterol (7C3) in vascular plants. A matrix of alternative routes along a main road is proposed. The structure of sterols is indicated by a number, a letter and a number. The first number represents a panel that gathers compounds with the same steroid nucleus (see Benveniste, 2004, for detailed translation of the codes). Cycloartenol (1A10) derives from 2(3)-oxidosqualene. The direction of the biosynthetic flow is indicated by the coordinate axes. The preferred biosynthetic pathway operating in most vascular plants is underlined by heavy arrows. CPI, cyclopropyl sterol isomerase; SMT, sterol methyltransferase; OBT14DM, obtusifoliol-14-demethylase; SMO, sterol 4-methyl oxidase; *CPH*, *CEPHALOPOD*; *CVP1*, *COTYLEDON VASCULAR PATTERN*; *DIM/DWF1*, gene encoding the Δ^5-sterol-Δ^{24}-reductase (isomerase); *FACKEL*, gene encoding the $\Delta^{8,14}$-sterol-Δ^{14}-reductase; *HYDRA1*, gene encoding the Δ^8–Δ^7-sterol isomerase; *DWF5*, gene encoding the $\Delta^{5,7}$-sterol-Δ^7-reductase; *DWF7*, Δ^7-sterol-C5(6)-desaturase. (Modified after Benveniste, 2004.)

studies using various commercial or experimental fungicides that have been found to interfere with plant sterol biosynthesis. Morpholine-type fungicides, e.g., inhibit cycloeucalenol isomerization, Δ^8/Δ^7-isomerization, Δ^{14}-reduction and Δ^7-reduction in the sterol pathway (Fig. 6.2), whereas azole fungicides were shown to block the 14α-demethylation step (see Section 6.2.1.3) (Rahier and Taton, 1997).

The biosynthesis of cholesterol in plants is not yet fully understood, but is probably similar to the formation of the 24-alkyl sterols, i.e. via cycloartenol, although in animals cholesterol is only formed via the lanosterol pathway. Exogenous cholesterol can be transformed by plants to various products, including pregnanes and other steroids (e.g. Bennett and Heftmann, 1966; Caspi *et al.*, 1966). Cholesterol is generally a minor sterol in plants; however, its concentration may be high in certain members of the Solanaceae. Overexpression of a sterol methyltransferase (SMT) in transgenic potato resulted in a marked reduction of cholesterol and glycoalkaloid levels, which supports the view to consider cholesterol as a precursor in steroid alkaloid biosynthesis (Arnqvist *et al.*, 2003). Cholesterol was also considered as a precursor of cardiac glycosides (see Section 6.3).

Microsomes prepared from maize (*Zea mays*) embryos or seedlings have proved to be an excellent biochemical system to study sterol biosynthesis in vitro. The development of molecular genetics tools, the availability of specific *Arabidopsis thaliana* mutants and the possibilities to genetically transform these mutants allowed the application of a new strategy to study sterol biosynthesis pathways (Benveniste, 2004). Some important results of both approaches are summarized above (Figs 6.2 and 6.3).

6.2.1.1 Sterol methyltransferases

The enzymes involved in C-24 alkylation in plant sterol formation have been described by Benveniste (1986, 2004). A full-length cDNA sequence was isolated from *Arabidopsis thaliana*, which contained features typical of methyltransferases in general and, in particular, showed 38% identity with a yeast gene encoding zymosterol-C-24-methyltransferase. A yeast mutant accumulating zymosterol, i.e. not capable of sterol C-24 alkylation, was transformed with the plant gene. As a result, several 24-ethyl and 24-ethylidene sterols were synthesized, indicating that the respective cDNA encodes a plant sterol C-methyltransferase able to perform two sequential methylations of the sterol side chain (Husselstein *et al.*, 1996). Microsomes prepared from the mutant expressing the *Arabidopsis thaliana* SMT possess *S*-adenosyl methionine-dependent sterol-C-methyltransferase activity. Delipidated preparations of these microsomes converted cycloartenol into 24-methylene cycloartanol and 24-methylene lophenol into 24-ethylidene lophenol. However, the catalytic efficiency of the expressed SMT was 17 times higher with 24-methylene lophenol than with cycloartenol. This was taken as evidence that the *Arabidopsis thaliana* cDNA ATSMT2-1 encodes a 24-methylene-lophenol-C-24^1-methyltransferase catalysing the second methylation step of plant sterol

biosynthesis (Bouvier-Navé *et al.*, 1997). cDNAs from *Glycine max* (Shi *et al.*, 1995, 1996), *Ricinus communis* (Bouvier-Navé *et al.*, 1997), *Zea mays* (Grebenok *et al.*, 1997), *Nicotiana tabacum*, *Oryza sativa* (Bouvier-Navé *et al.*, 1998) and *Arabidopsis thaliana* (Diener *et al.*, 2000; Schaeffer *et al.*, 2002) encode proteins that are about 80% identical in all possible combinations, but have only 40% identity with ATSMT2-1. A yeast mutant transformed with the tobacco SMT1 gene efficiently converted cycloartenol into 24-methylene cycloartanol, but not 24-methylene lophenol into 24-ethylidene lophenol, indicating that *NTSMT1* encodes a cycloartenol-C24 methyltransferase (Bouvier-Navé *et al.*, 1998). Meanwhile, the expression of *SMT2* and *SMT1* was also studied in plants (Schaller *et al.*, 1998; Schaeffer *et al.*, 2000; Schaeffer *et al.*, 2002; Holmberg *et al.*, 2002). *SMT1* controls the flux of carbon into sterol biosynthesis in tobacco (Holmberg *et al.*, 2002). *Arabidopsis thaliana* plants overexpressing a *35S::ATSMT2-1* transgene accumulated sitosterol at the expense of campesterol (Schaeffer *et al.*, 2002).

6.2.1.2 4,4-Dimethyl sterol 4-demethylase

Crucial steps in the conversion of cycloartenol to sterols are the events leading to the removal of the methyl groups at C-4 and C-14. C-4monodemethylation of 28-(^{3}H),24-methylene cycloartanol leads to the corresponding 4α-methyl sterol, cycloeucalenol. The demethylation process requires NADPH and molecular oxygen, and was shown to involve a 4-methyl, 4-hydroxymethyl derivative. From inhibitor studies, it was concluded that the C-4 demethylation of methylene cycloartanol results from a multi-step process, involving a terminal oxygenation system sensitive to cyanide that is distinct from cytochrome P450 (Pascal *et al.*, 1990). Immunoglobulin G (IgG), raised against plant cytochrome b_5, was used to characterize the electron-donating system further and it was found that the activities of 4,4-dimethyl sterol 4-demethylase (4,4-DMSO), 4α-methylsterol-4α-methyl oxidase and sterol Δ^7-sterol C-5(6)-desaturase (5-DES) (see Section 6.2.1.9) were completely inhibited by the antibody. These results suggest that membrane-bound cytochrome b_5 is carrying electrons from NAD(P)H to 4,4-DMSO, 4α-MSO and 5-DES (Rahier *et al.*, 1997).

Rahier and his co-workers also characterized the activities of a sterol C-4 methyl oxidase (SMO), a 4-carboxysterol-3-hydroxysteroid dehydrogenase/C-4 decarboxylase (3-HSD/D) and an NADPH-dependent 3-oxosteroid reductase in order to define the steps involved in C-4 demethylation in plants (Pascal *et al.*, 1993; Rondet *et al.*, 1999). Only recently, they have isolated two cDNAs from *Arabidopsis thaliana* encoding bifunctional 3-HSD/D. Transformation of a yeast ergosterol auxotroph mutant, which lacks 3-HSD/D activity, with either of these cDNAs restored ergosterol biosynthesis in the yeast mutant (Rahier *et al.*, 2006).

6.2.1.3 Cyclopropyl sterol isomerase

Already in 1974, Heintz and Benveniste (1974) reported the enzymatically catalysed opening of the cyclopropane ring of cycloeucalenol in bramble

(*Rubus fruticosus*) tissue cultures, in this way producing obtusifoliol. This step seems to be restricted to the plant kingdom and is catalysed by the cyclopropyl sterol isomerase (CPI), the catalytic mechanism of which has been thoroughly studied (Heintz and Benveniste, 1974; Rahier *et al.*, 1989) using sterol biosynthesis inhibitors as fungicides in agriculture. Expressing an *Arabidopsis thaliana* cycloartenol synthase cDNA in a yeast, LSS mutant provided a sterol auxotroph that could be genetically complemented with the isomerase. This cDNA was also expressed in *Escherichia coli* and it was shown by gas chromatography–mass spectrometry that protein extracts from this strain isomerized cycloeucalenol to obtusifoliol in vitro (Lovato *et al.*, 2000).

6.2.1.4 Obtusifoliol 14α-demethylase

In animals and fungi, the 14α-methyl group is the first of three methyls to be removed; however, in higher plants the 14α-methyl is only removed after one C-4 methyl was lost. The 14α-methyl group of obtusifoliol is then removed by the action of a cytochrome P450-containing monooxygenase system (Rahier and Taton, 1986). A-series of 7-oxo-obtusifoliol analogues and other compounds have been synthesized and investigated as potential inhibitors of the enzyme. Some of them were potent competitive inhibitors, binding 125–200 times more tightly than obtusifoliol. Feeding of one of the compounds synthesized, namely 7-oxo-24(25)-dihydro-29-norlanosterol, to cultured bramble cells resulted in a strong decrease of (^{14}C)-acetate incorporation into the demethyl-sterols fraction and in an accumulation of labelled obtusifoliol (Rahier and Taton, 1992). The *R*-(−) isomer of methyl 1-(2,2-dimethylindan-1-yl)imidazole-5-carboxylate (CGA 214372) inhibited obtusifoliol 14α-demethylase uncompetitively and was shown to have a high degree of selectivity for obtusifoliol 14α-demethylase (Salmon *et al.*, 1992).

Evidence is accumulating that obtusifoliol 14α-demethylase may be a good target for herbicides. For example, *Nicotiana tabacum* protoplasts have been transformed with the gene CYP51A1 encoding lanosterol-14-demethylase from *Saccharomyces cerevisiae*. Transgenic calli were killed by a phytotoxic fungicide inhibiting both plant obtusifoliol-14-demethylase and lanosterol-14-demethylase but were resistant to 7-ketotriazole, a herbicide which has been shown to inhibit obtusifoliol-14-demethylase only. It seems that lanosterol-14-demethylase can bypass the blocked obtusifoliol-14-demethylase, in this way causing the plant tissue to be resistant to a triazole herbicide (Grausem *et al.*, 1995). Screening of a wheat cDNA library with a heterologous CYP81B1 probe from *Helianthus tuberosus* led to the isolation of a cDNA coding for obtusifoliol 14-a-demethylase. The cDNA was expressed in *Saccharomyces cerevisiae,* and it was demonstrated that membranes isolated from yeast expressing the gene efficiently catalysed 14α-demethylation of obtusifoliol. From the molecular data, the enzyme was assigned to the CYP51 family (Cabello-Hurtado *et al.*, 1997). The respective CYP51 from *Sorghum bicolor* was cloned and expressed in *Escherichia coli* (Bak *et al.*,

1997). The plant enzymes (but not sterol 14-demethylases from fungal or human origin) showed strict substrate specificity towards obtusifoliol. The *Sorghum* enzyme, e.g., was not capable of demethylating various lanosterol derivatives, indicating that a demethylating sequence 4, 14, 4 is realized in plants (Lamb *et al.*, 1998).

cDNAs encoding lanosterol, eburicol and obtusifoliol 14-demethylases have been isolated from mammals (Aoyama *et al.*, 1994), fungi (Kalb *et al.*, 1986) and plants (Bak *et al.*, 1997; Cabello-Hurtado *et al.*, 1999), respectively. They share an amino acid identity ranging from 38 to 65% and were classified in the same family, namely CYP51 (Nelson *et al.*, 1996). The function of the *Nicotiana benthamiana* ortholog of *AtCYP51* has been demonstrated by silencing the endogenous *CYP51* with *potato virus X::NtCYP51-1* transcripts. This treatment resulted in a strong accumulation of obtusifoliol and other 14α-methyl sterols at the expense of campesterol and sitosterol (Burger *et al.*, 2003).

6.2.1.5 4α-Methylsterol demethylase

All reactions in the process of plant sterol demethylation appear to proceed via α-face attack. In fact, after the sequential oxidative 4α-demethylation of 4,4-dimethylsterols, a 4α-monomethyl sterol is produced. However, this compound cannot be demethylated further by the action of 4α-methylsterol demethylase, since this enzyme favours 4α-methyl sterols with rigid planar conformation. These structural requirements satisfy the Δ^7-sterols that are, however, formed only after sterol 14α-demethylation (see Section 6.2.1.4). Later on, the oxidative conversion of 24-methylene cycloartanol to cycloeucalenol was demonstrated in vitro. 4α-Carboxysterol decarboxylation shows an exclusive requirement for an oxidized pyridine nucleotide, with NAD+ being more efficient than NADP+. The decarboxylation reaction is independent of molecular oxygen. 4α-Carboxysterol-C3-dehydrogenase/C4-decarboxylase (4α-CD) is a microsome-bound protein (Rondet *et al.*, 1999). Obviously, demethylation at C4 of plant sterols is composed of two separate processes: an oxygen- and NAD(P)H-dependent oxidation of the 4α-methyl group to produce the 4α-carboxysterol metabolite followed by oxygen-independent dehydrogenation/decarboxylation to produce an obligatory 3-ketosteroid intermediate. Extensive substrate recognition and inhibitor studies have further established that in higher plants the demethylations occur in the sequence 4, 14, 4, in contrast to animals and yeast where the sequence is 14, 4, 4 (Taton *et al.*, 1994).

6.2.1.6 Sterone reductase

Microsomes prepared from maize embryos were also shown to catalyse the reduction of various sterones to produce the corresponding 3β-hydroxy derivatives. Based on studies concerning co-enzyme requirements and inhibitor susceptibility, the enzyme termed sterone reductase was classified as belonging to the family of ketone reductases. Since 4,4-dimethyl-sterones react poorly as compared to desmethyl- or 4α-monomethyl sterones, it was concluded

that the reductase is a component of the microsomal sterol 4-demethylation complex (Pascal *et al.*, 1993, 1994). The enzyme may be related to the hydroxysteroid oxidoreductases involved in cardenolide biosynthesis (see Section 6.3.1.2).

6.2.1.7 $\Delta^{8,14}$-Sterol Δ^{14}-reductase

This enzymatic double-bound reduction is thought to proceed through an electrophilic addition mechanism. Using an in vitro assay, ammonium and iminium analogues of the putative C-14 carbonium intermediate were shown to be potent inhibitors of the reduction reaction. The relative specificity of these different series of inhibitors towards cycloeucalenol–obtusifoliol isomerase, Δ^8–Δ^7-sterol isomerase (SI) and $\Delta^{8,14}$-sterol Δ^{14}-reductase was studied directly (Taton *et al.*, 1989). The *Arabidopsis thaliana* gene FACKEL (At3g52940) was shown to encode an integral membrane protein with eight to nine transmembrane segments related to the vertebrate lamin receptor and several sterol C-14 reductases, including yeast sterol C-14 reductase ERG24. Functional evidence was provided that FACKEL encodes a sterol C-14 reductase. GC/MS analysis confirmed that mutations in this gene lead to accumulation of intermediates in the biosynthetic pathway preceding the C-14 reductase step (Schrick *et al.*, 2000).

6.2.1.8 Δ^8–Δ^7-Sterol isomerase

When the 14α-methyl group is removed and the 14 double bond is reduced, the resulting Δ^8-sterols are isomerized to Δ^7-sterols. This process is catalysed by a Δ^8–Δ^7-isomerase. In plants, 4α-methyl-5α-ergosta-8,24(24^1)-dien-3β-ol is the substrate of this enzyme. An *Arabidopsis thaliana* Δ^8–Δ^7-SI cDNA has been isolated by functional complementation of the corresponding *Saccharomyces cerevisiae* sterol mutant (*erg2*) (Souter *et al.*, 2002). Mutants deficient in the Δ^8–Δ^7-isomerase gene (*HYDRA1;* At1g20050) are strongly depleted in campesterol and sitosterol.

6.2.1.9 Δ^7-Sterol C-5(6)-desaturase

During plant sterol synthesis, the Δ^5-bond is supposed to be introduced via the sequence Δ^7-sterol $\Rightarrow$ $\Delta^{5,7}$-sterol $\Rightarrow$ Δ^5-sterol. A microsomal enzyme system was identified that catalyses the conversion of Δ^7-sterols to their corresponding Δ^5-sterols. Part of the sequence is catalysed by a sterol desaturase (5-DES) requiring molecular oxygen and NADH. The enzyme appears to be specific for 4-desmethyl-Δ^7-sterols favouring sterols possessing a C-24 methylene or ethylidene substituent (Taton and Rahier, 1996). An *Arabidopsis thaliana* cDNA encoding a 5-DES was isolated and characterized by functional complementation of the yeast mutant *erg3* (Gachotte *et al.*, 1996). Overexpression of the *Arabidopsis thaliana* desaturase cDNA in transgenic *ste1* mutants (deficient in this particular gene) led to full complementation. Besides the 5-DES considered above (At3g02580), a second gene (At3g02590) coding for a 5-DES has been identified (Choe *et al.*, 1999).

6.2.1.10 $\Delta^{5,7}$-Sterol Δ^{7}-reductase

This enzyme catalyses the reduction of the Δ^{7}-double bond of the $\Delta^{5,7}$-sterols into Δ^{5}-sterols in vertebrates and higher plants. A microsomal preparation from seedlings of *Zea mays* catalysed the NADPH-dependent reduction of the Δ^{7}-bond of $\Delta^{5,7}$-cholestadienol, providing the first in vitro evidence for the intermediacy of $\Delta^{5,7}$-sterols in plant sterol biosynthesis (Taton and Rahier, 1996). The potent inhibition of the enzyme by ammonium-containing fungicides suggests a cationic mechanism involved in this reduction reaction (Taton and Rahier, 1991).

With a view to producing $\Delta^{5(6)}$-pregnenes in yeast, the Δ^{7}-reductase (7-RED) gene from *Arabidopsis thaliana* was engineered into *Saccharomyces cerevisiae* in order to overcome the dominance of endogenous $\Delta^{5(6),7}$ sterols, such as ergosterol. Coexpression of bovine side-chain cleavage $P450_{scc}$ (see Section 6.3.1.1), adrenodoxin and adrenodoxin reductase, led to the formation of pregnenolone, which was found to be totally absent from cell lysates or culture medium from control strains. Following additional coexpression of human NAD:Δ^{5}-3β-hydroxysteroid dehydrogenase, pregnenolone was further metabolized to progesterone. The majority of pregnenolone and progesterone produced remained sequestered in the yeast cells (Duport *et al.*, 1998).

6.2.1.11 Δ^{5}-Sterol Δ^{24}-reductase/isomerase

In higher plants, substrates for this enzyme are 24-methylene cholesterol and isofucosterol. Both sterols are probably isomerized in $\Delta^{24(25)}$-sterols prior to reduction. Feeding experiments using deuterium-labelled 24-methylenecholesterol and 24-methyl desmosterol demonstrated that the *Arabidopsis thaliana* protein *DIM/DWF1* is involved in both the isomerization and reduction of the $24(24^{1})$ bond and encodes a sterol $C24(24^{1})$ reductase isomerase (Klahre *et al.*, 1998). The peptide sequence of *DIM/DWF1* from *Arabidopsis thaliana* has 41% identity with a *Homo sapiens* ortholog (seladin-1), but no significant identity with the ERG4 gene of *Saccharomyces cerevisiae*. Thus, the C24 reduction step is performed by completely different enzymatic systems in higher plants and animals on one hand and yeast on the other hand.

6.2.1.12 Sterol 3-*O*-glucosyltransferase

Sterol 3-*O*-glucosyltransferases (SGTases) are membrane-bound enzymes and have been isolated from various sources. When investigating the localization of SGTase, it was found that the enzyme is only associated with the plasma membrane; therefore, SGTase is now being used as a marker enzyme for plasma membranes. It was shown that delipidated protein preparations showed no SGTase activity but that enzyme activity could be restored completely when phospholipids were added. The effect of different phospholipids on recovery of SGTase activity and the kinetic parameters of the reaction was studied using a delipidated and inactive enzyme preparation obtained from maize coleoptiles. Both phosphatidylcholine and phosphatidylglycerol

significantly decreased K_m and increased V_{max} (Ullman *et al.*, 1984, 1987). SGTase was reconstituted into unilamellar lipid vesicles. This was achieved by adding phospholipids, sterols and β-octylglucoside to the solubilized enzyme and passing the mixture through Sephadex G-50. An outward orientation for the active site of the enzyme was suggested and it was demonstrated that reconstituted SGTase activity is stimulated to a large extent by negatively charged phospholipids (Ury *et al.*, 1989).

SGTase was purified from *Avena sativa*. Polyclonal antibodies raised against *Avena* SGTase did not inhibit enzyme activity but are specifically bound to the native enzyme (Warnecke and Heinz, 1994). The purified SGTase has been used for the cloning of a corresponding cDNA from *Avena sativa*. Different fragments of the cDNA obtained were expressed in *Escherichia coli* and it was found that homogenates of the transformed cells exhibited sterol glucosyltransferase activity (Warnecke *et al.*, 1997).

SGTase was also detected in cell cultures and leaves of *Digitalis purpurea*. In the cultured cells, the enzyme was not associated with a specific subcellular fraction. However, almost 60% of the enzyme isolated from leaves was associated with the microsomal fraction. SGT was partially purified from both sources. Δ^5-Steroids were good substrates for the SGTase from *Digitalis purpurea*. 5α-Steroids, such as epiandrosterone and 5α-pregnan-3β-ol-20-one, were better substrates than their corresponding 5β-analogues. Digitoxigenin, a 5β-cardenolide genin (see Section 6.3), was only a poor substrate for the SGTase (Yoshikawa and Furuya, 1979).

Evidence is accumulating that at least two SGTases are present in potato: a membrane-bound enzyme with high affinity to sitosterol and a cytosolic enzyme with high affinity to solanidine, a steroid alkaloid (see Section 6.5.2). The membrane-bound enzyme glucosylated the substrates investigated in the following sequence: plant sterols > androstanes, pregnanes > steroid alkaloids (spirosolane type), steroid sapogenins > steroid alkaloids (solanidane type). The cytosolic SGTase clearly preferred steroid alkaloids of the solanidane type (Zimowski, 1992). cDNAs from *Avena sativa* and *Arabidopsis thaliana* have been identified that encode polypeptides of 608 (*Avena sativa*) and 637 (*Arabidopsis thaliana*) amino acid residues (Warnecke *et al.*, 1997). In vitro enzyme assays with cell-free extracts of *Escherichia coli* strains transformed with these cDNAs show UDP-glucose-dependent sterol glucosyltransferase activity using cholesterol, sitosterol and ergosterol as sterol acceptors (Warnecke *et al.*, 1999).

6.2.1.13 Sterol acyltransferase (SGTase) and steryl ester hydrolase (SEHase)

Unesterified sterols modulate the function of eukaryotic membranes. In human cells, sterol is esterified to a storage form by acyl-co-enzyme A (CoA):cholesterol acyltransferase (SGTase). In plants, free sterols are associated mainly with microsomal membranes, whereas the steryl esters are stored in lipid granules. The esterification process may, thus, allow regulation of the

amount of free sterols in membranes by subcellular compartmentation. Enzymes involved in the esterification of sterols and hydrolysis of steryl esters were investigated in tobacco. Results obtained with a sterol-overproducing mutant indicated that both SGTase and SEHase are involved in the control of the free sterol content and, more generally, in the homeostasis of free sterols in the plant cells (Bouvier-Navé and Benveniste, 1995).

Other enzymes involved in pregnane metabolism will be introduced when discussing cardenolide and BR biosynthesis (see Sections 6.3 and 6.4).

6.2.2 Biotransformation

Exogenous organic compounds can be modified by living cells. These modifications are generally referred to as 'biotransformations'. Plant cell suspension cultures can be used for biotransformation purposes (see, e.g. the comprehensive reviews of Kurz and Constabel, 1979; Reinhard and Alfermann, 1980). The supply of a suitable precursor may result in the formation of a product known from the intact plant or closely related compounds with interesting biological properties. In addition, the demonstration of a biotransformation reaction may be a first step in the elucidation of an enzyme-catalysed conversion.

The transformations of cholesterol, progesterone, pregnenolone and pregnanes have been studied extensively with cell cultures of *Atropa belladonna*, *Brassica napus*, *Catharanthus roseus*, *Capsicum frutescens*, *Cheiranthus cheiri*, *Digitalis lanata*, *D. lutea*, *D. purpurea*, *Dioscorea deltoidea*, *Glycine max*, *Hedera helix*, *Lycopersicum esculentum*, *Nicotiana rustica*, *N. tabacum*, *Parthenocissus* spp., *Rosa* spp., *Solanum tuberosum* and *Sophora angustifolia*. The biotransformation reactions observed include: reduction of double bonds; reduction of the 3-keto function; oxidation of the 3-hydroxyl group; reduction of the 20-keto group; 6β-, 11α- and 14α-hydroxylation; as well as 3-*O*-glucoside and 3-*O*-palmitate formation (Kurz and Constabel, 1979; Reinhard and Alfermann, 1980).

Mucuna pruriens cell cultures are known to hydroxylate a variety of phenolic compounds (Pras, 1990). The solubility of the phenolic steroid, 17β-estradiol (Fig. 6.1), is only 12 μM in culture medium and no biotransformation products could be detected after administration to freely suspended cells, immobilized cells or partially purified *Mucuna phenoloxidase*. Complexation with β-cyclodextrin dramatically enhanced the solubility of 17β-estradiol. Alginate-entrapped cells, cell homogenates and the phenoloxidase were able to *o*-hydroxylate 17β-estradiol when supplied as the cyclodextrin complex, the most efficient biotransformation being achieved with the isolated enzyme (Woerdenbag *et al.*, 1990).

A green cell suspension culture of *Marchantia polymorpha*, a liverwort, was shown to convert testosterone (Fig. 6.4) to 6β-hydroxytestosterone and epitestosterone to androst-4-ene-3,17-dione (Hamada *et al.*, 1991). The same culture was able to reduce the C-17 carbonyl of androst-4-ene-3,17-dione. It

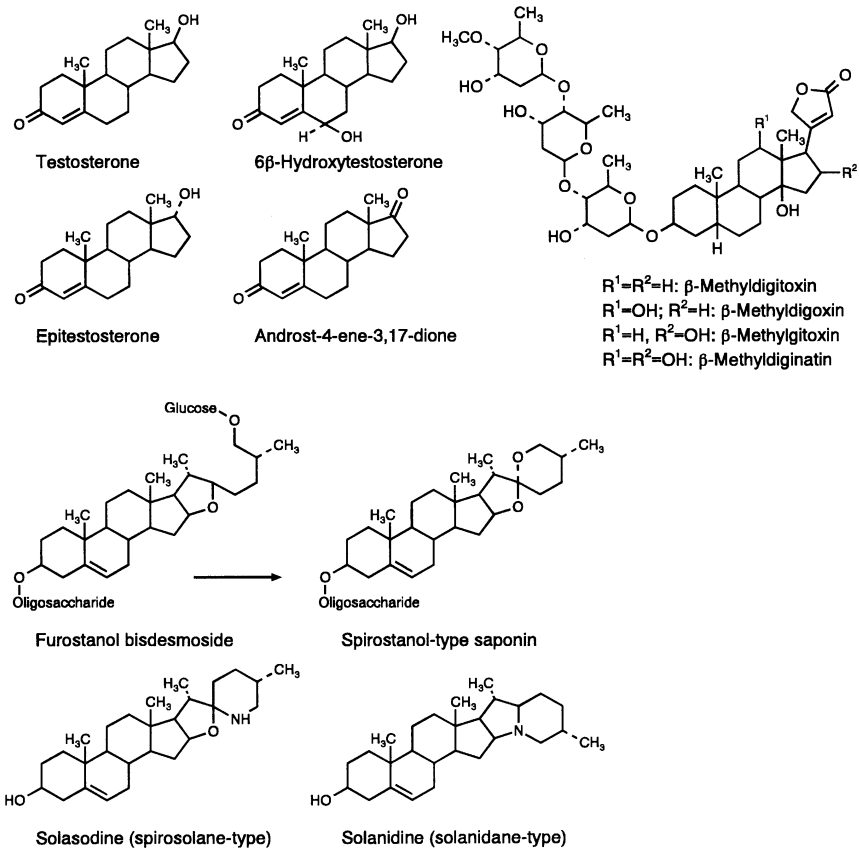


Figure 6.4 Chemical structures of estranes, androstanes, cardiac glycosides steroid saponins and steroid alkaloids mentioned in the text.

seems that the enzymes responsible for the 6β-hydroxylation of testosterone and the oxidation of C-17 hydroxyls exhibit strict substrate specificity.

With a view to synthesizing isotopically labelled cardenolide precursors, the metabolism of 5β-pregnan-3β-ol-20-one was investigated in *Nerium oleander* cell cultures. This particular pregnane was oxidized and epimerized to its 3-keto- and the 3α-hydroxyderivative, respectively (see Fig. 6.6). The latter compound was further biotransformed to its glucoside, 5β-pregnan-20-one-3α-*O*-glucoside. Interestingly, the 3β-isomer, which might be an intermediate in cardenolide biosynthesis, was not glucosylated (Paper and Franz, 1990).

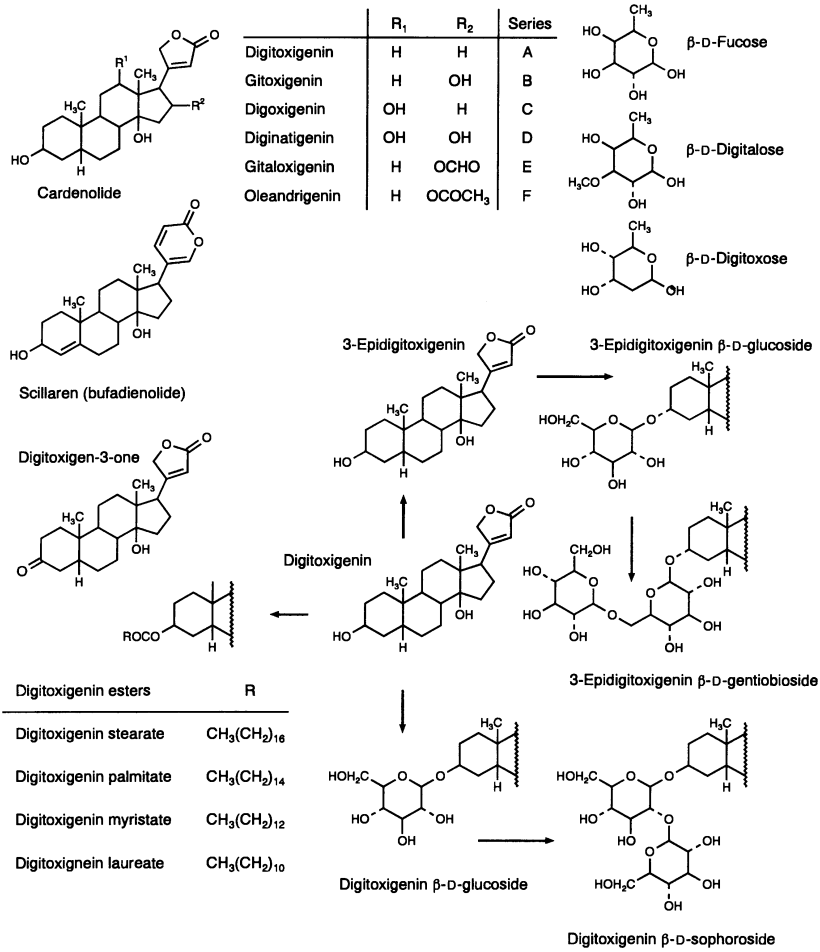
6.3 Cardiac glycosides

Cardiac glycosides are secondary plant metabolites scattered in several unrelated angiosperm families, e.g. Apocynaceae, Asclepiadaceae,

Table 6.2 Occurrence of cardenolides in the plant kingdom

Genus	Family	Order	Reference
Acokanthera	Apocynaceae	Gentianales	Hauschild-Rogat *et al*. (1967)
Calotropis	Apocynaceae	Gentianales	Lhinhatrakool and Sutthivaiyakit (2006)
Cerbera	Apocynaceae	Gentianales	Laphookkhieo *et al*. (2003)
Coronilla	Fabaceae	Fabales	Hembree *et al*. (1979)
Crossopetalum	Celastracae	Celastrales	Ankli *et al.* (2000)
Cryptolepis	Asclepiadaceae	Gentianales	Venkateswara *et al.* (1989)
Cryptostegia	Asclepiadaceae	Gentianales	Kamel *et al.* (2001)
Digitalis	Plantaginaceae	Lamiales	Luckner and Wichtl (2000)
Elaeodendron	Celastraceae	Celastrales	Kupchan *et al.* (1977)
Erysimum	Brassicaceae	Brassicales	Lei *et al.* (2000)
Euonymus	Celastraceae	Celastrales	Bliss and Ramstad (1957)
Glossostelma	Asclepiadaceae	Gentianales	Reichstein *et al.* (1967)
Gomphocarpus	Asclepiadaceae	Gentianales	Warashina and Noro (2000)
Isoplexis	Plantaginaceae	Lamiales	Spengel *et al.* (1967)
Kanahia	Asclepiadaceae	Gentianales	Kapur *et al.* (1967)
Lepidium	Brassicaceae	Brassicales	Hyun *et al.* (1995)
Lophopetalum	Celastraceae	Celastrales	Habermeier (1980)
Mallotus	Euphorbiaceae	Malpighiales	Roberts *et al.* (1963)
Maquira	Moraceae	Rosales	Shrestha *et al.* (1992)
Margaretta	Asclepiadaceae	Gentianales	Sierp *et al.* (1970)
Mimosa	Fabaceae	Fabales	Yadava and Yadav (2001)
Nerium	Apocynaceae	Gentianales	Tschesche *et al.* (1964)
Nierembergia	Solanaceae	Solanales	Gil *et al.* (1995)
Ornithogalon	Liliaceae	Liliales	Ghannamy *et al.* (1987)
Oxystelma	Asclepiadaceae	Gentianales	Srivastava *et al.* (1991)
Parepigynum	Apocynaceae	Gentianales	Hua *et al.* (2003)
Pergularia	Asclepiadaceae	Gentianales	Hamed *et al.* (2006)
Periploca	Asclepiadaceae	Gentianales	Spera *et al.* (2007)
Prosopis	Fabaceae	Fabales	Yadava (1999)
Rhodea	Liliaceae	Liliales	Kuchukhidze and Komissarenko (1977)
Securigera	Fabaceae	Fabales	Zatula *et al.* (1963)
Speirantha	Liliaceae	Liliales	Pauli (1995)
Streblus	Moraceae	Rosales	Saxena and Chaturvedi (1985)
Streptocaulon	Asclepiadaceae	Gentianales	Zhang *et al.* (2007)
Strophanthus	Apocynaceae	Gentianales	Jäger *et al.* (1964)
Terminalia	Combretaceae	Myrtales	Yadava and Rathore (2000)
Thevetia	Apocynaceae	Gentianales	Kyerematen *et al.* (1985)
Trewia	Euphorbiaceae	Malpighiales	Kang *et al.* (2005)
Tupistra	Liliaceae	Liliales	Deng *et al.* (1965)
Xysmalobium	Asclepiadaceae	Gentianales	Ghorbani *et al.* (1997)

Convallariaceae, Fabaceae, Hyacynthaceae, Ranunculaceae and Scrophulariaceae (Table 6.2). Some of the cardiac glycosides are important pharmaceuticals in the treatment of heart insufficiency. Cardiac glycosides consist of a steroid nucleus and a sugar side chain of variable length. The C and D rings of the steroid nucleus are connected *cis*, in contrast to most other steroids.



	R_1	R_2	Series
Digitoxigenin	H	H	A
Gitoxigenin	H	OH	B
Digoxigenin	OH	H	C
Diginatigenin	OH	OH	D
Gitaloxigenin	H	OCHO	E
Oleandrigenin	H	$OCOCH_3$	F

Figure 6.5 Structures of cardiac glycosides mentioned in the text and cardenolide esters and glycosides produced by biotransformation. Formation of the sugar side chain of *Digitalis* cardenolides.

Another common structural feature is a hydroxyl group in position C-14β. Cardiac glycosides are divided into two groups:

1. the cardenolides, carrying a five-membered lactone ring, and
2. the bufadienolides, carrying a six-membered lactone ring in position C-170 (Fig. 6.5).

6.3.1 Biosynthesis

The putative biosynthetic pathway (Fig. 6.6) leading to the cardiac glycosides is basically deduced from studies using radiolabelled precursors. For more

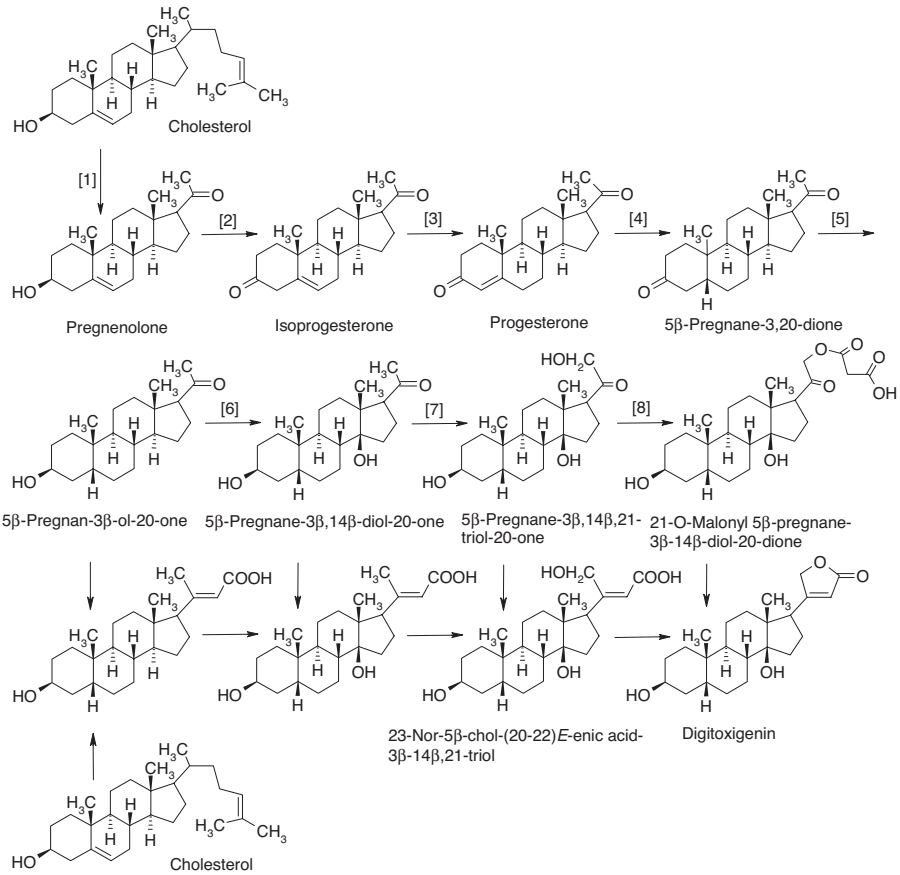


Figure 6.6 Routes for cardenolide genin formation in *Digitalis*. The 'classical' pathway is shown in the upper part, the alternative route via norcholenic acids is depicted in the lower part. Cholesterol (or another phytosterol) is assumed to be the starting point for both pathways.

details, the reader is referred to reviews by Grunwald (1980), Schütte (1987) and Kreis *et al.* (1998). The more recent identification and characterization of various enzymes involved in pregnane and cardenolide metabolism have further clarified the pathway. Since little is known about enzymes involved in the formation of bufadienolides, their biosynthesis will not be considered here in depth. Most of the more recent studies concerning the biosynthesis of cardiac glycosides have been conducted with enzymes isolated from *Digitalis* plants and tissue cultures. The *Digitalis* glycosides are cardenolides classified according to the substitution patterns of their steroid moieties. The A-type glycosides (digitoxigenin derivatives) are the most abundant and the C-type glycosides (digoxigenin derivatives) are the most important cardenolides (Fig. 6.5). The sugar side chain attached in position C-3β of the steroid part is composed of up to five sugar residues, including rare 6-deoxy

and 2,6-dideoxy sugars, such as D-fucose, D-digitalose and D-digitoxose (Fig. 6.5). The so-called primary glycosides carry a terminal glucose.

6.3.1.1 Side-chain cleavage cytochrome $P450_{scce}$

In mammals, the first and limiting step in the biosynthesis of all C_{21} and C_{20} steroids is the conversion of cholesterol into pregnenolone. Cholesterol is also supposed to be a precursor of pregnanes, cardenolides and steroid saponins in plants. Analogous to the formation of steroids in animals, this reaction is thought to be catalysed by side-chain cleavage cytochrome $P450_{scc}$ (SCCE).

Several studies have indicated that a route via cholesterol and progesterone is not the most significant cardenolide-forming pathway (see Kreis *et al.*, 1998). For example, Maier *et al.* (1986) found that Δ^5-norcholenoic acids (C_{23} steroids) are incorporated into cardenolides. Further indirect evidence for a main route not involving cholesterol was provided by studies in which 5-azacycloartanol, a specific inhibitor of the *S*-adenosyl-L-methionine (SAM):cycloartenol 24-methyltransferase, was fed to *Digitalis lanata* shoot cultures. As a result, the endogenous pool of cholesterol increased, whereas the cardenolides decreased. The decrease of cardenolides was in the same range as the decrease of 24-alkylsterols, indicating that one of these sterols, but not cholesterol, may be a precursor fuelling the cardenolide pathway (Milek *et al.*, 1997), and stigmasterol, the main phytosterol in cardenolide-producing tissues, may be a good candidate as a cardenolide precursor. In this context, it is interesting to note that in addition to the mammalian pathway from cholesterol to pregnenolone, another route from Δ^{22}-sterols may be operative (Kerr *et al.*, 1995). In this case, the $P450_{scc}$ is not necessarily involved in pregnenolone formation.

In analogy to the formation of steroids in animals, this reaction is thought to be catalysed by $P450_{scc}$ which, however, has never been characterized in detail in plants. The enzyme activity was determined by measuring either the decrease of cholesterol (Pilgrim, 1972), the radioactivity of the C_6 fragment formed from the cleavage of [26-^{14}C]-cholesterol (Palazon *et al.*, 1995) or quantification of the product pregnenolone by a sophisticated GC-MS method (Lindemann and Luckner, 1997). The latter found the enzyme associated with mitochondria and microsomal fractions of proembryogenic masses, somatic embryoids and leaves of *D. lanata*. Pregnenolone formation was highest with sitosterol as the substrate; however, cholesterol, 20α-hydroxycholesterol and 22*S*-hydoxycholesterol were also accepted.

Finally, it cannot be excluded that enzymes similar to *Arabidopsis thaliana* CYP90B1 (6-oxocampestanol 22α-hydroxylase) are involved in the side-chain degradation (Choe *et al.*, 1998).

6.3.1.2 Δ^{5}-3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-ketosteroid isomerase

NAD:Δ^{5}-3β-hydroxysteroid dehydrogenase (*3β-HSD*) The conversion of pregnenolone into progesterone involves two steps. The first reaction is the

NAD-dependent oxidation of the 3β-hydroxy group, yielding Δ^5-pregnen-3-one catalysed by the Δ^5-3β-hydroxysteroid dehydrogenase. The double bond is shifted from position 5 to position 4 by the action of Δ^5-Δ^4-ketosteroid isomerase (3-KSI) 3β-HSD was isolated from *Digitalis lanata* cell suspension cultures and characterized by Seidel and co-workers (1990), and was purified (Finsterbusch *et al.*, 1999). Using pregnenolone and NAD as the substrate and co-substrate, respectively, considerable progesterone formation was seen.

Deduced oligonucleotide primers from peptide fragments, obtained from the digestion of the 3β-HSD isolated from *D. lanata* leaves (Finsterbusch *et al.*, 1999), were used for the amplification of 3β-HSD gene fragments. Subsequently, Lindemann *et al.* (2000) amplified and sequenced a 700-nucleotide cDNA fragment for a putative 3β-HSD. Based on these reports, Herl *et al.* (2006a) generated primers for PCR amplification of the *D. lanata* 3β-HSD gene. For comparison, PCR amplification of the fragments was performed with DNA templates from several *Digitalis* species. All genes were found to be of similar sizes and they did not differ much from one another or from their genomic fragments. The genomic sequences contained a 90 bp intron at the 3′ end of the gene causing the differences in size.

Lindemann *et al.* (2000) observed that the 3β-HSD from *Digitalis lanata* shows some sequence similarities with microbial hydroxysteroid dehydrogenases and contains a conserved putative short-chain dehydrogenase/reductase (SDR) domain. The *Digitalis* 3β-HSD genes also share some similarities with putative alcohol dehydrogenase genes of *Arabidopsis thaliana*, *Lycopersicon esculentum*, *Oryza sativa*, *Nicotiana tabacum* and *Solanum tuberosum* and, even more exciting, (−)-isopiperitenol dehydrogenase from *Mentha x piperita* (Ringer *et al.*, 2005) and secoisolariciresinol dehydrogenase from *Forsythia x intermedia* (Xia *et al.*, 2001). No obvious similarities with the animal 3β-HSD/KSI were seen.

Molecular cloning and heterologous expression of 3β-HSD from *D. lanata* was reported by Herl *et al.* (2007). In *Digitalis*, 3β-HSD is a soluble enzyme and shares this property with other members of the SDR family (Janknecht *et al.*, 1991; Oppermann and Maser, 1996). In the presence of NAD, r*Dl*3β-HSD converts pregnenolone to isoprogesterone. Progesterone was produced as well. Besides pregnenolone, several steroids with 3β-hydroxy group were tested. Steroids with 3α-hydroxy group were tested as well. Testosterone (4-androsten-17β-ol-3-one), a C_{17}-steroid with a 3-carbonyl group and a 17β-hydroxy group, was converted to 4-androstene-3,17-dione. This indicates that r3β-HSD possesses 3β- as well as 17β-dehydrogenase activity (Herl *et al.*, 2007). A 3β/17βHSD with a broad substrate spectrum was also reported to occur in the bacterium *Comamonas testosteroni*, whereas other HSDs display stricter substrate specificities (see Benach *et al.*, 2002, for more details).

The r*Dl*3β-HSD was also able to catalyse the reduction of 3-ketosteroids when NADH was used as a co-substrate. Pregnane-3,20-diones without Δ^4- or Δ^5-double bond like 5β-pregnane-3,20-dione and 5α-pregnane-3,20-dione were accepted. 4-Androstene-3,17-dione was also accepted as a substrate;

however, not the 3-keto but the 17-keto function was reduced. Isomerization of Δ^4- or Δ^5-double bond was not observed under these conditions. A clear preference for NAD (NADH for reduction) as co-substrate(s) was observed. NADP and NADPH, respectively, were also accepted, but were less efficient. In many aspects the r*Dl*3β-HSD behaves like the hydroxysteroid oxidoreductases supposed to be involved in cardenolide metabolism (Warneck and Seitz, 1990; Seitz and Gaertner, 1994). It was presumed (Finsterbusch *et al.*, 1999; Herl *et al.*, 2007) that 3β-HSD catalyses at least two steps in cardenolide biosynthesis, namely the dehydrogenation of pregnenolone and the reduction of 5β-pregnane-3,20-dione (Fig. 6.6).

Dehydrogenase activity could clearly be separated from a ketosteroid isomerase (see below) indicating that r*Dl*3β-HSD is related to microbial HSDs of the short-chain dehydrogenase/reductase (SDR) family but not with mammalian HSDs, which are multifunctional enzymes.

Δ^{5}-3-Ketosteroid isomerase (*3-KSI*). This enzyme catalyses the allylic isomerization of the 5,6 double bond of Δ5-3-ketosteroids to the 4,5 position by stereospecific intramolecular transfer of a proton. The enzyme has been isolated from bacteria, and especially the 3-KSIs from *Comamonas testosteroni* and *Pseudomonas putida* have been investigated (Smith *et al.*, 1980). The gene coding for the 3-KSI of *Pseudomonas putida* biotype B has been cloned and its nucleotide sequence determined (Kim *et al.*, 1994).

3-KSI was isolated from a *D. lanata* cell suspension culture and it was found that KSI did not co-purify with 3β-HSD (see above) (Meitinger and Kreis, unpublished). However, it is not yet finally clear whether 3-KSI activity is also associated with the 3β-HSD, although circumstantial evidence implies that this is not the case. The spontaneous isomerization of 4-pregnene-3,20-dione represents a crucial problem and this may explain why 5-pregnene-3,20-dione was also found when 5-pregnene-3β-ol,20-one was used as a substrate for the *D. lanata* or recombinant 3β-HSD (Finsterbusch *et al.*, 1999; Herl *et al.*, 2006a). Since, on the other hand, the occurrence of 3-KSI has been demonstrated unambiguously, the isomerase step is now included in the putative cardenolide pathway as an individual biosynthetic step (Figs 6.6 and 6.7).

6.3.1.3 Progesterone 5β-reductase

Progesterone 5β-reductase (5β-POR) catalyses the transformation of progesterone into 5β-pregnane-3,20-dione; i.e. the rings A and B of the steroid are then connected *cis*. Therefore, one of the important structural characteristics of the *Digitalis* cardenolides appears to be accomplished at this stage and, hence, 5β-POR is sometimes referred to as a key enzyme in the biosynthesis of 5β-cardenolides. Progesterone was the preferred substrate, whereas the relative conversion rates for other steroids, such as testosterone, cortisone and cortisol, were much lower. The enzyme was purified to homogeneity from the cytosolic fraction of shoot cultures of *D. purpurea* (Gärtner *et al.*, 1990). The enzyme has been partially sequenced by Gärtner *et al.* (1994).

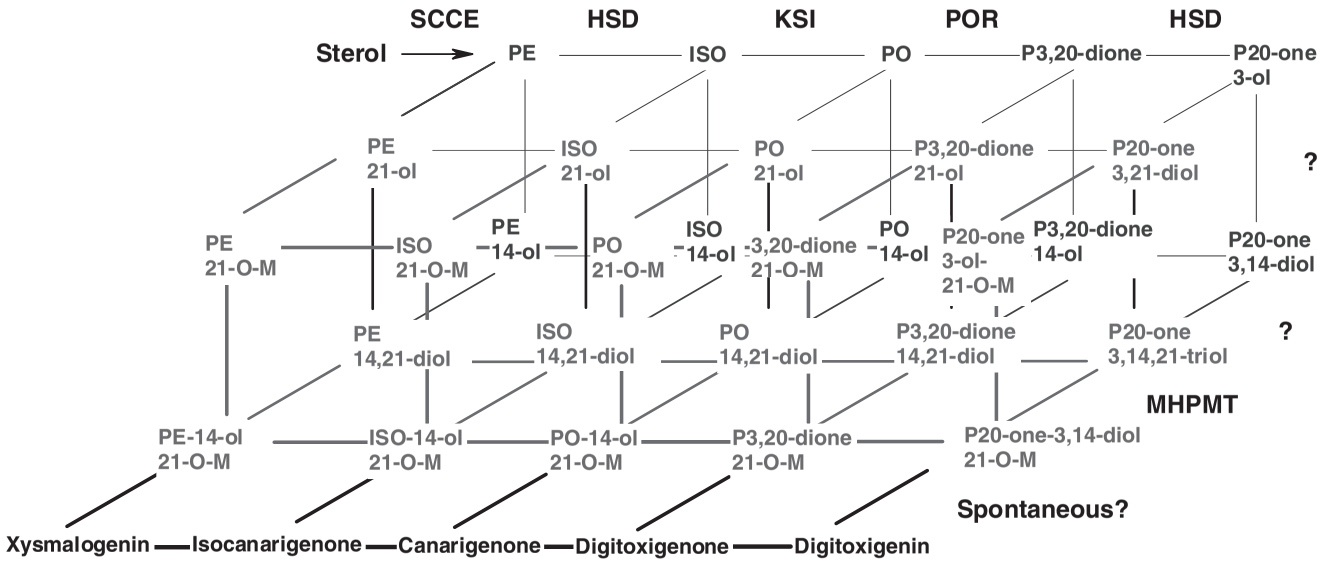


Figure 6.7 Biosynthesis of 5β-cardenolides and $\Delta^{5,6}$-cardenolides. A matrix of alternative routes in the 'classical' pathway is proposed. PE, pregnenolone; ISO, isoprogesterone; PO, progesterone; P, 5β-pregnane; M, malonyloxy residue. The numbers refer to the skeleton carbon to which substituents (hydroxyls or carbonyls) are attached. SCCE, side-chain cleavage cytochrome $P450_{scce}$; HSD, $\Delta^{5\text{-}}$3β-hydroxysteroid dehydrogenase; KSI, $\Delta^{5\text{-}}$3-ketosteroid isomerase (3-KSI); POR, progesterone 5β-reductase; MHPMT, 21-hydroxypregnane 21-*O*-malonyltransferase; ?, enzymes/genes not known.

The gene for 5β–POR of *Digitalis obscura* (*Dop5βr*) was first identified by Roca-Pérez *et al.* (2004). Herl *et al.* (2006a) reported the cloning and heterologous functional expression of 5β-POR from leaves of *D. lanata* Ehrh. (*Dl*5β-POR) and the biochemical characterization of the recombinant enzymes. A high degree of sequence identity was seen when the nucleotide sequence of the cDNA was analysed *in silico* and compared with 5β-POR genes of 20 other *Digitalis* (incl. *Isoplexis*) species (Herl *et al.*, 2006a, b). The deduced 5β-POR protein sequences were found similar to those of *Oryza sativa* (about 58%) and *Populus tremuloides* (about 64%). Interestingly, no obvious similarities were found with the pulegone reductase of *Mentha piperita*, described as a medium-chain dehydrogenase/reductase (Ringer *et al.*, 2003), or animal Δ^4-3-ketosteroid-5β-reductase, described as an aldo-keto-reductase (Kondo *et al.*, 1994), implying very different evolutionary origins in spite of the similarity of the reactions catalysed or even substrates used. The r*Dl*5β-POR did not only accept progesterone but also testosterone, 4-androstene-3,17-dione, cortisol and cortisone. Other substrates, such as pregnenolone, 21-OH-pregnenenolone and isoprogesterone were not accepted by r*Dl*5β-POR. NADPH is the co-substrate. Essential structural elements for substrates of r*Dl*5β-POR are the carbonyl group at C-3 and the double bond in conjugation to it, less important is the side chain at C-17 and the substitution pattern of the steroid ring system (Herl *et al.*, 2006a).

Only recently, 5β-POR was chosen as a genetic marker (Herl *et al.*, 2008) and compared to the previously applied nuclear *ITS* and plastid *trnL-F* sequences (Bräuchler *et al.*, 2004). The results from separate analyses show high congruence within the genus *Digitalis* and support the conclusion that all species of *Isoplexis* have a common origin and are embedded now in the genus *Digitalis*.

Egerer-Sieber *et al.* (2006) reported on the purification and crystallization of recombinant 5β-POR from *D. lanata*. Later on, Gavidia *et al.* (2007) predicted that the 5β-POR belongs to the SDR family (Oppermann *et al.*, 1997). Finally, Thorn *et al.* (2008) fully characterized the crystal structure and found that the progesterone reductase from *D. lanata* defines a novel class of short-chain dehydrogenases/reductases.

6.3.1.4 Progesterone 5α-reductase

Progesterone 5α-reductase (5α-POR), which catalyses the reduction of progesterone to 5α-pregnane-3,20-dione, probably in a competitive situation with the 5β-POR, was isolated and characterized (Warneck and Seitz, 1990). It was found to be located in the endoplasmic reticulum. At temperatures below 45 °C, the product of the enzyme reaction, 5α-pregnane-3,20-dione, was enzymatically reduced to 5α-pregnan-3β-ol-20-one. 5α-Cardenolides have been described in *Xysmalobium* (Asclepidiaceae) and *Digitalis* (incl. *Isoplexis*) (Plantaginaceae). Finasteride, an inhibitor of animal and human testosterone-5α-reductase, at 180 μM inhibited 5α-POR of *D. lanata* completely, but left 5β-POR of the same source unaffected (Grigat, 2005). Feeding finasteride to *D. lanata* shoot cultures resulted in an increased cardenolide formation

indicating that 5α-POR may compete with 5β-POR for its substrate and, as a consequence, 5α-POR-related pathway(s) with the 5β-cardenolide pathway. In *Arabidopsis thaliana* the DET2 gene (see Section 6.4.1.2) encodes a protein similar to mammalian steroid 5α-reductases. The DET protein is probably involved in BR biosynthesis. Therefore, it might well be that 5α-POR is a DET2 homolog.

6.3.1.5 3-hydroxysteroid 5-oxidoreductases (5-HSORs)

Finsterbusch *et al.* (1999) discussed that the reactions summarized below may also be catalysed by the 3β-HSD (see Section 6.3.1.2), although they were assigned to putative enzymes, termed 3β-hydroxysteroid 5α-oxidoreductase, 3β-hydroxysteroid 5β-oxidoreductase and 3α-hydroxysteroid 5β-oxidoreductase. This issue has to be examined further before clear conclusions concerning the role of individual *5-HSOR* enzymes in the cardenolide pathway can be drawn.

NADPH: 3β-hydroxysteroid 5β-oxidoreductase (3β-HS-5β-OR) The 3β-HS-5β-OR catalyses the conversion of 5β-pregnane-3,20-dione to 5β-pregnane-3β-ol-20-one. It was found to be a soluble protein (Gärtner and Seitz, 1993). The reverse reaction was observed, yielding 5β-pregnane-3,20-dione when using 5β-pregnane-3β-ol,20-one and NADP as a substrate and co-substrate, respectively.

NADPH: 3α-hydroxysteroid 5β-oxidoreductase (3α-HS-5β-OR) This microsomal enzyme catalyses the conversion of 5β-pregnane-3,20-dione to 5β-pregnan-3α-ol-20-one (Stuhlemmer *et al.*, 1993a). In a situation similar to that described for the progesterone reductases, the hydroxysteroid 5β-oxidoreductases may compete for 5β-pregnane-3-ones and, in the cardenolide pathway, part of these putative intermediates will be withdrawn due to the action of the 3α-HS–5β-OR. The 3α-HS–5β-OR seems to be specific for 5β-pregnane-3-ones; 5α-pregnane-3-ones and Δ^4–Δ^5-pregnenes were not accepted as substrates.

6.3.1.6 Pregnane hydroxylases

The enzymes involved in pregnane 21-hydroxylation and pregnane 14β-hydroxylation in the course of cardenolide or bufadienolide formation have not yet been described. Concerning steroid 14β-hydroxylation, it was found that labelled 3β-hydroxy-5β-pregnan-20-one was incorporated by *Digitalis purpurea* plants into digitoxin, while 3β-hydroxy-5β-pregn-8(14)-en-20-one was not. From this and previous studies, it was concluded that a route via Δ8(14) or Δ8(9) pregnenes, 14β-steroids or an 8,14-epoxide (Tschesche and Kleff, 1973; Anastasia and Ronchetti, 1977) does not appear to be operative in the cardenolide pathway. Therefore, direct hydroxylation with a change in configuration at C-14 seems to be the most probable mechanism of 14β-hydroxylation. *Arabidopsis thaliana* CYP 85A1 hydroxylates the steroid

nucleus at C-6 (Shimada *et al.*, 2001) and similar enzymes/genes may also be involved in cardenolide genin hydroxylation.

Digitoxin 12β-hydroxylase This microsomal cytochrome P450-dependent monooxygenase is capable of converting digitoxigenin-type cardenolides to their corresponding digoxin-type cardenolides (Petersen and Seitz, 1985). Digitoxin, β-methyldigitoxin and α-acetyldigitoxin, as well as digitoxigenin-type cardenolides with shorter or no sugar side chain were hydroxylated (Petersen *et al.*, 1988). Gitoxigenin, k-strophanthin-β and cymarin, on the other hand, were not accepted. After immobilization in alginate, the enzyme retained 70% of its original activity. The kinetic data of digitoxin 12β-hydroxylase (D12H) immobilized in alginate were the same as for the enzyme in freely suspended microsomes (Petersen *et al.*, 1987).

6.3.1.7 Malonyl-co-enzyme A:21-hydroxypregnane 21-*O*-malonyltransferase

With regard to the formation of the butenolide ring, it is hypothesized that the condensation of 5β-pregnane-3β,14β,21-triol-20-one with a dicarbon unit yields digitoxigenin. However, when the 3-β-*O*-acetate of 5β-pregnane-3β,14β,21-triol-20-one was incubated together with malonyl-co-enzyme A in cell-free extracts of cardenolide-producing plants, the malonyl hemiester of the substrate was formed (Stuhlemmer and Kreis, 1996). Malonyl-CoA and acetoacetyl-CoA were accepted as co-substrates, whereas no 21-*O*-ester formation was observed with acetyl-CoA or succinyl-CoA. Pregnen-21-ol-20-one, cortexone, 5β-pregnan-21-ol-3,20-dione and 5β-pregnane-3β,21-diol-20-one were only very poor substrates (Stuhlemmer and Kreis, 1996). Kuate *et al.* (2008) reported the purification and characterization of malonyl-co-enzyme A: 21-hydroxypregnane 21-*O*-malonyltransferase (*Dp*21MaT) from leaves of *Digitalis purpurea*. A 'cardenolide synthase'; i.e., an enzyme forming the very butendolide ring has not been described as yet. However, non-enzymatic ring closure of the 21-*O*-malonyl hemiester of 5β-pregnane-3β,14β,21-triol-20-one has been observed (Pádua and Kreis, unpublished). Butenolide formation was also studied in *Asclepias curassavica* (Groeneveld *et al.*, 1990). Excised defoliated stems incorporated radioactive acetate into various lipids, including cardenolides. Labelled cardenolides, biosynthesized from (1,2-^{13}C)-acetate were isolated. The construction of the butenolide ring by the condensation of a pregnane derivative with one molecule acetate, as proposed for the *Digitalis* cardenolides, was not confirmed by the ^{13}C NMR data. In summary, butenolide ring formation in cardenolide biosynthesis is still far from being elucidated. Cumalin ring formation in bufadienolide biosynthesis has been studied scarcely, but it may be assumed that three carbons from oxaloacetate can be incorporated by an esterification/aldol reaction sequence similar to that proposed for butenolide ring formation (Dewick, 2002) (Fig. 6.8).

As already mentioned, little is known about the biosynthetic sequence leading to bufadienolides. It may be similar to the cardenolide pathway as far

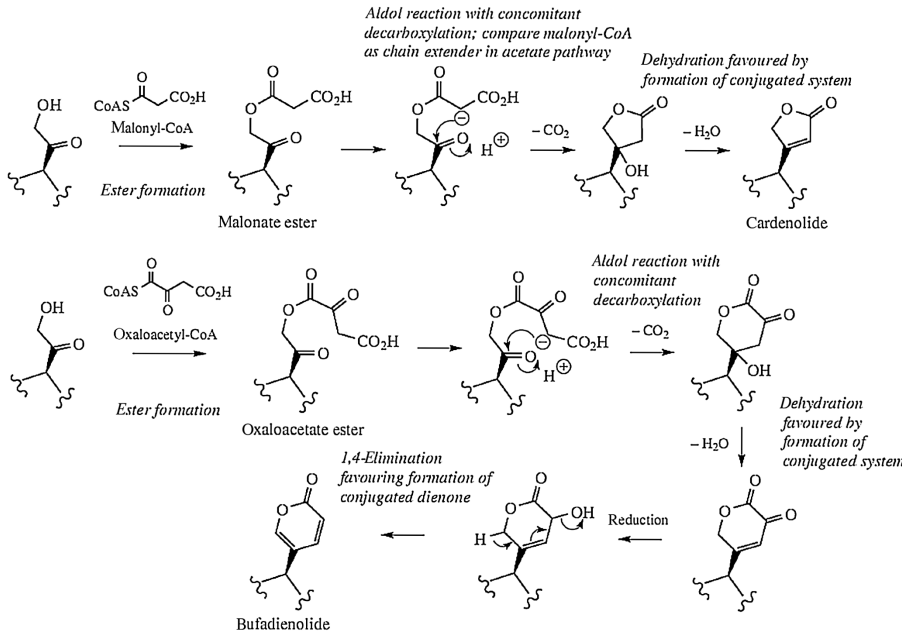


Figure 6.8 Proposed mechanism for lactone ring formation. Cardenolides: an intermediate malonate ester is involved, and ring formation probably occurs via an aldol addition process giving the cardenolide digitoxigenin, the carboxyl carbon of the malonate ester being lost by decarboxylation. Bufadienolides: three carbons from oxaloacetate can be incorporated by a similar esterification/aldol reaction sequence to yield the cumaline ring system. (From Dewick, 2002.)

as the sequence leading to 5β-pregnane-3β,14β,21-triol-20-one is concerned. With regard to the final step, α-pyrone formation, it was reported that administration of radiolabelled oxaloacetate to *Urginea maritima* plants yielded labelled scillirosid. Chemical degradation of scillirosid indicated that the α-pyrone ring of bufadienolides is formed by the condensation of a pregnane derivative, such as 5β-pregnane-3β,14β,21-triol-20-one, with oxaloacetic acid (Galagovsky *et al.*, 1984).

The putative cardenolide pathway implies that the various sugars are attached at the cardenolide aglycone stage, although it cannot be ruled out that pregnane glycosides are obligate intermediates in cardenolide formation. Some results indicate that digitoxose is formed from glucose without rearrangement of the carbon skeleton (Franz and Hassid, 1967) and that nucleotide-bound deoxysugars are present in cardenolide-producing plants (Bauer *et al.*, 1984). Groeneveld *et al.* (1992) have shown high incorporation of ^{14}C-labelled malonate into cardenolides, but one-third of the radioactivity disappeared after acid hydrolysis of the cardiac glycosides and was, therefore, postulated to be incorporated into the carbohydrate side chain.

To study cardenolide genin glycosylation in more detail, digitoxigenin was fed to light-grown and dark-grown *D. lanata* shoot cultures, as well as to suspension-cultured cells (Theurer *et al.*, 1998). In either system, the substrate was converted to digoxigenin (Fig. 6.5), digitoxigen-3-one, 3-epidigitoxigenin, digitoxigenin 3-*O*-β-D-glucoside, 3-epidigitoxigenin 3-*O*-β-D-glucoside (Fig. 6.5), glucodigifucoside (Fig. 6.10) and additional cardenolides. Digitoxosylation was not observed in these studies. Moreover, administration of cardenolide mono- and bisdigitoxosides or cardenolide fucosides did not lead to the formation of cardenolide tridigitoxosides. These results support the hypothesis that cardenolide fucosides and digitoxosides may be formed via different biosynthetic routes and that glycosylation may be an earlier event in cardenolide biosynthesis than previously assumed. Luta *et al.* (1998) synthesized a set of pregnane and cardenolide fucosides and they have shown that feeding of the 3-*O*-β-D-fucoside of 21-hydroxypregnenolone to *D. lanata* shoot cultures leads to a 25-fold increase in the formation of glucodigifucoside, when compared to a control where the respective aglycone was fed (Luta *et al.*, 1997). The enzyme-catalysed reactions involved in the formation or modification of the sugar side chain of *Digitalis* cardenolides are summarized in Fig. 6.9.

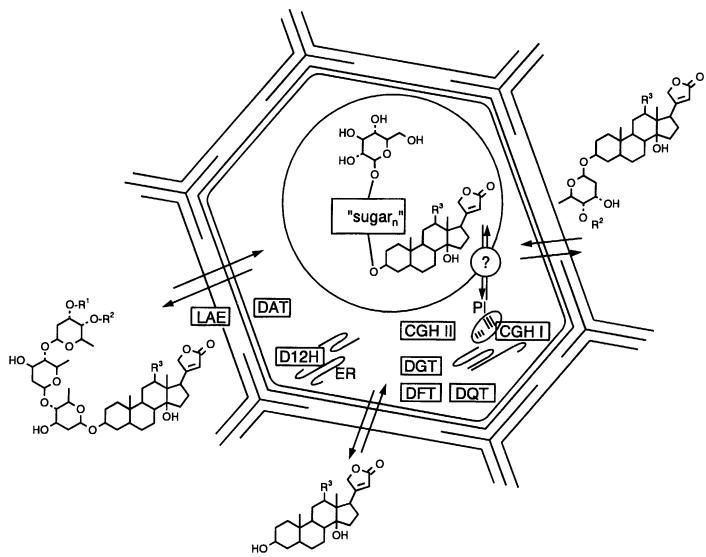


Figure 6.9 Cellular organization of cardenolides glycoside uptake, modification and storage. Exogenous cardenolides enter the cell by diffusion, after which they can be modified in several ways. Only those possessing a terminal glucose are stored in the vacuole, probably involving active transport across the tonoplast. Abbreviations: LAE, lanatoside 15′-*O*-acetylesterase; DAT, digitoxin 15′-*O*-acetyltransferase; D12H, digitoxin 12β-hydroxylase; ER, endoplasmic reticulum; CGH I and CGH II, cardenolide 16′-*O*-glucohydrolase I and II, respectively; DGT, digitoxin 16′-*O*-glycosyltransferase; DFT, digitoxigenin 3-*O*-fucosyltransferase; DQT, digitoxigenin 3-*O*-quinovosyltransferase.

6.3.1.8 Digitoxin 16′-*O*-glucosyltransferase

The enzymatic glucosylation of secondary glycosides to their respective primary glycosides was first demonstrated by Franz and Meier (1969) in particulate preparations from *D. purpurea* leaves and was investigated in more detail in cell cultures of *D. lanata* (Kreis *et al.*, 1986). The UDP-glucose:digitoxin 16′-*O*-glucosyltransferase (DGT) requires two substrates: a secondary cardiac glycoside and a sugar nucleotide. Of six sugar nucleotides tested, only UDP-α-D-glucose served as a glycosyl donor; other glucose nucleotides (Kreis *et al.*, 1986) and UDP-α-D-fucose (Faust *et al.*, 1994) were not accepted. The DGTs of different *Digitalis* species differed considerably with regard to their substrate preferences. Although 15′-*O*-acylated glycosides do not occur in *D. purpurea*, they were glucosylated to their corresponding primary glycosides by enzyme preparations from *D. purpurea* cell cultures (Kreis *et al.*, 1986). Cardenolide monodigitoxosides, such as evatromonoside, were accepted very well, whereas cardenolide genins or bisdigitoxosides were glucosylated at a much slower rate. Glucosylation was not observed when digiproside (digitoxigenin fucoside) was tried as the glucosyl acceptor, indicating that DGT accepts only substrates with an equatorial OH group in the 4′ position (Faust *et al.*, 1994).

6.3.1.9 Digitoxigenin 3-*O*-fucosyltransferase and Digitoxigenin 3-*O*-quinovosyltransferase

UDP-fucose:digitoxigenin 3-*O*-fucosyltransferase (DFT) is a soluble enzyme in *D. lanata* leaves and catalyses the transfer of the sugar moiety of UDP-α-D-fucose to cardenolide genins. Gitoxigenin and digitoxigenin were much better substrates than digoxigenin (Faust *et al.*, 1994). Incubation of crude protein extracts together with digitoxigenin and UDP-α-D-fucose resulted not only in the formation of digiproside but also of digitoxigenin quinovoside, its 4′-epimer, which is a minor glycoside in *D. lanata*. It was demonstrated that the sugar is epimerized at the sugar nucleotide level and not at the glycoside stage. Neither UDP-quinovose:digitoxigenin 3-*O*-quinovosyltransferase (DQT) nor epimerase activity was present in purified DFT preparations.

6.3.1.10 Digiproside 4′-*O*-glucosyltransferase

Glucodigifucoside was formed by a soluble enzyme from young leaves of *D. lanata* in the presence of UDP-α-D-glucose and digiproside (Faust *et al.*, 1994). The enzyme is not identical with the glucosyltransferases described above; it has not yet been characterized in detail. Glucodigifucoside is a major cardenolide in *D. lanata* leaves during all stages of development and may be regarded as the end-product of the 'fucose pathway'.

6.3.1.11 Digitoxin 15′-*O*-acetyltransferase

This soluble, cytosolic enzyme catalyses the 15′-*O*-acetylation of cardenolide tri- and tetrasaccharides. Using acetyl co-enzyme A as the acetyl donor, acetyl co-enzyme A: digitoxin 15′-*O*-acetyltransferase (DAT) activity was detected

in partially purified protein extracts from *D. lanata* and *D. grandiflora*, both known to contain lanatosides (Sutor *et al.*, 1993).

6.3.1.12 Lanatoside 15′-*O*-acetylesterase

An esterase converting acetyldigitoxose-containing cardenolides to their corresponding nonacetylated derivatives was demonstrated in *D. lanata* cell suspension cultures and leaves (Sutor *et al.*, 1990). The lanatoside 15′-*O*-acetylesterase (LAE) was shown to be bound to the cell wall. LAE was present in *D. lanata* leaves and cell cultures (Sutor *et al.*, 1990) but was not detectable in cell suspension cultures of *D. grandiflora* and *D. purpurea* (Kreis *et al.*, 1993), and in leaves of *D. purpurea* and *D. heywoodii* (Sutor *et al.*, 1990). Lanatosides, as well as their corresponding secondary glycosides, were good substrates; α,β-diacetyldigoxin was deacetylated to some extent, yielding small amounts of β-acetyldigoxin but not the respective α-derivative. Apigenin 7-*O*-acetylglucoside was not deacetylated. Therefore, LAE seems to be a site-specific cardenolide acetylesterase capable of removing the 15′-acetyl group of lanatosides and their deglucosylated derivatives. Meanwhile, LAE was isolated, purified and partially sequenced (Sutor and Kreis, 1996; Kandzia *et al.*, 1998). A fragment obtained by Lys-C digestion showed partial homology to other hydrolases and apoplasmic proteins. It included the probable location of an active site histidine (Kandzia *et al.*, 1998).

6.3.1.13 Cardenolide glucohydrolases (CGH)

*Cardenolide 16-*O*-glucohydrolase (CGH I)* CGH I was found to be associated with plastids (Bühl, 1984) and could be solubilized from leaves of various *Digitalis* species using buffers containing Triton X-100 or other detergents (Kreis and May, 1990). Considerable variations in substrate preferences were observed among the cardenolide 16′-*O*-glucosidases of the three species investigated. The enzyme of *D. lanata*, termed CGH I, was purified from young leaves (May and Kreis, 1997; Schöninger *et al.*, 1998). Purified CGH 1 was digested and the resulting fragments were sequenced. One fragment had the typical amino acid sequence of the catalytic centre of family 1 of glycosyl hydrolases. Cardenolide 16′-*O*-glucohydrolase, like the other members of this enzyme family, appeared to have a glutamic acid residue directly involved in glycosidic bond cleavage as a nucleophile (Schöninger *et al.*, 1998).

A clone of cardenolide 16′-*O*-glucohydrolase cDNA (CGH I) was obtained from *D. lanata*. The amino acid sequence derived from CGH I showed high homology to a widely distributed family of β-glucohydrolases (glycosyl hydrolases family 1). The recombinant CGH I protein produced in *Escherichia coli* had CGH I activity. CGH I mRNA was detected in leaves, flowers, stems and fruits of *D. lanata* (Framm *et al.*, 2000).

The coding sequence for the *D. lanata* CGH I was inserted downstream of the 35S promoter in the binary vector pBI121 resulting in plant expression vector pBI121cgh (Shi and Lindemann, 2006). Explants excised from seedlings of

Cucumis sativus were transformed using *Agrobacterium rhizogenes* harbouring pBI121cgh. Hairy roots were obtained from infected explants. Glycolytic activity of the transgenic CGH I was demonstrated by HPLC using lanatosides as the substrates.

Cardenolide glucohydrolase II Another CGH, termed CGH II, was isolated from *D. lanata* and *D. heywoodii* leaves and cell cultures. This soluble enzyme hydrolyses cardenolide disaccharides with a terminal glucose and appears to be quite specific for glucoevatromonoside, which is supposed to be an intermediate in the formation of the cardenolide tetrasaccharides. The tetrasaccharides, deacetyllanatoside C and purpureaglycoside A, which are rapidly hydrolysed by CGH I (see Section 6.3.1.13) were very poor substrates for CGH II (Hornberger *et al.*, 2000).

6.3.1.14 Cardenolide β-D-fucohydrolase

A β-D-fucosidase was isolated from young *D. lanata* leaves. This soluble enzyme catalyses the cleavage of digiproside and synthetic pregnane 3β-*O*-D-fucosides to D-fucose (6-deoxygalactose) and the respective genin. Digitoxigenin 3β-*O*-D-galactoside was not hydrolysed by the enzyme. It is not identical with the CGHs described above, which do not accept β-D-fucosides as substrates (Luta *et al.*, 1997).

6.3.2 Transport and storage

As the SCCE described above (Section 6.3.1.1) may be part of a protein complex in the mitochondria, more effort was directed to study the possible interaction partners, especially the peripheral-type benzodiazepine receptor (PBR) (Papadopoulos *et al.*, 1997; Koch, 2002) and the acyl-CoA-binding protein (ACBP; Metzner *et al.*, 2000). The ACBPs bind to the peripheral-type PBR present in the envelope of mitochondria (Garnier *et al.*, 1994). This interaction stimulates the transport of cholesterol into mitochondria (Papadopoulos and Brown, 1995). The cholesterol taken up into the mitochondria is available as a substrate to the side-chain cleavage enzyme which transforms cholesterol into pregnenolone (Papadopoulos *et al.*, 1997). Because of its interaction with PBR, ACBP is also described as diazepam-binding inhibitor or endozepine. Some isoforms of the latter were isolated and characterized from *D. lanata* (Metzner *et al.*, 2000). Lindemann and Luckner (1997) speculated that cardenolide formation is regulated mainly by the availability of cholesterol and its transport into mitochondria, where the $P450_{scc}$ is assumed to be located.

Cell suspension cultures established from different plants producing cardiac glycosides did not produce cardenolides or bufadienolides, whereas embryoids, morphogenic clumps and shoot-differentiating cultures generally contained low amounts of cardiac glycosides (Luckner and Diettrich, 1985; Seidel and Reinhard, 1987; Stuhlemmer *et al.*, 1993b). Plants obtained by organogenesis or somatic embryogenesis were found to contain the cardiac

glycosides characteristic of the parent plant. Several studies have reported a positive correlation between light, chlorophyll content and cardenolide production (e.g. Hagimoro *et al*., 1982). However, chloroplast development is not sufficient for expression of the cardenolide pathway, since photomixotrophic cell cultures where shown to be incapable of producing cardenolides (Reinhard *et al.*, 1975). *Digitalis* roots cultivated in vitro are not capable of producing cardenolides, although they do contain these compounds in situ.

Suspension-cultured *Digitalis* cells, which do not synthesize cardenolides de novo (Kreis *et al.*, 1993), as well as roots or shoots cultivated in vitro (Theurer *et al.*, 1998), are able to take up exogenous cardenolides and modify them. It has been demonstrated that cardenolides may enter and leave the cells by diffusion. Only the primary cardenolides, i.e. those containing a terminal glucose, are actively transported across the tonoplast and stored in the vacuole. A model comprising the events leading to cardenolide storage has been proposed (Fig. 6.9) (Kreis *et al.*, 1993). Cardiac glycoside transport was also investigated at the organ and whole plant levels. The long-distance transport of primary cardenolides from the leaves to the roots or to etiolated leaves was demonstrated. It was established that the phloem, but not the xylem, is a transporting tissue for cardenolides (Christmann *et al.*, 1993). To summarize, it seems that primary cardenolides may serve both as the transport and the storage form of cardenolides. After their synthesis they are either stored in the vacuoles of the source tissue or loaded into the sieve tubes and transported to various cardenolide sinks, such as roots or flowers. The mechanisms involved in remetabolization and phloem loading and unloading have not yet been investigated.

6.3.3 Biotransformations

During the 1970s and 1980s, investigations concerning the ability of cultured plant cells to modify exogenous cardenolides were carried out (Reinhard and Alfermann, 1980; Suga and Hirata, 1990; Ramachandra Rao and Ravishankar, 2002). In these studies, cell cultures of the cardenolide-producing species *Digitalis cariensis*, *D. dubia*, *D. grandiflora*, *D. lanata*, *D. leucophaea*, *D. lutea*, *D. mertonensis*, *D. parviflora*, *D. purpurea*, *Strophanthus amboensis*, *S. intermedius*, *S. gratus*, *Thevetia neriifolia*, as well as of various cardenolide-free species, were employed.

6.3.3.1 Biotransformation of cardenolide genins

To summarize these studies, oxidation and epimerization of the 3β-hydroxyl and 5β-hydroxylation and glucosylation of the 3-hydroxyl appear to be quite common reactions, whereas other stereospecific hydroxylations as well as conjugation with deoxysugars are probably more species specific. The combination of the biosynthetic potential of unrelated plant species and the formation of novel cardenolides by biotransformation was achieved by Kawaguchi *et al.* (1990) who administered digitoxigenin to hairy root cultures

of *Panax ginseng*. Four esters, namely digitoxigenin stearate, digitoxigenin palmitate, digitoxigenin myristate and digitoxigenin laureate, as well as two new glycosides, 3-epidigitoxigenin *O*-D-gentiobioside and digitoxigenin *O*-D-sophoroside, were isolated, together with six known cardenolides (Fig. 6.5).

Digitoxigenin was fed to light-grown and dark-grown *Digitalis lanata* shoot cultures. In either system, the substrate was converted to digoxigenin (Fig. 6.5), digitoxigen-3-one, 3-epidigitoxigenin, digitoxigenin *O*-D-glucoside, 3-epidigitoxigenin *O*-D-glucoside (Fig. 6.5) and glucodigifucoside. Interestingly, fucosylated and digitalosylated cardenolides were formed in light-grown shoots, whereas digitoxosylation was not observed (Theurer *et al.*, 1998).

Biotransformation of cardiac glycosides The biotransformation of cardiac glycosides has been studied extensively using *Digitalis lanata* cell and organ cultures. Side-chain glucosylation, deglucosylation, acetylation, deacetylation and steroid 12β-hydroxylation have been reported (Reinhard and Alfermann, 1980). Most important is the ability of cultured *Digitalis* cells to biotransform cardenolide tridigitoxosides of the A-series into the respective 12β-hydroxylated C-series glycosides (Fig. 6.5). Cell lines with high 12β-hydroxylation capacity have been selected by cell-aggregate-cloning and by protoplast-cloning techniques (Reinhard and Alfermann, 1980; Baumann *et al.*, 1990; Kreis and Reinhard, 1990a). A cell culture process was developed in which a commercial digoxin-type cardenolide, namely β-methyldigoxin, can be prepared with good yields and almost no side reactions from β-methyldigitoxin (Fig. 6.4) (Alfermann *et al.*, 1983; Reinhard *et al.*, 1989). Alternative approaches using *Di lanata* cells to produce C-series cardenolides have been tried as well. Special emphasis was laid on the use of digitoxin as the substrate for biotransformation. For example, a two-stage cultivation method was employed to develop a semicontinuous biotransformation process for the production of deacetyllanatoside C on the 20 L scale using two airlift bioreactors, one for cell growth and another for deacetyllanatoside C production (Kreis and Reinhard, 1990b).

6.4 Brassinosteroids

Brassinosteroids (BRs) are hydroxylated derivatives of cholestane and their structure variations comprise substitutions pattern on ring A, B and the C-17 side chain (Fig. 6.10). The BRs are classified as C_{27}, C_{28}, or C_{29} BRs, depending on the substitutions and the length of the side chain. More than 70 BRs as well as more than 42 BR metabolites have been isolated and identified (Bajguz and Tretyn, 2003).

BRs are specific plant steroid hormones that are essential for normal plant development (Adam and Schneider, 1999; Adam *et al.*, 1999; Bishop and Koncz, 2002; Asami *et al.*, 2005). They act on different levels in multiple

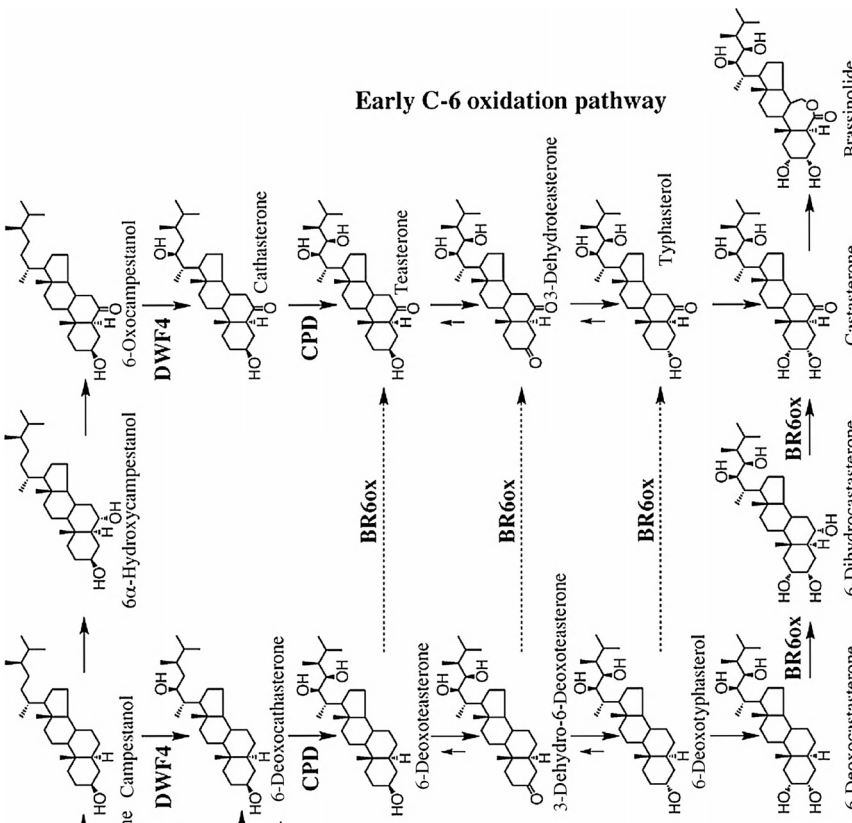


Figure 6.10 Biosynthesis of Brassinolide from campesterol. Enzymes identified from *Arabidopsis thaliana* are shown. Enzymes from other plants are identified in the text (Fujioka and Yokota, 2003).

developmental processes, including cell division, cell elongation, vascular differentiation and reproductive development, and they cause changes in gene expression. BRs also confer resistance to plants against various abiotic and biotic stresses (Yokota, 1999). Surprisingly high similarities exist to the animal steroid hormone biosynthesis (Fujioka and Yokota, 2003). Most BR deficient plants have a characteristic dwarf phenotype and may be rescued to a wild-type phenotype when supplemented with minute amounts of BRs applied exogenously. It was shown that the expression of hundreds of genes is significantly altered after BR treatment. The identification of numerous BR-regulated genes provides the basis for the identification of *cis*-acting elements in promoters that mediate BR effects (Müssig *et al.*, 2002; Müssig, 2005).

6.4.1 Biosynthesis

Initially, the BR biosynthetic pathway was elucidated in *Catharanthus roseus* cell cultures by analysing the conversion products and intermediates (Fujioka *et al.*, 1997). More recently, the biosynthesis of BRs has mainly been studied in *Arabidopsis thaliana*. Many of the genes encoding BR biosynthetic enzymes have been cloned using BR biosynthesis mutants of *Arabidopsis thaliana*, pea, tomato and rice. These mutants are BR deficient and revert to a wild-type phenotype following treatment with exogenous BRs.

The biosynthesis and the metabolism of BRs were reviewed several times (e.g. Fujioka and Yokota, 2003; Asami *et al.*, 2005). The enzymes for BR biosynthesis appear to be located within the cell and to be associated with the endoplasmic reticulum, in particular. Ohnishi *et al.* (2006) concluded from their data when analysing cytochrome P450 enzymes in the model plant *Arabidopsis thaliana* that more than one BR pathway may exist in plants. Their results highlighted the need for refining the BR biosynthetic pathway (Bishop, 2007). It is now well established that two parallel routes, the early and the late steps, are connected and linked to a complex network pathway (Fujioka and Yokota, 2003) which is shown in Fig. 6.10. Campesterol may be taken as the starting point for BR biosynthesis. Originally, the conversion of campesterol to campestanol (CN) was thought to be a single reaction (Suzuki *et al.*, 1995). However, when this pathway was investigated in detail it was shown that the conversion comprises four reactions. Fujioka *et al.* (2002) identified many novel 22-hydroxylated C_{27} and C_{28} BRs in cultured *C. roseus* cells and *Arabidopsis thaliana* seedlings and in parallel metabolic studies elucidated a new subpathway.

BR biosynthesis inhibitors are potentially valuable tools for studying BR biosynthesis (Asami and Yoshida, 1999). Triazoles are known BR biosynthesis inhibitors; however, they are not very specific and also suppress the synthesis of gibberellic acid (Rademacher, 2000) or cardenolides (see above). A more specific BR biosynthesis inhibitor, brassinazole, was synthesized by modifying uniconazole (Min *et al.*, 1999). Treatment of *Arabidopsis* seedlings with brassinazole resulted in a phenotype typical of BR-deficient mutants (Asami

et al., 2000). Similar effects were also observed with cress, tomato, pea and tobacco seedlings (Asami and Yoshida, 1999; Min *et al.*, 1999; Asami *et al.*, 2000). Brassinazole rather specifically blocks the C-22α hydroxylation step (Asami *et al.*, 2001). More specific inhibitors of BR biosynthesis have been synthesized and have been utilized to identify new BR mutants (Wang *et al.*, 2002).

6.4.1.1 3β-Hydroxysterol dehydrogenase

The *sax1* (hypersensitive to abscisic acid and auxin) mutant probably has a defect in the oxidation and isomerization of 3β-hydroxy-Δ^5 precursors to 3-oxo-Δ^4 steroids (Ephritikhine *et al.*, 1999). AtHSD1 (At5g50600) encodes a protein with homology to animal 11-β-hydroxysteroid dehydrogenase (Li *et al.*, 2007); however, no obvious sequence similarities exist with the 3β-HSD involved in cardenolide biosynthesis (see Section 6.3.1.2).

6.4.1.2 Sterol 5α-reductase

DET2 is considered to catalyse a major rate-limiting step in BR biosynthesis. The DET2 gene encodes a protein similar to mammalian steroid 5α-reductase (Li *et al.*, 1996). The DET2 enzyme can catalyse 5α-reduction of many sterols. Treating cultured cotton ovules with finasteride, a steroid 5α-reductase inhibitor, reduced fibre elongation. It may thus well be that the progesterone 5α-reductase described in the biosynthesis of cardenolides (see above) is rather involved in BR metabolism than in cardenolide formation. Pea *lk* is an extreme dwarf that is BR deficient because of loss of 5α-reductase activity and is an ortholog of *Arabidopsis thaliana* DET2 (e.g. Yokota *et al.*, 1997; Li and Chory, 1999).

6.4.1.3 C-22 Hydroxylases

The *DWF4* gene encodes a cytochrome P450 monooxygenase (CYP90B1). CYP90B1 converted CN to 6-deoxocathasterone, confirming that CYP90B1 is a steroid C-22 hydroxylase. The substrate specificity of CYP90B1 indicated that sterols with a double bond at positions C-5 and C-6 are preferred substrates compared with stanols, which have no double bond at the position. In addition, CYP90B1 showed C-22 hydroxylation activity towards various C(27–29) sterols. Cholesterol (C27 sterol) is the best substrate, followed by CR (C28 sterol), whereas sitosterol (C29 sterol) is a poor substrate (Fujita *et al.*, 2006).

6.4.1.4 C-23 Hydroxylases

The *CPD* gene encodes a cytochrome P450 (CYP90A1), which was the first P450 found in BR biosynthetic pathways (Szekeres *et al.*, 1996). It is responsible for C-23 hydroxylation of the steroid side chain. CYP90C1 and CYP90D1 are redundant BR C-23 hydroxylases. In vitro biochemical assays revealed that both CYP90C1 and CYP90D1 catalyse C-23 hydroxylation of various

22-hydroxylated BRs with markedly different catalytic efficiencies. It was thus proposed that C-23 hydroxylation shortcuts can bypass campestanol, 6-deoxocathasterone and 6-deoxoteasterone and lead directly from (22*S*,24*R*)-22-hydroxy-5α-ergostan-3-one and 3-epi-6-deoxocathasterone to 3-dehydro-6-deoxoteasterone and 6-deoxotyphasterol (Ohnishi *et al.*, 2006). From the evidence available it may be assumed that the tomato *dpy* mutant is also deficient in the conversion of 6-deoxocathasterone to 6-deoxoteasterone. (Koka *et al.*, 2000).

6.4.1.5 C-6 Oxidase

C-6 oxidation genes play a key role in the biosynthesis of BRs. They control BR activation, which involves the C-6 oxidation of 6-deoxocastasterone (6-DeoxoCS) to castasterone (CS) and in some cases the further conversion of CS to brassinolide (BL). C-6 oxidation is controlled by the CYP85A family of cytochrome P450 enzymes, and to date, two CYP85As have been isolated in tomato, two in *Arabidopsis thaliana*, one in rice and one in grape (Nomura *et al.*, 2005; Jager *et al.*, 2007). The tomato *Dwarf* gene encodes CYP85A1. Functional expression of *Dwarf* in yeast established that it catalyses the two-step oxidation of 6-deoxoCS to CS (Bishop *et al.*, 1999). It was found that the Dwarf enzyme has a broad substrate specificity, catalysing C-6 oxidation of a whole set of 6-deoxobrassinosteroids. This is also the case for the orthologous genes *Arabidopsis BR6 ox* (Shimada *et al.*, 2001) and rice *OsDwarf* (Hong *et al.*, 2002).

6.4.1.6 Oxido-reductases

The genes for the enzymes catalysing the reversible conversion between (6-deoxy)teasterone and (6-deoxy)typhasterol have not been identified as yet. Using enzyme extracts from a cytosolic fraction of *Marchantia polymorpha*, 3β-dehydrogenase activity converting teasterone to 3-dehydroteasterone and 3α-reducatase activity catalysing the further conversion of 3-dehydroteasterone to typhasterol have been demonstrated (Park *et al.*, 1999). Furthermore, enzymes involved in the reversible conversion between 24-epiteasterone and 24-epityphasterol were also investigated (Winter *et al.*, 1999; Stuendl and Schneider, 2001). 3β-Dehydrogenase of *Arabidopsis thaliana* and tomato and 3α-reductase of tomato were both cytosolic and required NAD and NADH, respectively. A 3α-dehydrogenase from tomato and *Arabidopsis thaliana* that catalyses the metabolism of 24-epityphasterol to 3-dehydro-24-epiteasterone is a cytosolic enzyme requiring NAD. However, the 3β-reductase that catalyses the conversion of 3-dehydro-24-epiteasterone to 24-epiteasterone is a microsomal enzyme requiring NADPH. These findings indicate that two or more enzymes are involved in these reactions. These conversions resemble those seen in the cardenolide pathway (see Section 6.3.1) and it might be interesting to check the cardenolide biosynthesis enzymes for their ability to accept BL pathway intermediates as substrates.

6.4.1.7 C2-Oxidase

A cytochrome P450, DDWF1 (CYP92A6), was claimed to be a 2-hydroxylase. However, the function of this P450 remains ambiguous (Kang *et al.*, 2001). The involvement of the 2,3-epoxybrassinosteroids secasterone and 2,3-diepisecasterone in the biosynthesis of castasterone (CS) has been demonstrated in seedlings of *Secale cereale*. Deuterated secasterone, upon administration to rye seedlings, was incorporated into CS and its 2β- and 3β-epimers. Administration of deuterated 2,3-diepisecasterone resulted in CS and 2-epicastasterone (Antonchick *et al.*, 2005).

6.4.1.8 Brassinolide synthase

Brassinolide (BL) has a seven-membered lactone ring that is formed by a Baeyer–Villiger oxidation of its immediate precursor CS. Tomato CYP85A3 catalysed the Baeyer–Villiger oxidation to produce BL from CS in yeast, in addition to the conversion of 6-deoxocastasterone to CS (Nomura *et al.*, 2005). *Arabidopsis* CYP85A2, which was initially characterized as CS synthase (see above), also has BL synthase activity. A microsomal enzyme preparation from cultured cells of *Phaseolus vulgaris* catalysed a conversion from CS to BL. This enzyme preparation also catalysed the conversions of 6-deoxocastasterone and typhasterol to CS.

6.4.2 Transport

BRs must move from the interior of the cell, namely the site of its synthesis, to the exterior, where they are perceived by the same cell or by neighbouring cells. On the other hand, BRs are widely distributed throughout reproductive and vegetative plant tissues. This raises the question of whether or not BRs are transported over long distances between these tissues. Several lines of evidence indicate that this is not the case but that, although BRs do not undergo long-distance transport, they may influence long-distance signalling by altering auxin transport (Symons *et al.*, 2008).

6.5 Phytoecdysteroids

Phytoecdysteroids are a family of more than 200 plant steroids related in structure to the invertebrate steroid hormones (20-hydroxyecdysone). Most phytoecdysteroids possess a cholest-7-en-6-one carbon skeleton, a 14α-hydroxy-7-en-6-one chromophore and A/B-*cis* ring fusion (5β-H). The carbon number can vary between C19 and C29 (sometimes C30). Their biological significance is still under discussion. Two main hypotheses are described: first, that they have a hormonal role within the plant; second, they may participate in the defence of plants against non-adapted phytophagous invertebrates. A number of other specific roles have been demonstrated as well for individual plants. To clarify the final role(s) of phytoecdysteroids much more experimental data

have to be collected. Biological aspects of phytoecdysteroids have been reviewed by Kubo and Hanke (1986), Adler and Grebenok (1999), Dinan (2009) and Dinan and Lafont (2006).

The first phytoecdysteroids were isolated from *Podocarpus nakaii* (Nakanishi *et al.*, 1966). Meanwhile it became apparent that phytoecdysteroids are rather widespread in more than 100 plant families covering the whole plant kingdom from ferns to angiosperms (Lafont and Wilson, 1996). Their content in plants range from minute amounts to typically 0.1%, some organs contain up to 3.2%, e.g in *Diploclistsia glaucescenes* (Bandara *et al.*, 1989). A typical chemical structure of a phytoecdysteroid is shown in Fig. 6.11.

If one considers combinations of possible modification of the chemical structure, it may be assumed that there are more than 1000 individual structures which may occur *in planta* (Dinan *et al.*, 1999). Many of the modifications found in phytoecdysteroids are also found in other classes of plant triterpenoids (e.g. BRs, sterols). This fact raises the possibility that enzymes of the biosynthetic pathways may be common to plants producing these various classes of metabolites. A lack of specificity of these enzymes may cause the generation of many diverse metabolites or biosynthetic products without massive genetic redundancy of the corresponding enzymes involved.

The largest concentrations of phytoecdysteroids were located in fruits, flowers, bark, rhizomes, roots and seeds (Dinan, 2009). Phytoecdysteroids are highest in tissues which are most important for the survival of the plant. Evidently, a clear correlation between the accumulation in specific organs and the proposed biological function as protective compounds may be drawn.

6.5.1 Biosynthesis

The understanding of the biosynthetic pathway(s) for phytoecdysteroids is limited (Fig. 6.11). A summary of the knowledge of the biosynthesis has been reviewed by several authors (e.g. Rees, 1995; Lafont, 1997). Studies by Adler and co-workers have demonstrated that active biosynthesis of phytoecdysteroids takes place in developing tissue and that these compounds are transported to other organs (Grebenok and Adler, 1991; Tomás *et al.*, 1993). Phytoecdysteroids are synthesized in plants from mevalonic acid via cholesterol and/or lanosterol (for detail see Tomás *et al.*, 1992; Adler and Grebenok, 1999). The authors conclude that two major pathways, side-chain dealkylation (C29→C28, but not C29→C27) and 5β-hydroxylation, are operating leading to two series of related C29/C28/C27 compounds. The extent to which both reactions are operational is very much depending on the conditions, and this goes a long way to explaining the highly variable phytoecdysteroid profiles found in *Ajuga reptans* (Tomás *et al.*, 1992). The diverse profiles and the use of common intermediates open a new insight into the metabolic network existing in the plants.

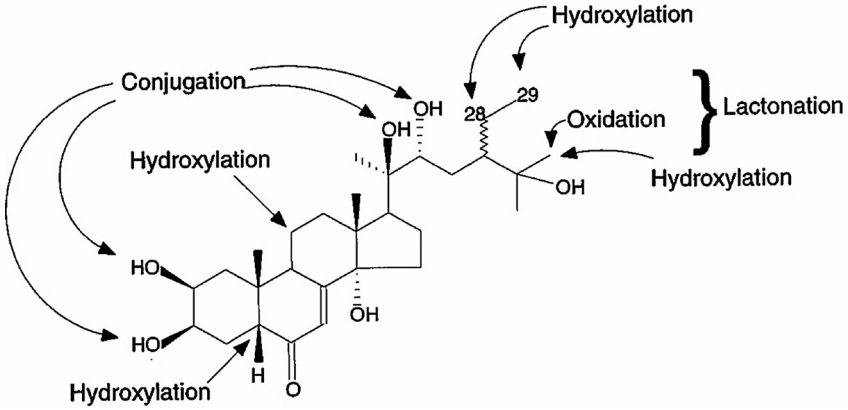


Figure 6.11 Structure and sites of biochemical modification of phytoecdysteroids. The structures of the most commonly reported phytoecdysteroid, 20-hydroxyecdysone, and a 24-alkylphytoecdysteroid, cyasterone. Some common sites of biochemical modifications reported for phytoecdysteroids. (From Adler and Grebenok, 1999.)

6.5.2 Biotransformations

PE-free callus from *Polypodium vulgare* was shown to biotransform ecdysone to 20-hydroxyecdysone, which is the last step in the biosynthetic pathway of the main plant PE. This hydroxylation is catalysed by a cytochrome P450 enzyme which was subsequently purified from that source (Canals *et al.*, 2005). In another study, Reixach *et al.* (1999) have shown that 25-deoxy-20-hydroxyecdysone was transformed efficiently in both tissues into 20-hydroxyecdysone, but no 25-deoxyecdysteroids such as pterosterone and inokosterone were formed. Likewise, incubation of 2-deoxyecdysone produced exclusively ecdysone and 20E, indicating a high 2-hydroxylase activity in both tissues.

6.6 Steroid saponins and steroid alkaloids

6.6.1 Steroid saponins

Saponins may be classified into two groups, the triterpenoid saponins, which will not be considered here (for reviews, see Mahato *et al.*, 1992; Conolly *et al.*, 2008; Abe, 2007; Vincken *et al.*, 2007; Liang and Zhao, 2008), and the steroid saponins. Steroid alkaloids behave like saponins but are sometimes treated as 'alkaloids', although these compounds are formed from intermediates of the steroid saponin pathway. Steroid saponins constitute a vast group of plant-borne glycosides present almost exclusively in the monocotyledonous angiosperms and occurring in only a few dicotyledonous families, such as the

Fabaceae and Plantaginaceae. When dissolved in water, saponins form soapy solutions and can therefore be used as detergents in the preparation of galenicals and cosmetics. Saponins can increase the permeability of biomembranes and may thus exhibit cytotoxic, haemolytic and antiviral properties; most of them are highly toxic for fish. Moreover, steroid saponins are important starting materials for the commercial production of steroid hormones.

6.6.1.1 Biosynthesis

As far as the enzymatic and genetic aspects are concerned, the biosynthesis of steroid saponins (including the steroid alkaloids) has not been studied extensively. The C_{27}-steroid saponins (including the steroid alkaloids) are probably formed from cholesterol in such a way that ultimately one (furostanes) or two heterocyclic rings (spirostanes, spirosolanes, solanidanes) connected to C-16 and C-17 are attached to the steroid ring system. Side-chain hydroxylations at C-26 or C-27 with subsequent *O*-glycosylation may be important steps in spirostane-type saponin formation. For example, 26-*O*-glycosylated oligofurostanosides may be regarded as direct precursors of dioscin and related saponins or even as 'preformed spirostanes'; once the glucose is removed, intramolecular ketalization and spiroether formation can be accomplished (Fig. 6.1). A crucial step in steroid alkaloid formation is the replacement of one of the side-chain hydroxyl groups by an amino group. Subsequently, the amino nitrogen is 'trapped' by ring closure. Their biosynthesis has been reviewed by Haralampidis *et al.* (2002).

The saponin genins are linked to sugars at the 3-hydroxy group. Frequently, several sugar moieties are attached forming a branched oligosaccharide chain. Little is yet known about the regulation of saponin biosynthesis and the enzymes involved in saponin formation in plants. The accumulation of, e.g., glycoalkaloids can be inhibited by the sterol synthesis inhibitor, tridemorph (Bergenstrahle *et al.*, 1992b). Tetcyclacis, a plant growth retardant, caused a significant increase in the cholesterol content of the roots of fenugreek but a decrease of their sapogenin content. Since tetcyclacis was shown to be only a poor inhibitor of the SAM:cycloartenol-C-24-methyltransferase, cholesterol accumulation does not result from the inhibition of the sterol side-chain-alkylating enzyme (Cerdon *et al.*, 1995). As in the case of cardenolides (see Section 6.3.1), it remains to be determined why the increase of a putative precursor does not enhance secondary metabolite formation.

Furostanol glycoside 26-O-β-glucosidase (F26G) Some plants contain biologically inactive, bisdesmosidic furostanol saponins (Fig. 6.1). Upon tissue damage, these saponins can come in contact with a β-glucosidase, which removes the glucose molecule attached to C-26, resulting in the formation of highly active spirostanol-type saponins. These metabolites may also be formed from furostanol glycosides during postharvest treatment or storage. The F26G involved in this conversion was purified from *Costus speciosus* rhizomes. The enzyme was highly specific for cleavage of the C26-bound glucose moiety

of furostanol glycosides. The purified F26G is dimeric (subunits: 54 and 58 kDa). The *N*-terminal sequence of the 54 kDa protein has a high similarity to the sequences found in *N*-terminal regions of known plant β-glucosidases (Inoue and Ebizuka, 1996). Using primers based on sequences of F26G cDNA fragments, 5′- and 3′-end clones were isolated by rapid amplification of cDNA ends (RACE). The entire coding portion of F26G cDNA was cloned by using primers designed from sequences of the RACE products, and cell-free extracts of *Escherichia coli* expressing F26G cDNA showed F26G activity (Inoue *et al.*, 1996). F26G activity was also detected in other plant materials, e.g. the inflorescenses of *Allium erubescens* (Vardosanidze *et al.*, 1991).

6.6.2 Steroid alkaloids

Steroid alkaloids are reported to be involved in chemical defence against herbivores and microbes and to have a variety of adverse as well as beneficial effects in cells, animals and humans.

6.6.2.1 Biosynthesis

Steroid alkaloids are derived from cholesterol with appropriate side-chain modifications. The amino group is probably derived from *L*-arginine. They may be regarded as nitrogen analogues of steroid saponins (Dewick, 2002). Their biosynthesis on the enzyme or gene level has poorly been investigated. *UDP-glucose:solanidine 3-O-β-D-glucosyltransferase (solanidine-GTase)* and *UDP-glucose:solanodine 3-O-β-D-glucosyltransferase (solasosodinGTase).* The glycosylations of the spirostanol alkaloid, solanidine (Fig. 6.1), are considered to be the terminal steps in the synthesis of the potentially toxic glycoalkaloids, α-solanine and α-chaconine. As mentioned previously, at least two different enzymes responsible for steroid glucosylation are present in potato (Zimowski, 1992), and it was found that the cytosolic glucosyltransferase, termed solanidine-GTase, glycosylated solanidine with a high yield (Zimowski, 1991). Concomitant to the accumulation of glycoalkaloids in freshly cut potato tubers was an increase in the specific activity of the solanidine-GTase, whereas the activity of the sterol-specific SGTase (see above) was unaffected by either tuber slicing or addition of ethephon (Bergenstrahle *et al.*, 1992b). The accumulation of glycoalkaloids can be inhibited by the ethylene-releasing substance, ethephon. Discs incubated at high levels of ethephon had a very low glycoalkaloid content and also a lower activity of solanidine-GTase than control discs. Thus, solanidine-GTase may well be involved in initiation and regulation of glycoalkaloid biosynthesis.

Solanidine-GTase was purified to near homogeneity from potato sprouts. The isolation of this enzyme was complicated by its copurification with patatin. Separation of the two proteins was finally achieved by binding the glycosylated patatin to concanavalin A, under conditions where the solanidine-GTase did not bind. In this study, no enzyme activity was detected

when UDP-galactose was used as a substrate (Stapleton *et al.*, 1991). This is in contrast to other reports where soluble enzyme preparations from potato tubers were shown to catalyse solanidine galactosylation, although with a much lower yield, using UDP-galactose as the sugar donor (Zimowski, 1991; Bergenstrahle *et al.*, 1992a). After purification, solanidine glucosylating and galactosylating activities were recovered in the same fractions but with loss of most of the galactosyltransferase activity (Bergenstrahle *et al.*, 1992a). With respect to substrate specificity, it was shown that the spirosolane alkaloids tomatidine and solasodine were glucosylated even better than solanidine, whereas 3β-hydroxy steroids lacking a ring nitrogen, such as cholesterol, diosgenin, digoxigenin and β-sitosterol, did not serve as glucose acceptors. UDP-galactose was found to be a competitive inhibitor of the solanidine glucosyltransferase of potato (Bergenstrahle *et al.*, 1992a).

Spirosolane-type steroid alkaloids were glucosylated by a soluble 55 kDa protein from *Solanum melongena* much better than solanidane-type compounds. The enzyme was, therefore, termed solasodine-GTase, although it may be closely related to the solanidine-GTase described above. In order to distinguish between glucosyltransferase and galactosyltransferase activity, UDP-xylose was used to block UDP-glucose 4-epimerase when using UDP-galactose as a glycosyl donor. Interestingly, spirosolane-type sapogenins, such as diosgenin, tigogenin, yamogenin and hecogenin, were also glycosylated. Sterols, on the other hand, were not glycosylated by the cytosolic enzyme(s) (Paczkowski and Woiciechowski, 1994; Paczkowski *et al.*, 1997). Kohara *et al.* (2005) were the first to clone a glucosyltransferase involved in steroid alkaloid biosynthesis. Later on the same group characterited and engineered steroid alkaloid glucosyltransferases and their homologs. They successfully converted a non-functional homolog into an active glucosyltransferase (Kohara *et al.* 2007).

6.6.3 Withanolides

The withanolides (Fig. 6.1) are C_{28}-steroids and biogenetically related to the steroid saponins in that they are derived from ergostane-type sterols, in which C-22 and C-26 are oxidized and become part of a lactone (reviewed by Glotter, 1991). These compounds appear to be specific for the Solanaceae. Their biosynthesis has not yet been studied at the enzyme level. Tracer studies have indicated that C-26 is directly derived from C-2 of mevalonolactone. From the relative incorporation rates, it was concluded that the side chain of the sterol precursor had been partially cleaved during the biosynthetic process (Veleiro *et al.*, 1985). There is no direct evidence whether the major biosynthetic oxidative processes begin in ring A (C-1) or in the side chain (C-22 and C-26). However, since all the withanolides have the side chain, in one or other of its final forms, it is reasonable to assume that the elaboration of the latter precedes the first step in the functionalization of the carbocyclic system. Recently, Sangwan *et al.* (2008) found that ^{14}C from [2-^{14}C]-acetate and [U-^{14}C]-glucose

was incorporated into withanolide A in roots of *Withania somnifera*, and the authors concluded that withanolide A is de novo synthesized within roots.

6.6.4 Transport and storage

Radiolabelled diosgenin-type saponins were isolated from different parts, such as stem, leaf, seeds, flowers and rhizomes, of *Costus speciosus* after feeding ^{14}C-labelled precursors. The results indicated that: (1) diosgenin is biosynthesized in leaves and then translocated to all the parts of the plant and (2) glycosidation of diosgenin takes place in all parts of the plant and diosgenin glycosides are stored in rhizomes, seeds and flowers. Saponin deglycosidation was observed only in the rhizomes (Akhila and Gupta, 1987).

6.7 Conclusions

Plant sterols are products of primary metabolism, but they may also be regarded as direct precursors of many secondary plant metabolites, such as the cardiac glycosides, saponins and steroid alkaloids. All of the compounds mentioned share the same basic skeleton; therefore, the accumulation of a particular compound can only be achieved if (1) enzymes with a high degree of substrate specificity are involved in their biosynthesis, (2) metabolites can be channelled efficiently to the respective pathways and (3) products can be transported, sequestered and/or stored in specific compartments.

A detailed knowledge of the localization, properties and substrate preferences of the different enzymes involved in steroid formation in plants is necessary to understand the various pathways, their regulation and the biosynthetic relationships among the various groups of steroids. With regard to ring formation and annealing, and the biosynthetic sequence realized, initial conformation and conformational changes accomplished during biosynthesis are of utmost importance. This has been elaborated exceptionally well for sterol formation, in which specific enzymes are involved that can act only on molecules with appropriate conformation. It is most likely that similar restrictions apply to, e.g., 5β-cardenolide formation, where specific conformational changes are accomplished by progesterone 5β-reduction and 14β-hydroxylation, although this has not yet been clarified unambiguously. Since pregnenes are assumed to be intermediates in various pathways, several steroid-modifying enzymes, such as 3-hydroxysteroid dehydrogenases, 3-oxidoreductases and Δ^5-steroid reductases (see Section 6.3.1), may compete for the same substrate. Therefore, the various pregnane-modifying enzymes isolated from *Digitalis* may not necessarily be operative in the cardenolide pathway(s) only; progesterone 5α-oxidoreductase and progesterone 5β-oxidoreductase share the same substrate as do 3α-hydroxysteroid 5β-oxidoreductase and 3β-hydroxysteroid 5β-oxidoreductase. Moreover, one part of the intermediate pool which qualifies for further use in a specific

pathway, e.g. cardenolide biosynthesis, may be removed and funneled into known or hitherto unknown pathways.

The storage forms of plant sterols as well as of most of the secondary plant products derived from the cycloartenol pathway have sugars attached to the hydroxyl group at C-3 of the steroid skeleton. Some of the glycosyltransferases and glycosidases involved in the formation of various steroids have been demonstrated to exhibit a high degree of substrate specificity. Due to these modifications, the respective molecules may be tagged, so as to be recognized and channelled into the different pathways. In fact, a branched cardenolide pathway was postulated to be operative in *Digitalis lanata* and it was assumed that cardenolide digitoxosylation has to occur at the C-21 stage of the pathway, whereas fucosylation can be accomplished at the C-21 and/or the C-23 stage. These and other findings indicate that steroid glycosylation may take place at various stages and should no longer be regarded as terminal biosynthetic steps that can only be accomplished after the formation of the steroid skeleton.

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