ANNUAL PLANT REVIEWS VOLUME 40

Biochemistry of Plant Secondary Metabolism: Second Edition Edited by Michael Wink
 ISBN: 978-1-405-18397-0

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Biochemistry of Plant Secondary Metabolism

Second Edition

Edited by

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WILEY-BLACKWELL
A John Wiley & Sons, Ltd., Publication

Annual Plant Reviews

This edition first published 2010

Blackwell Publishing was acquired by John Wiley & Sons in February 2007. Blackwell's publishing programme has been merged with Wiley's global Scientific, Technical, and Medical business to form Wiley-Blackwell.

Registered office
John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, United Kingdom

Editorial offices
9600 Garsington Road, Oxford, OX4 2DQ, United Kingdom
2121 State Avenue, Ames, Iowa 50014-8300, USA

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Library of Congress Cataloging-in-Publication Data

Biochemistry of plant secondary metabolism / edited by Michael Wink. – 2nd ed.
p. cm. – (Annual plant reviews ; v. 40)
Includes bibliographical references and index.
ISBN 978-1-4051-8397-0 (hardback : alk. paper) 1. Plants–Metabolism.
2. Metabolism, Secondary. 3. Botanical chemistry. I. Wink, Michael.
QK881.B54 2010
572′.42–dc22 2009038730

A catalogue record for this book is available from the British Library.

Set in 10/12 pt Palatino by Aptara® Inc., New Delhi, India
Printed in Singapore

1 2010

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PREFACE

A characteristic feature of plants is their capacity to synthesize and store a wide variety of low molecular weight compounds, the so-called *secondary metabolites* (SMs) or natural products. The number of described structures exceeds 100 000; the real number in nature is certainly much higher because only 20–30% of plants have been investigated in phytochemistry so far. In contrast to primary metabolites, which are essential for the life of every plant, the individual types of SMs usually occur in a limited number of plants, indicating that they are not essential for primary metabolism, i.e. anabolism or catabolism.

Whereas SMs had been considered to be waste products or otherwise useless compounds for many years, it has become evident over the past three decades that SMs have important roles for the plants producing them: they may function as signal compounds within the plant, or between the plant producing them and other plants, microbes, herbivores, predators of herbivores, pollinating or seed-dispersing animals. More often SMs serve as defence chemicals against herbivorous animals (insects, molluscs, mammals), microbes (bacteria, fungi), viruses or plants competing for light, water and nutrients. Therefore, SMs are ultimately important for the fitness of the plant producing them. Plants usually produce complex mixtures of SMs, often representing different classes, such as alkaloids, phenolics or terpenoids. It is likely that the individual components of a mixture can exert not only additive but certainly also synergistic effects by attacking more than a single molecular target. Because the structures of SMs have been shaped and optimized during more than 500 million years of evolution, many of them exert interesting biological and pharmacological properties which make them useful for medicine or as biorational pesticides.

In this volume of *Annual Plant Reviews*, we have tried to provide an up-to-date survey of the biochemistry and physiology of plant secondary metabolism. A companion volume – M. Wink (ed.) *Functions of Plant Secondary Metabolites and Biotechnology* – published simultaneously provides overviews of the modes of action of bioactive SMs and their use in pharmacology as molecular probes, in medicine as therapeutic agents and in agriculture as biorational pesticides.

In order to understand the importance of SMs for plants, we need detailed information on the biochemistry of secondary metabolism and its integration into the physiology and ecology of plants. Important issues include

characterization of enzymes and genes of corresponding biosynthetic pathways, and of transport and storage mechanisms, regulation in space/time and compartmentation of both biosynthesis and storage. The study of secondary metabolism has profited largely from the recent progress in molecular biology and cell biology and the diverse genome projects. Although *Arabidopsis thaliana* is not an excellent candidate to study secondary metabolism on the first view, the genomic analyses, EST-libraries, mutants and other tools of *A. thaliana* have been extremely helpful to elucidate secondary metabolism in other plants.

The present volume is the second edition of a successful first edition which was published in 1999 and which has received many positive responses from its readers. To achieve a comprehensive and up-to-date summary, we have invited scientists who are specialists in their particular areas to update their previous chapters. This volume draws together results from a broad area of plant biochemistry and it cannot be exhaustive on such a large and diverse group of substances. Emphasis was placed on new results and concepts which have emerged over the last decades.

The volume starts with a bird's eye view of the biochemistry, physiology and function of SMs (M. Wink), followed by detailed surveys of the major groups of SMs: alkaloids and betalains (M.F. Roberts *et al.*); cyanogenic glucosides, glucosinolates and non-protein amino acids (D. Selmar); phenyl propanoids and related phenolics (M. Petersen *et al.*); terpenoids, such as mono-, sesqui-, di- and triterpenes, cardiac glycosides and saponins (M. Ashour *et al.*, W. Kreis and F. Müller-Uri). The final chapter discusses the evolution of secondary metabolism (M. Wink *et al.*). The structural types of SMs are often specific and restricted in taxonomically related plant groups. This observation was the base for the development of 'chemotaxonomy'. A closer look indicates that a number of SMs have a taxonomically restricted distribution. Very often, we find the same SMs also in other plant groups which are not related in a phylogenetic context. There is evidence that some of the genes, which encode key enzymes of SM formation, have a much wider distribution in the plant kingdom than assumed previously. It is speculated that these genes were introduced into the plant genome by horizontal gene transfer, i.e. via bacteria that developed into mitochondria and chloroplasts (endosymbiont hypothesis). Evidence is presented that a patchy distribution can also be due to the presence of endophytic fungi, which are able to produce SMs.

The book is designed for use by advanced students, researchers and professionals in plant biochemistry, physiology, molecular biology, genetics, agriculture and pharmacy working in the academic and industrial sectors, including the pesticide and pharmaceutical industries.

The book brought together contributions from friends and colleagues in many parts of the world. As editor, I would like to thank all those who have

taken part in writing and preparation of this book. I would like to thank Theodor C. H. Cole for help, especially in preparation of the index. Special thanks go to the project editor Catriona Dixon from Wiley-Blackwell and her team for their interest, support and encouragement.

Michael Wink
Heidelberg

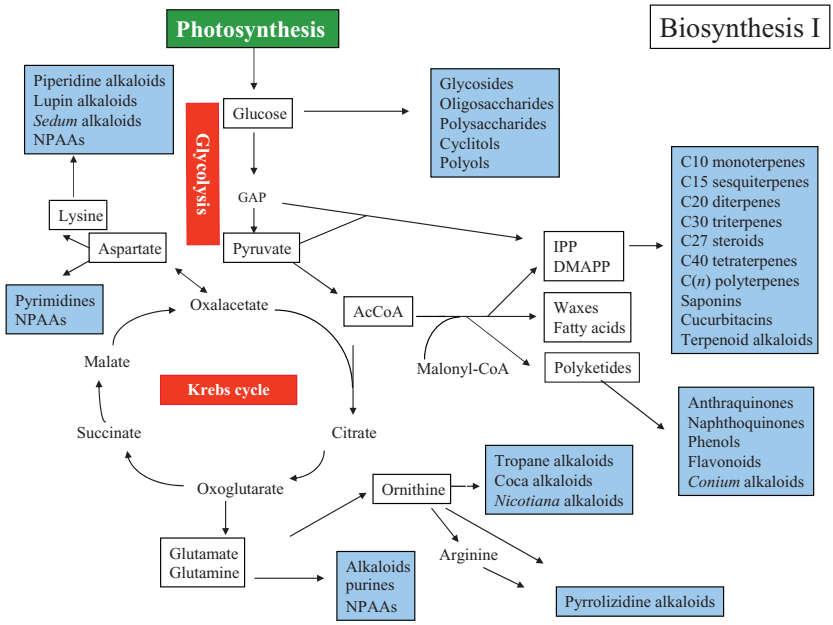

Plate 1 Main pathways leading to secondary metabolites. Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethyl allyl diphosphate; GAP, glyceraldehyde-3-phosphate; NPAAs, non-protein amino acids; AcCoA, acetyl coenzyme A. (Fig. 1.2, p. 7)

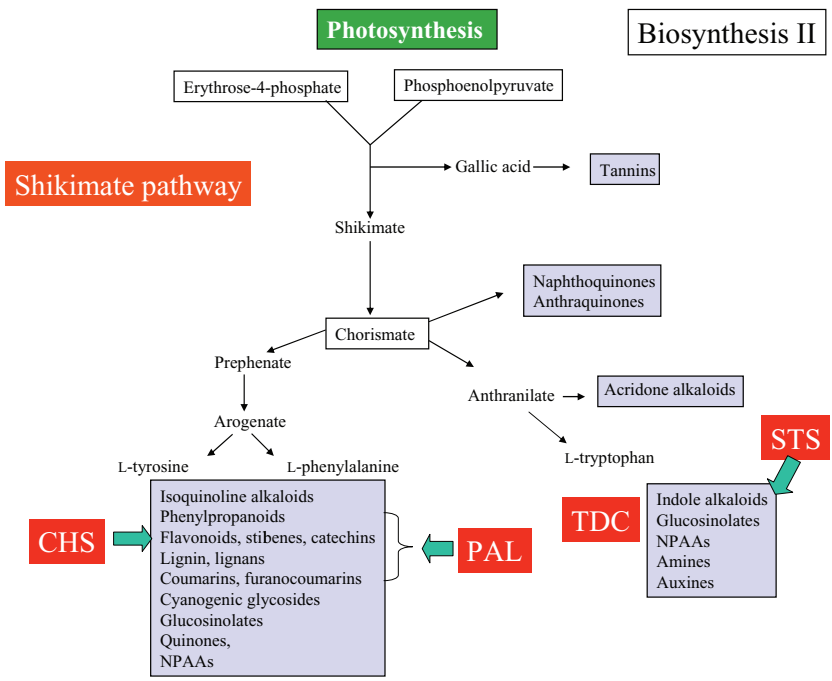

Plate 2 Several pathways of secondary metabolites derive from precursors in the shikimate pathway. Abbreviation: NPAAs, non-protein amino acids; PAL, phenylalanine ammonia lyase; TDC, tryptophan decarboxylase; STS, strictosidine synthase; CHS, chalcone synthase. (Fig. 1.3, p. 8)

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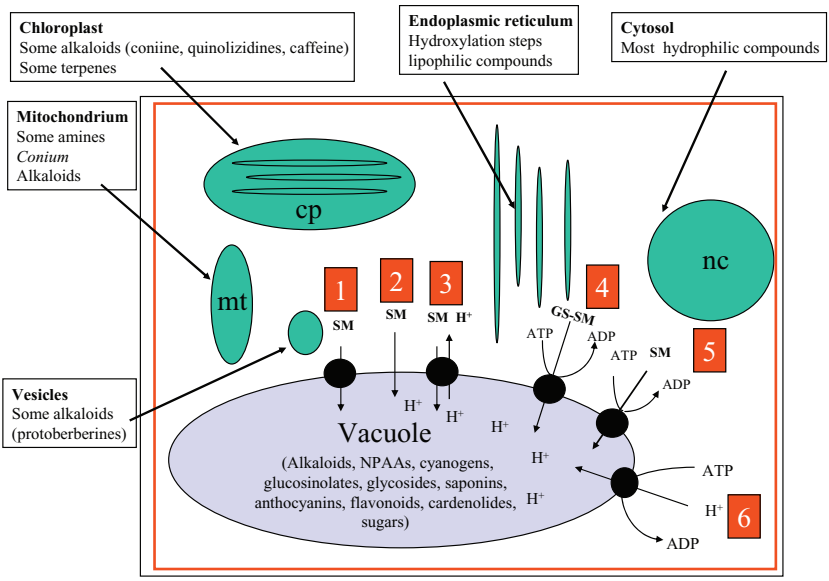

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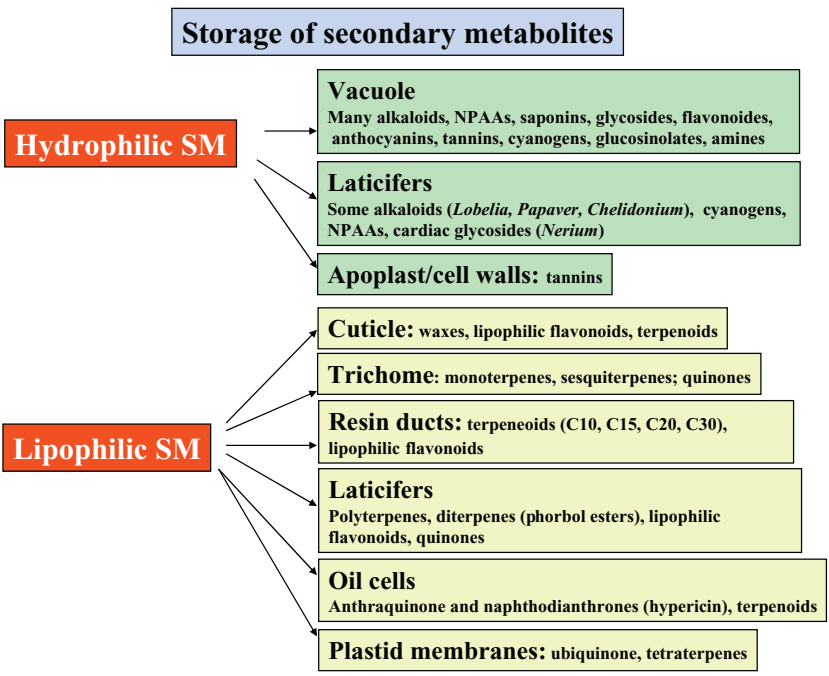

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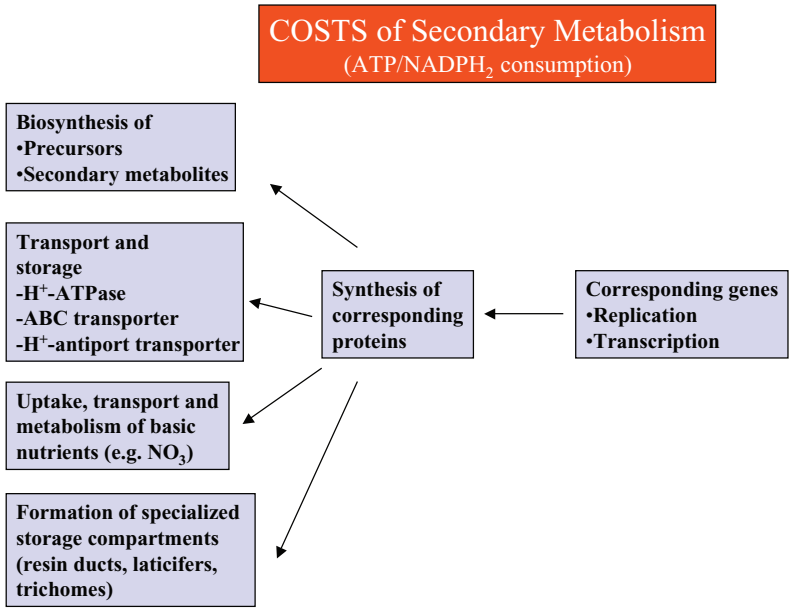

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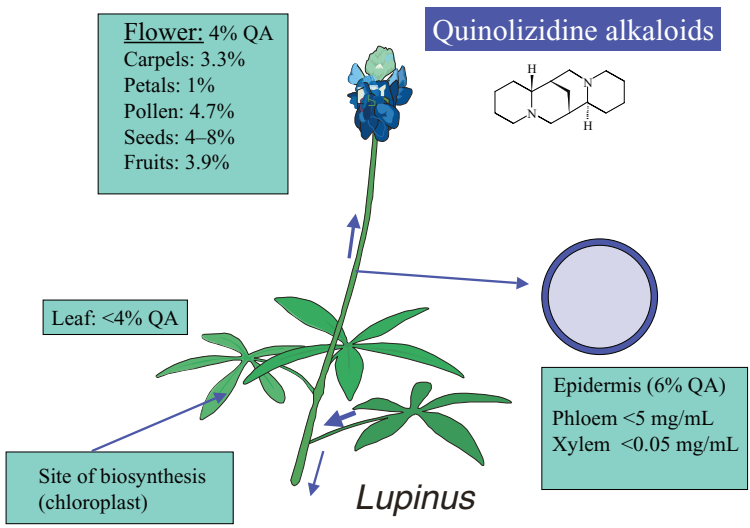

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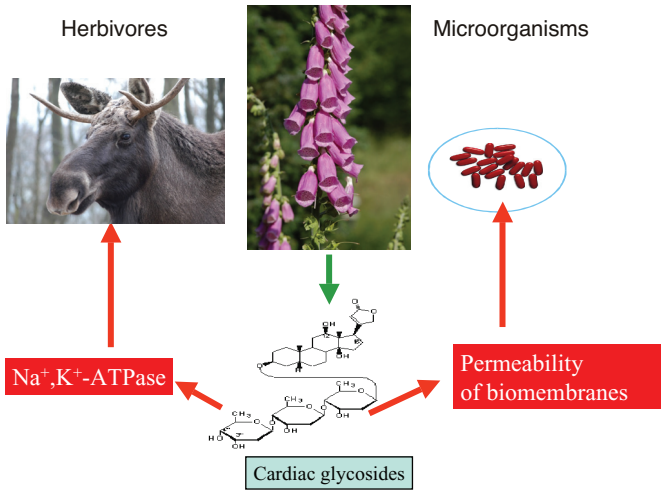
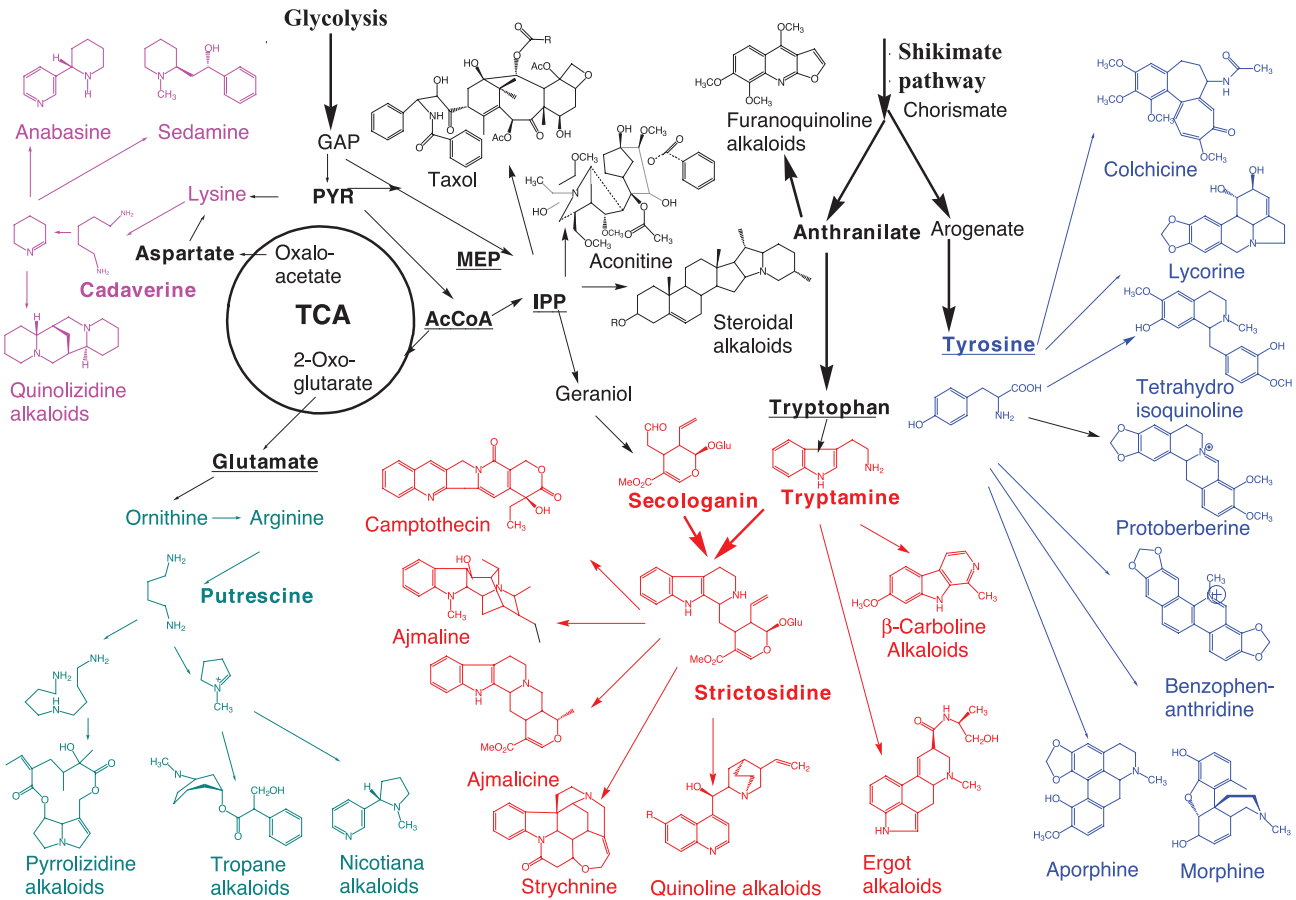


Plate 7 Schematic view of the ecological roles of plant SM. Foxglove (*Digitalis purpurea*) produces cardiac glycosides, which are very toxic to animals (vertebrates, insects) because they inhibit Na^+, K^+-ATPase, one of the most important transporters in animal cells. Cardiac glycosides are additionally toxic to microbes because the molecules have detergent properties and disturb membrane fluidity. (Fig 1.8, p. 16)



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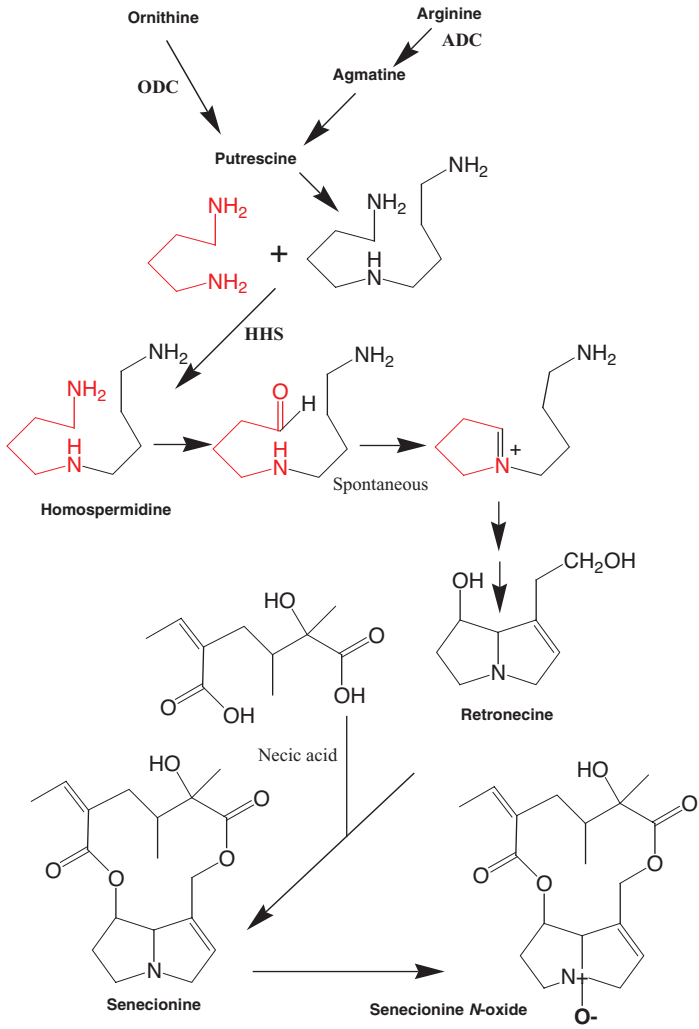

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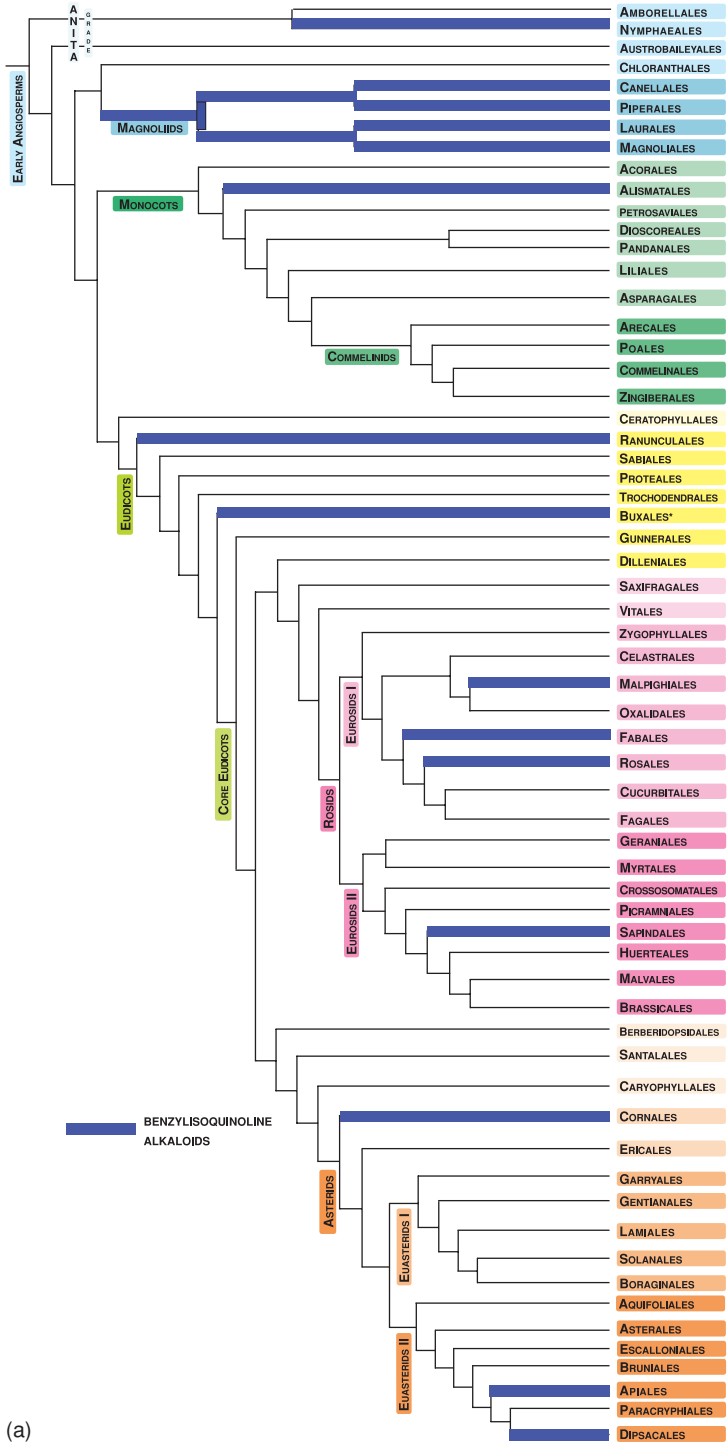
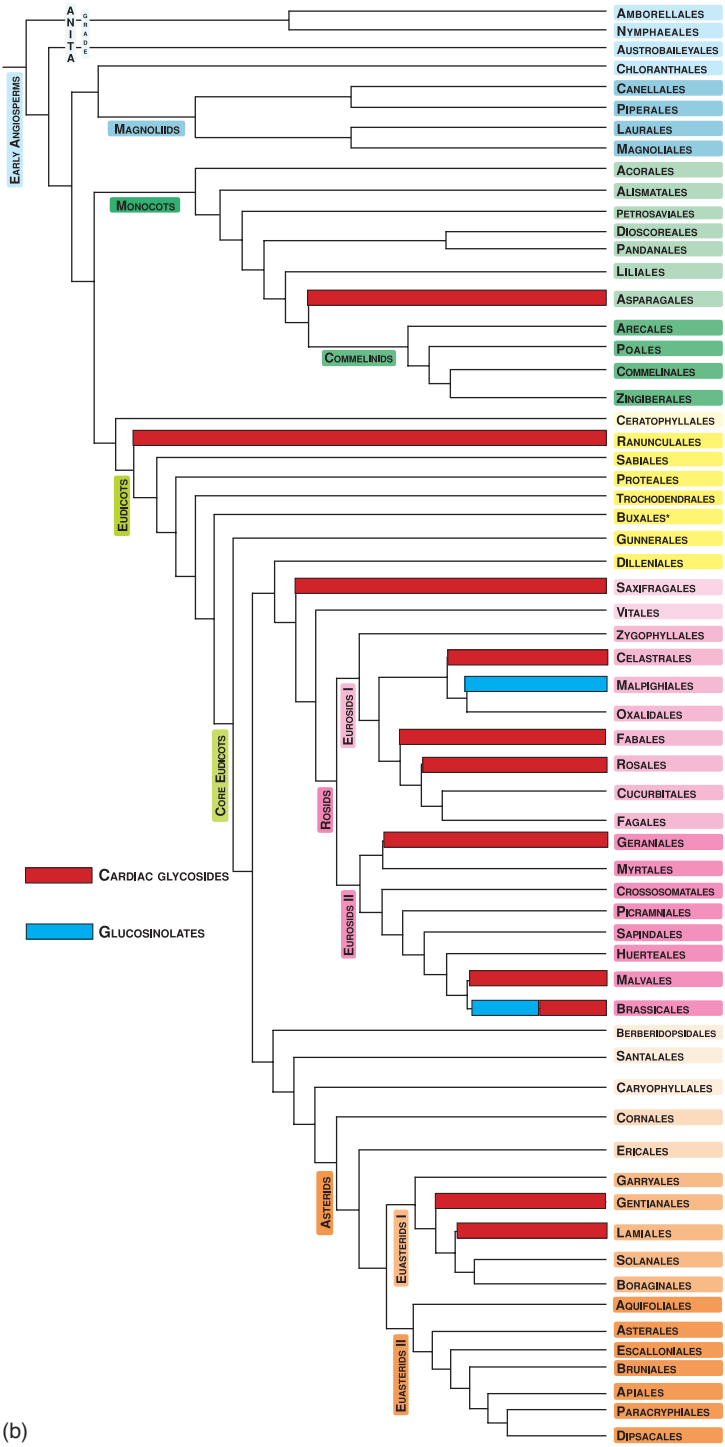


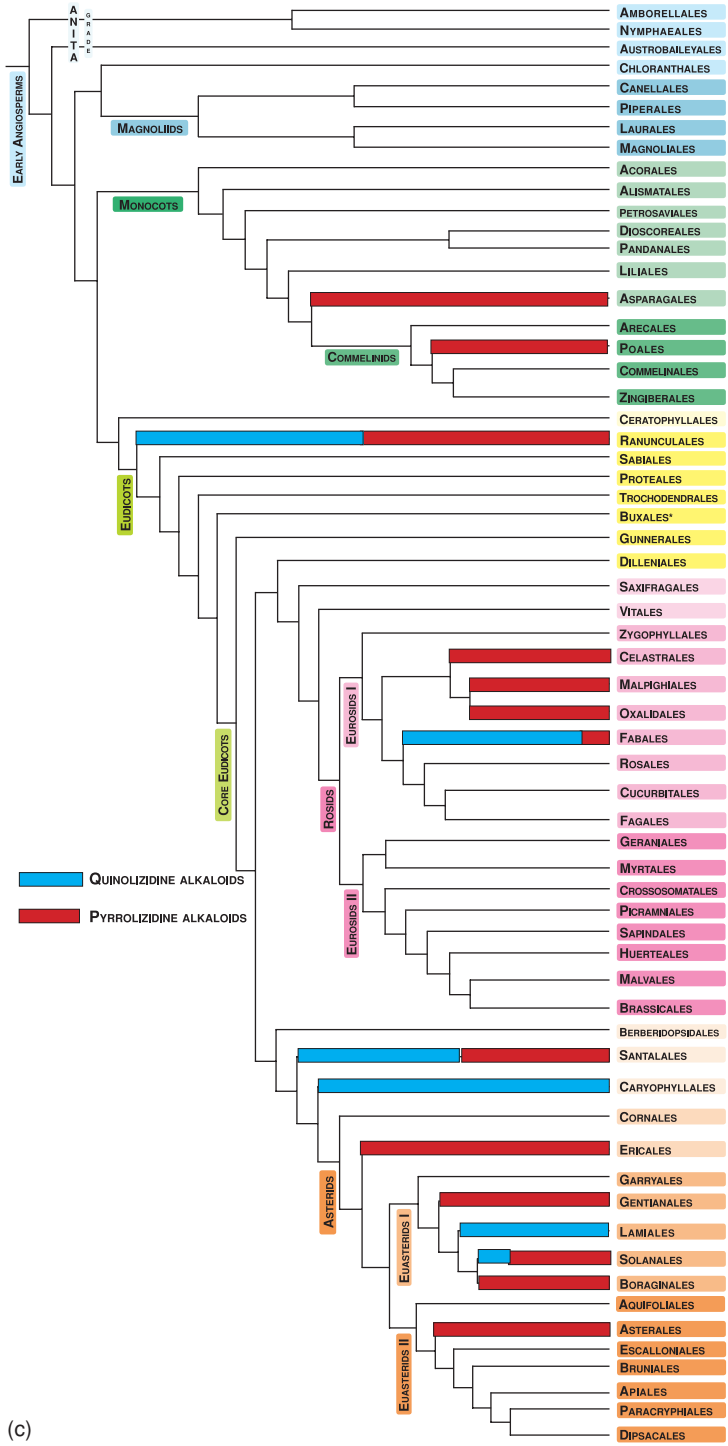
(a)

Plate 10 (a) Families and orders of higher plants, placed in a phylogenetic framework according to APG II. Branches leading to families, which accumulate benzylisoquinoline alkaloids are highlighted in colour. (Fig. 7.8a, p. 375)



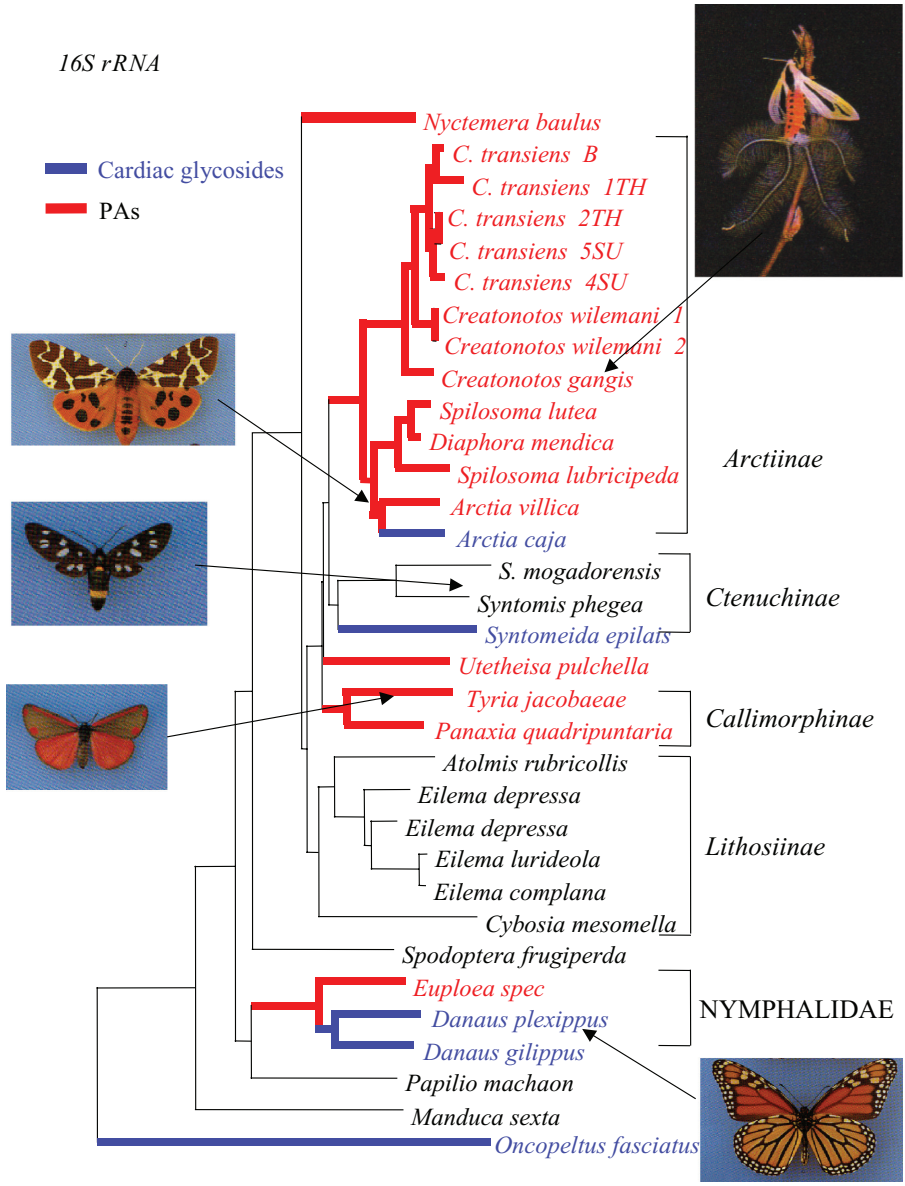
(b)

Plate 11 (b) Families and orders of higher plants, placed in a phylogenetic framework according to APG ll. Branches leading to families, which accumulate glucosinolates, cardiac glycosides are highlighted in colour. (Fig. 7.8b, p. 376)



(c)

Plate 12 (c) Families and orders of higher plants, placed in a phylogenetic framework according to APG ll. Branches leading to families, which accumulate pyrrolizidine and quinolizidine alkaloids and are highlighted in colour. (Fig. 7.8c, p. 377)



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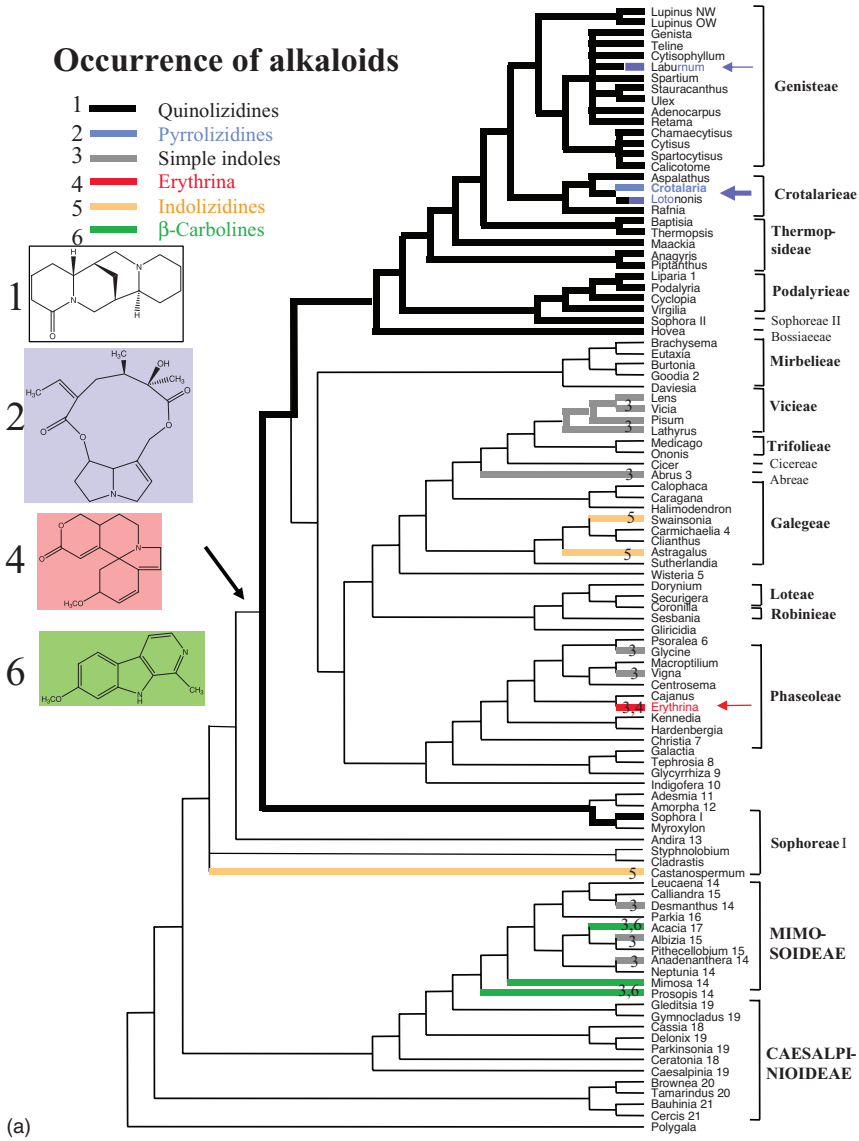

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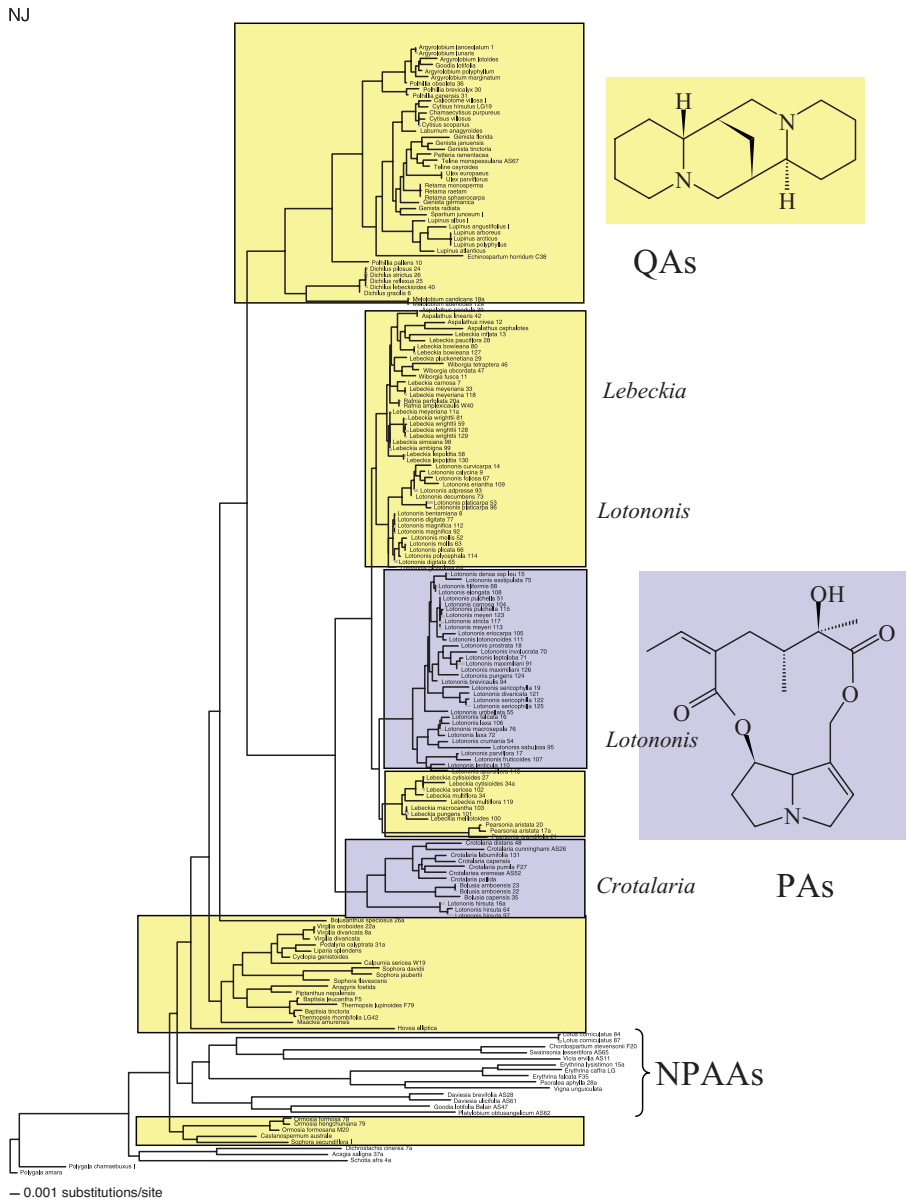
Occurrence of QAs and PAs in legumes



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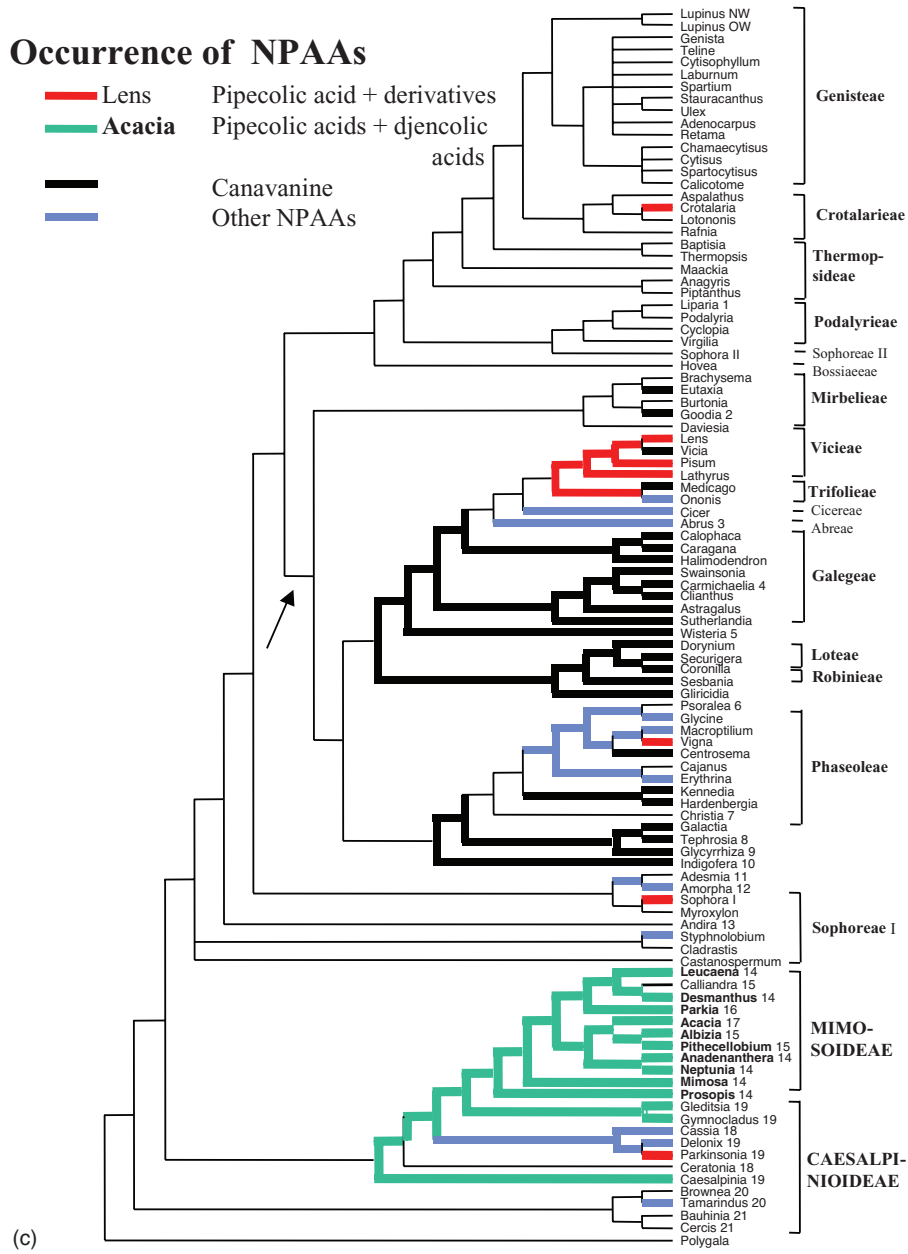

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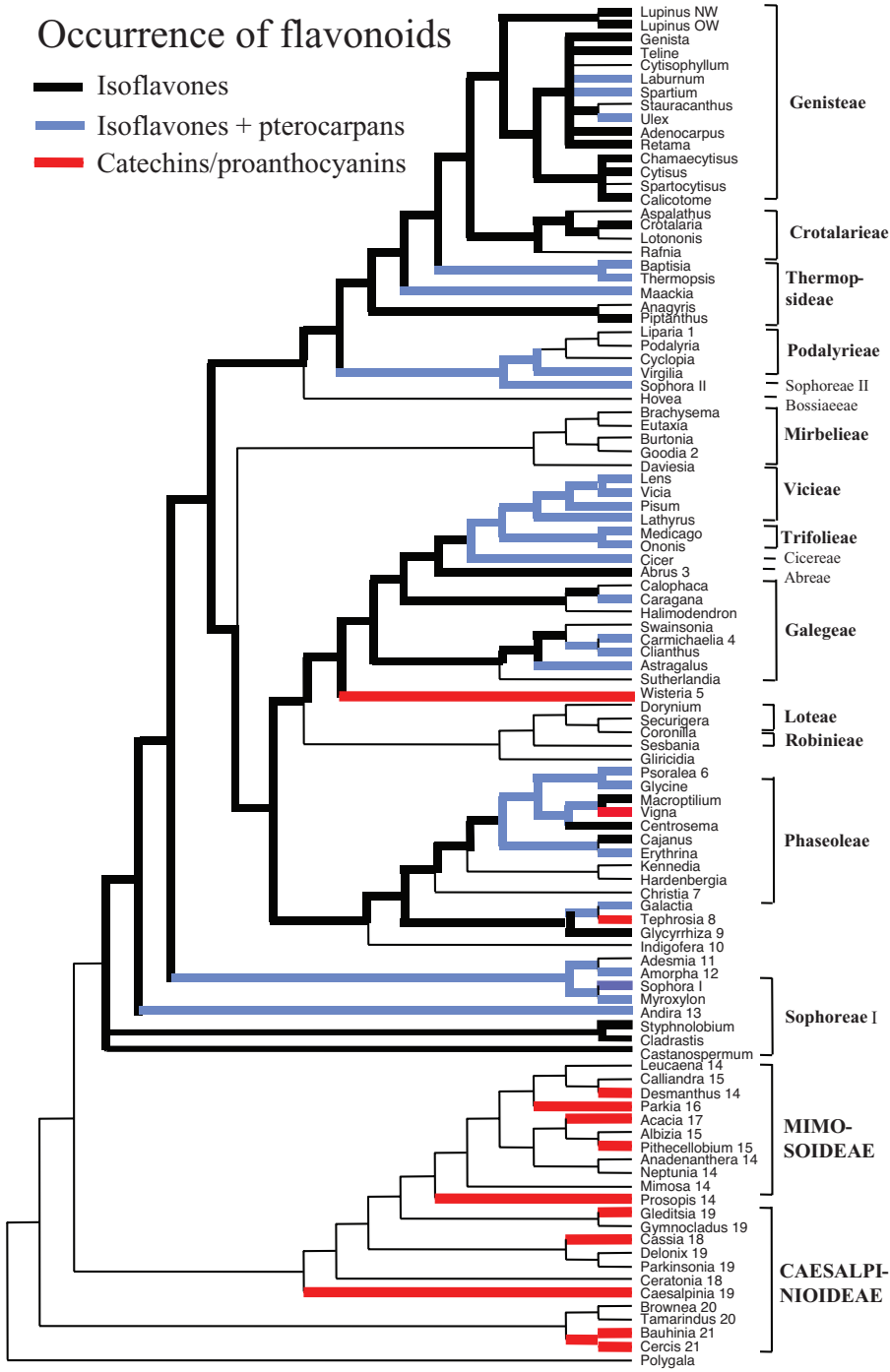
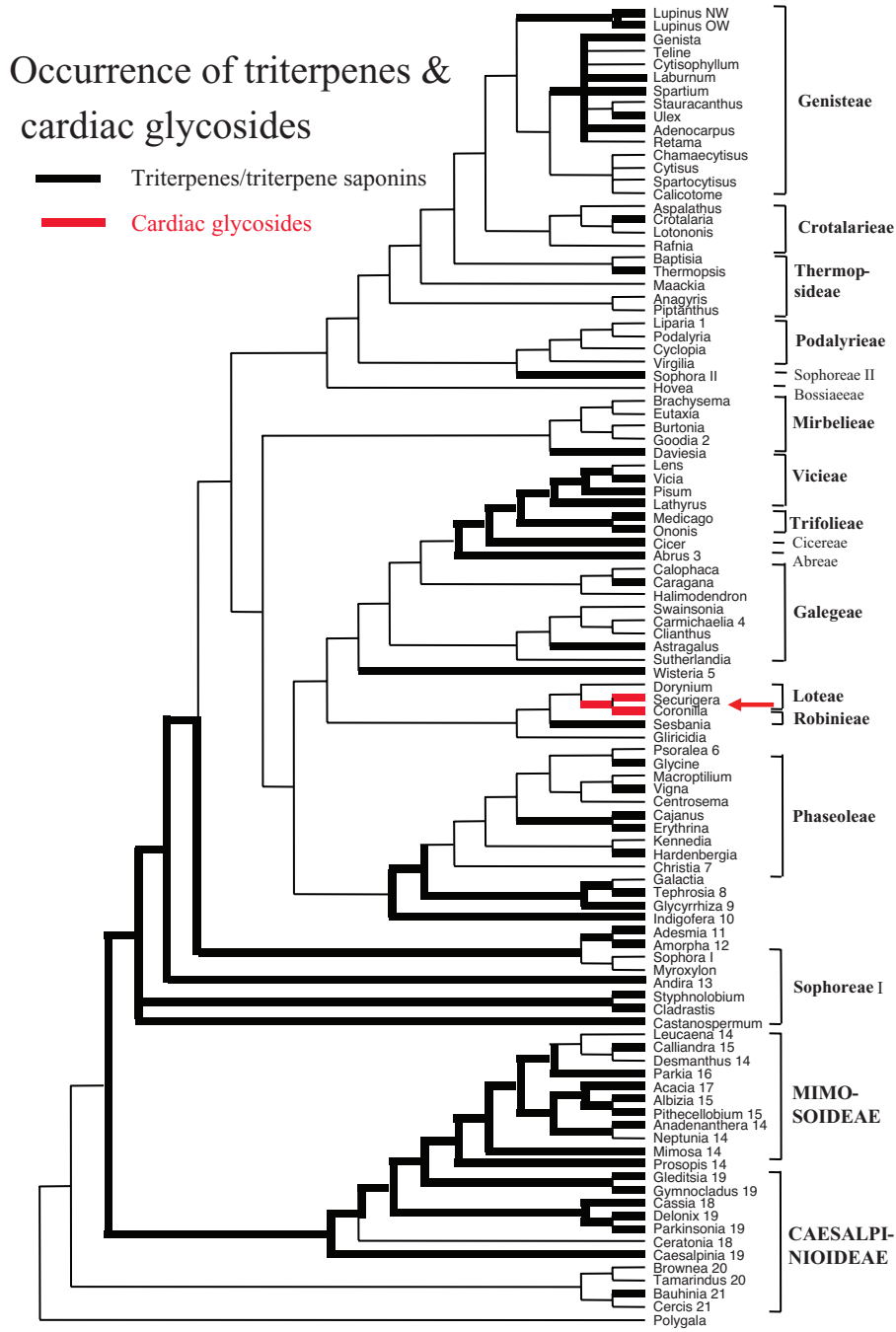


(f)

Plate 17 (f) Occurrence of flavonoids. Key to branches leading to families that accumulate: isoflavones; isoflavones and pterocarpans; catechins/proanthocyanins. See also legend (a). (Fig. 7.11f, p. 394)



(g)

Plate 18 (g) Occurrence of triterpenes and cardiac glycosides. Key to branches leading to families that accumulate: triterpenes/triterpene saponins; cardiac glycosides. See also legend to (a). (Fig. 7.11g, p. 395)

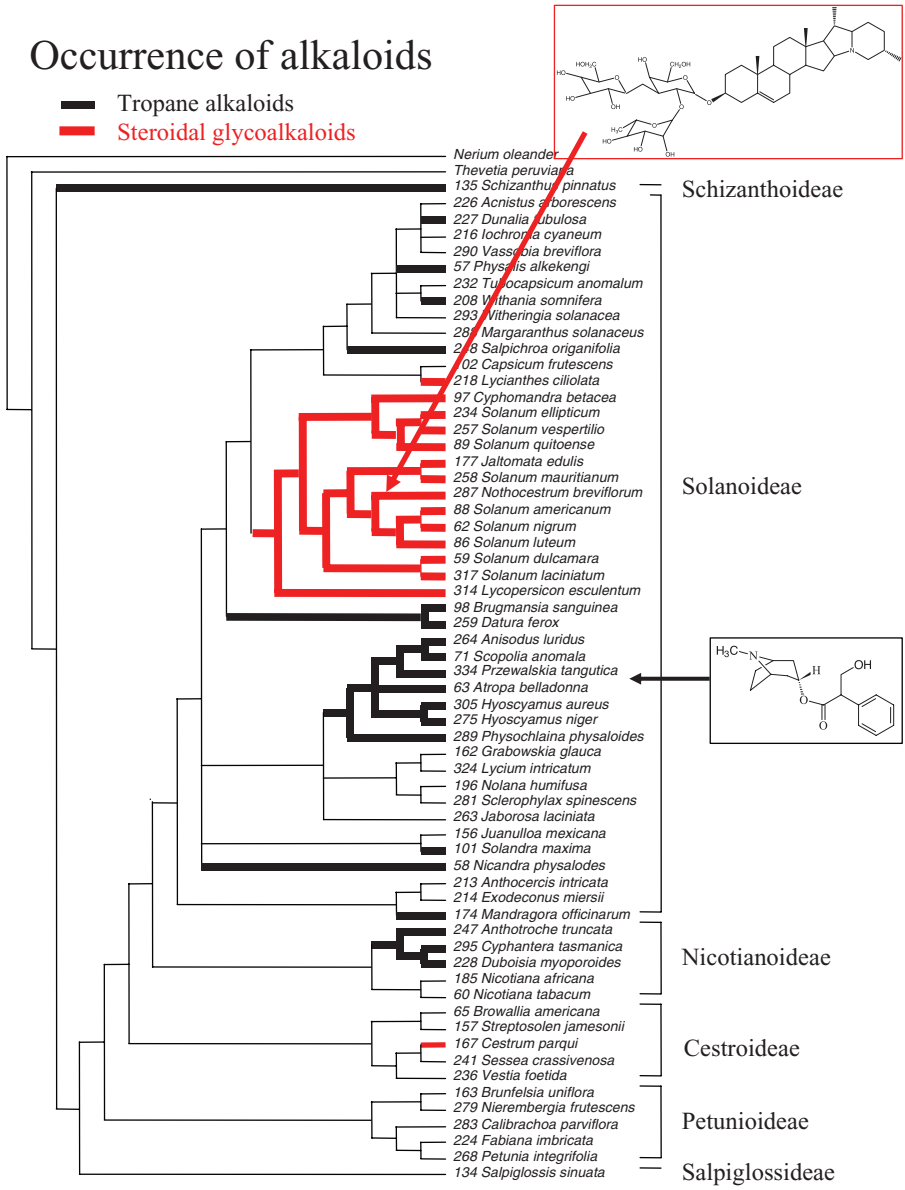


Plate 19 Distribution of tropane and steroidal glycoalkaloids in the family Solanaceae. (After Wink, 2003.) (Fig. 7.13, p. 404)

Occurrence of iridoid glycosides in Lamiaceae

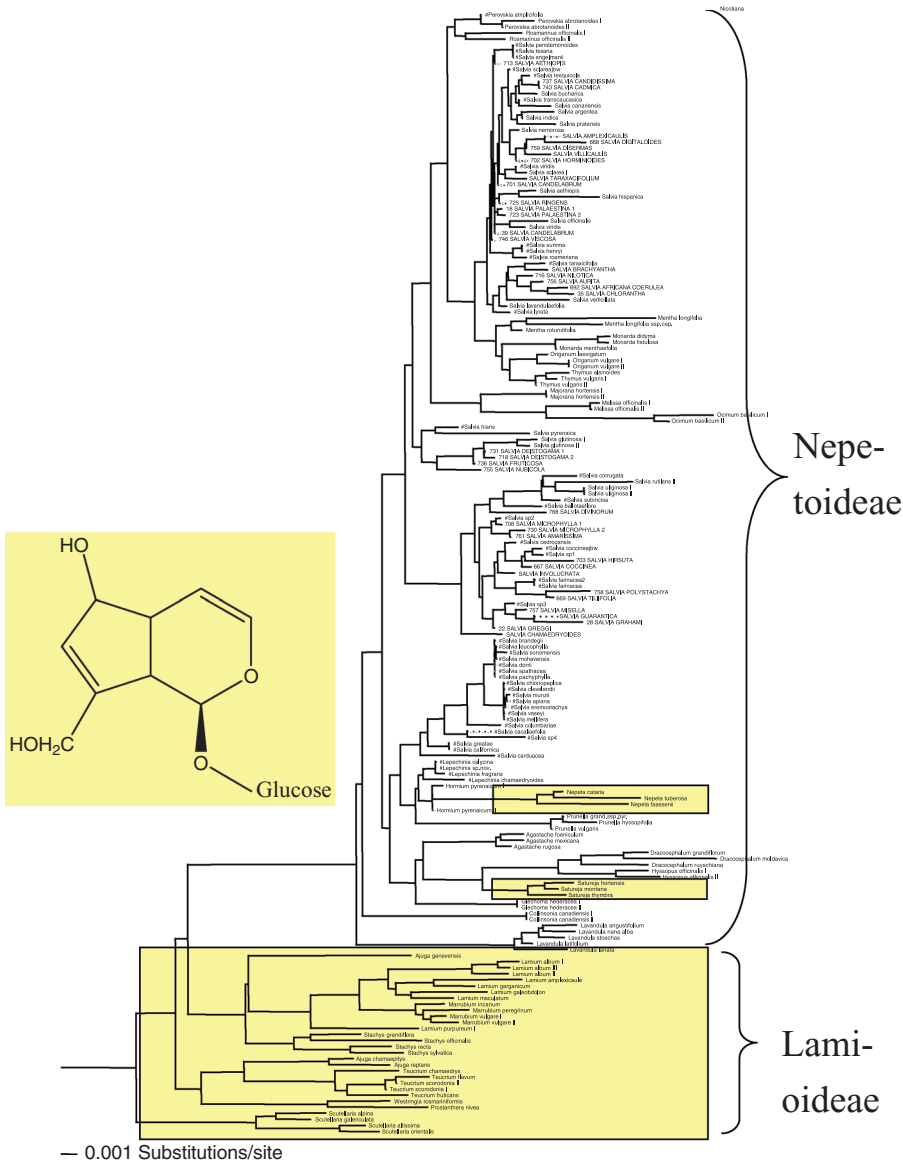


Plate 20 Distribution of iridoid glycosides in the family Lamiaceae, reconstructed from a *rbc*L data set. (After Wink and Kaufmann, 1996.) (Fig. 7.14, p. 405)

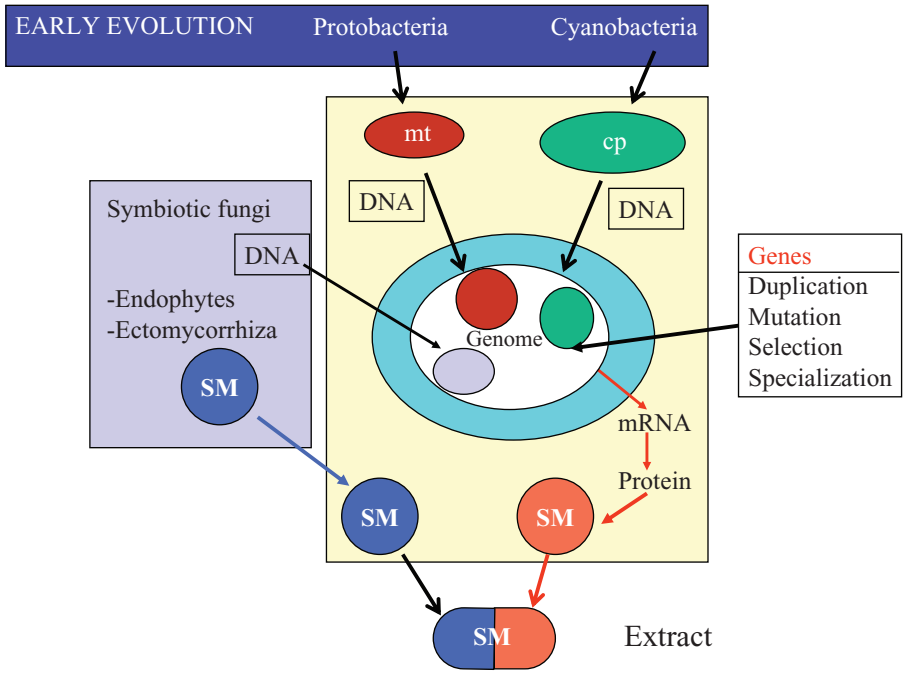


Plate 21 Schematic illustration of a possible origin of SM in plants. (Fig. 7.18, p. 424)

Annual Plant Reviews (2010) **40**, 1–19
doi: 10.1002/9781444320503.ch1

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Chapter 1

INTRODUCTION: BIOCHEMISTRY, PHYSIOLOGY AND ECOLOGICAL FUNCTIONS OF SECONDARY METABOLITES

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Abstract: Secondary metabolites (SM) occur in plants in a high structural diversity. The different classes of SM and their biosynthetic pathways are summarized in this introduction. A typical feature of SM is their storage in relatively high concentrations, sometimes in organs which do not produce them. A long-distance transport via the phloem or xylem is then required. Whereas hydrophilic substances are stored in the vacuole, lipophilic metabolites can be found in latex, resin ducts, oil cells or cuticle. SM are not necessarily end products and some of them, especially if they contain nitrogen, are metabolically recycled. Biosynthesis, transport and storage are energy-dependent processes which include the costs for the replication and transcription of the corresponding genes and the translation of proteins. The intricate biochemical and physiological features are strongly correlated with the function of SM: SM are not useless waste products (as assumed earlier), but important tools against herbivores and microbes. Some of them also function as signal molecules to attract pollinating arthropods or seed-dispersing animals and as signal compounds in other plant – plant, plant – animal and plant – microbe relationships.

Keywords: secondary metabolites (SM); biosynthesis; transport; storage; turnover; costs; ecological functions

1.1 Introduction

A characteristic feature of plants and other sessile organisms, which cannot run away in case of danger or which do not have an immune system to combat pathogens, is their capacity to synthesize an enormous variety of

 ISBN: 978-1-405-18397-0

Table 1.1 Number of known secondary metabolites from higher plants

Type of secondary metabolite	Number[a]
Nitrogen-containing	
Alkaloids	21 000
Non-protein amino acids (NPAAs)	700
Amines	100
Cyanogenic glycosides	60
Glucosinolates	100
Alkamides	150
Lectins, peptides, polypeptides	2000
Without nitrogen	
Monoterpenes (C10)[b]	2500
Sesquiterpenes C15)[b]	5000
Diterpenes (C20)[b]	2500
Triterpenes, steroids, saponins (C30, C27)[b]	5000
Tetraterpenes (C40)[b]	500
Flavonoids, tannins	5000
Phenylpropanoids, lignin, coumarins, lignans	2000
Polyacetylenes, fatty acids, waxes	1500
Polyketides	750
Carbohydrates, organic acids	200

[a]Approximate number of known structures.
[b]Total of terpenoids number exceeds 22 000 at present.

low molecular weight compounds, the so-called secondary metabolites (SM). Although only 20–30% of higher plants have been investigated so far, several tens of thousands of SM have already been isolated and their structures determined by mass spectrometry (electron impact [EI]-MS, chemical ionization [CI]-MS, fast atom bombardment [FAB]-MS, electrospray ionization liquid chromatography [ESI-LC]-MS), nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR) or X-ray diffraction (Harborne, 1993; DNP, 1996; Eisenreich and Bacher, 2007; Marston, 2007). In Table 1.1, an estimate of the numbers of known SM is given. Representative structures are presented in Fig. 1.1. Within a single species 5000 to 20 000 individual primary and secondary compounds may be produced, although most of them as trace amounts which usually are overlooked in a phytochemical analysis (Trethewey, 2004).

1.2 Biosynthesis

Despite the enormous variety of SM, the number of corresponding basic biosynthetic pathways is restricted and distinct. Precursors usually derive from basic metabolic pathways, such as glycolysis, the Krebs cycle or the shikimate pathway. A schematic overview is presented in Figs 1.2 and 1.3. Plausible hypotheses for the biosynthesis of most SM have been published (for overviews see Bell and Charlwood, 1980; Conn, 1981; Mothes *et al.*,

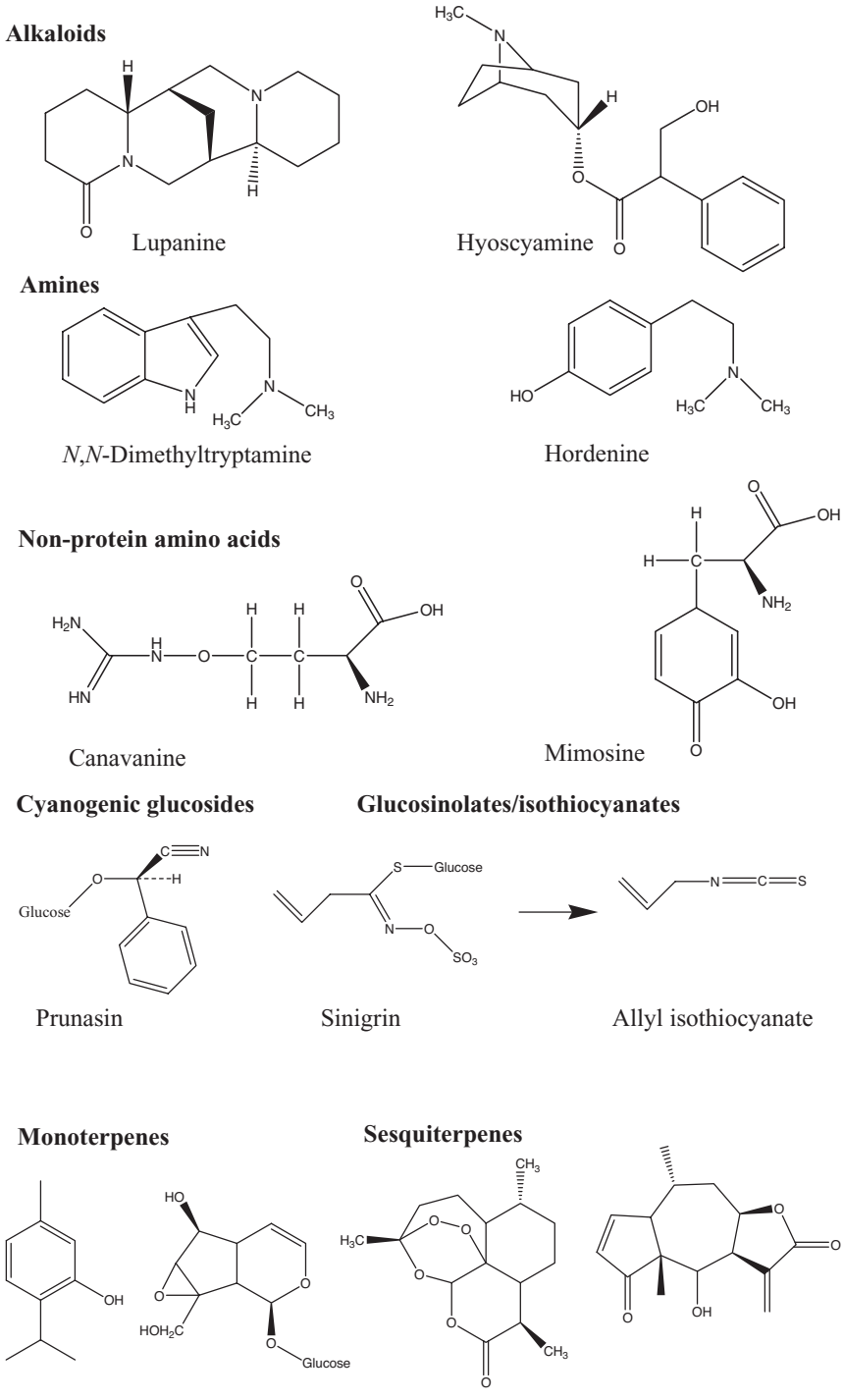


Figure 1.1 Structures of selected secondary metabolites.

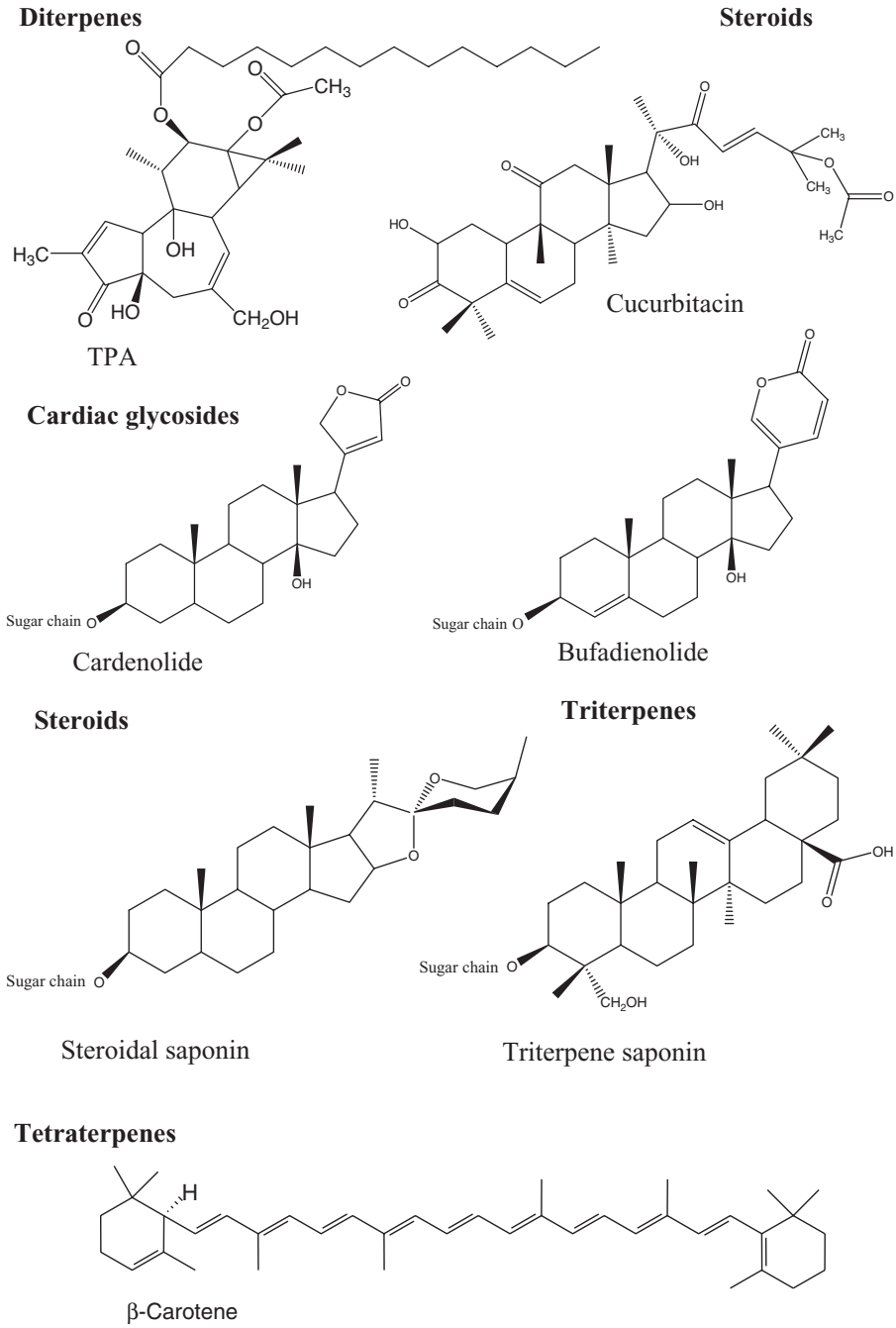
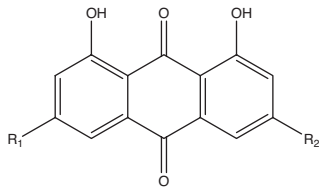
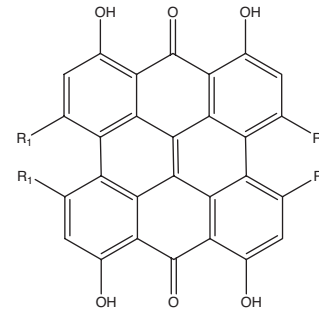


Figure 1.1 (*Continued*)

Polyketides

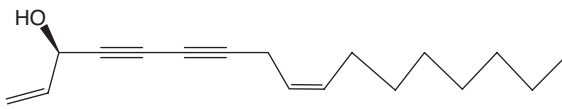


Anthraquinone

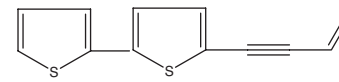


Naphthodianthrone

Polyacetylenes

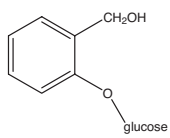


Falcarinol

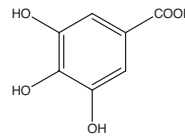


Thiophene (bbt)

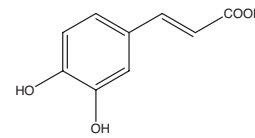
Simple phenolics



Salicin

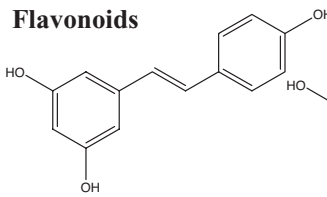


Gallic acid

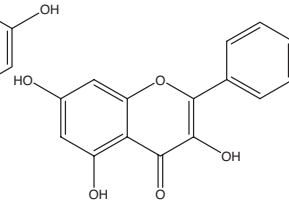


Caffeic acid

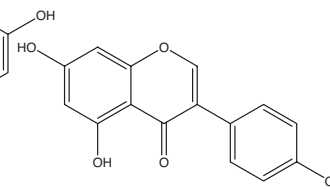
Flavonoids



Stilbene (resveratrol)



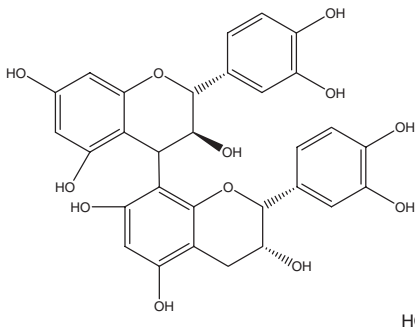
Flavonol (quercetin)



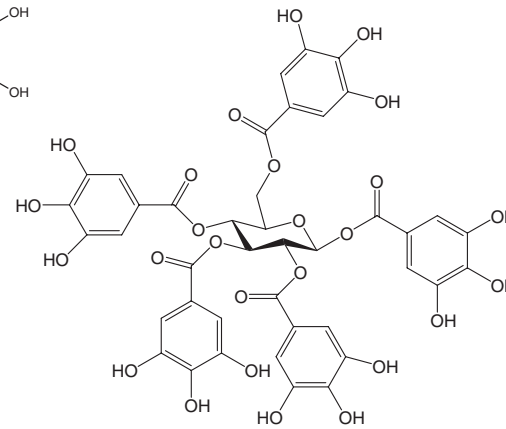
Isoflavone (genistein)

Figure 1.1 (*Continued*)

Tannins



Catechins (procyanidin)



Gallotannin

Figure 1.1 (*Continued*)

1985; Luckner, 1990; Dey and Harborne, 1997; Seigler, 1998; Dewick, 2002) that are based, at least in part, on tracer experiments. In addition, genetic tools to knock out genes become important to dissect plant secondary pathways (Memelink, 2005). For pathways leading to cyanogenic glycosides, glucosinolates, some alkaloids and non-protein amino acids (NPAAs), amines, flavonoids and several terpenes, the enzymes which catalyse individual steps, have been identified. In pathways leading to isoquinoline, indole, pyrrolidine, pyrrolizidine and tropane alkaloids, flavonoids, coumarins, NPAAs, mono-, sesqui- and triterpenes, some of the genes, which encode biosynthetic enzymes, have already been isolated and characterized (Kutchan *et al.*, 1991; Kutchan, 1995; Saito and Murakoshi, 1998; Dewick, 2002; Facchini *et al.*, 2004; Kutchan, 2005; Petersen, 2007; Zenk and Juenger, 2007; Schäfer and Wink, 2009). Whereas, earlier this century, it was argued that SM arise spontaneously or with the aid of non-specific enzymes, we now have good evidence that biosynthetic enzymes are highly specific in most instances and most have been selected towards this special task (although they often derive from common progenitors with a function in primary metabolism or from prokaryotic genes imported to plant cells through chloroplasts and mitochondria). As a consequence of specific enzymatic synthesis, final products nearly always have a distinct stereochemistry. Only the enzymes that are involved in the degradation of SM, such as glucosidases, esterases and other hydrolases, are

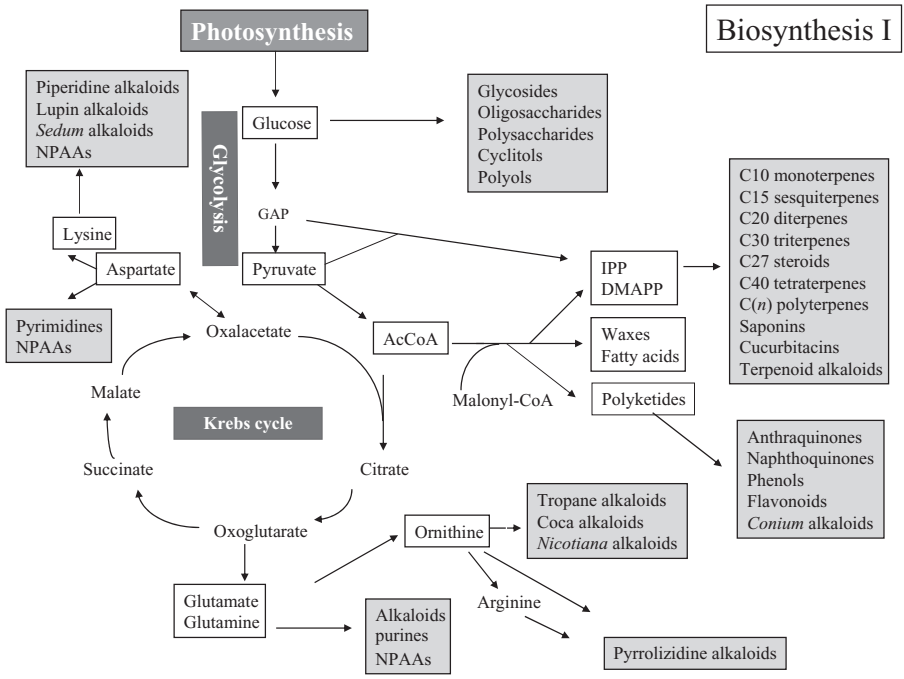


Figure 1.2 Main pathways leading to secondary metabolites. Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethyl allyl diphosphate; GAP, glyceraldehyde-3-phosphate; NPAAs, non-protein amino acids; AcCoA, acetyl coenzyme A. (See Plate 1 in colour plate section.)

less substrate specific. The biosynthesis of SM is a highly coordinated process, which includes metabolon formation and metabolic channelling. Channeling can involve different cell types and cellular compartmentation. These processes guarantee a specific biosynthesis and avoid metabolic interferences (Winkel, 2004; Jörgensen *et al*., 2005).

Some SM are produced in all tissues, but their formation is generally organ-, tissue-, cell- and often development-specific. Although, in most instances, details have not been elucidated, it can be assumed that the genes of secondary metabolism are also regulated in a cell-, tissue- and development-specific fashion (as are most plant genes that have been studied so far). This means that a battery of specific transcription factors needs to cooperate in order to activate and transcribe genes of secondary metabolism. Master regulators (transcription factors by nature) are apparently present, which control the overall machinery of biosynthetic pathways, transport and storage.

Sites of biosynthesis are compartmentalized in the plant cell. While most biosynthetic pathways proceed (as least partially) in the cytoplasm, there is evidence that some alkaloids (such as coniine, quinolizidines and caffeine), furanocoumarins and some terpenes (such as monoterpenes, diterpenes,

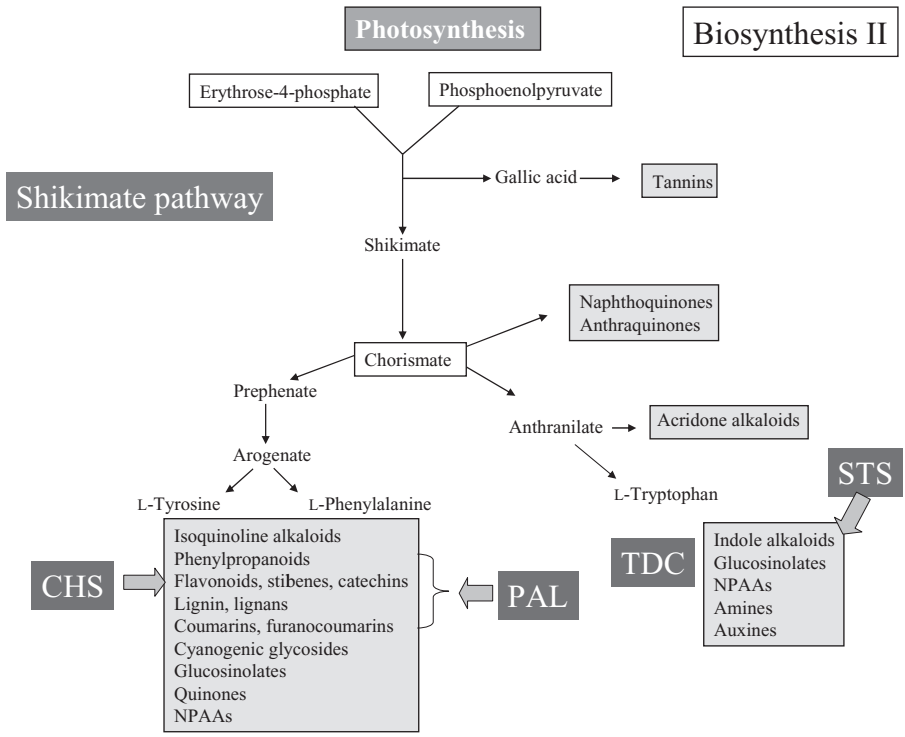


Figure 1.3 Several pathways of secondary metabolites derive from precursors in the shikimate pathway. Abbreviation: NPAAs, non-protein amino acids; PAL, phenylalanine ammonia lyase; TDC, tryptophan decarboxylase; STS, strictosidine synthase; CHS, chalcone synthase. (See Plate 2 in colour plate section.)

phytol and carotenoids that are formed in the pyruvate/glyceraldehyde phosphate pathway) are synthesized in the chloroplast (Roberts, 1981; Wink and Hartmann, 1982; Kutchan, 2005). Sesquiterpenes, sterols and dolichols are produced in the endoplasmic reticulum (ER) or cytosolic compartment. A schematic overview is presented in Fig. 1.4. Coniine and amine formation has been localized in mitochondria (Roberts, 1981; Wink and Hartmann, 1981) and steps of protoberberine biosynthesis in vesicles (Amann *et al.*, 1986; Kutchan, 2005; Zenk and Juenger, 2007). Hydroxylation steps are often catalysed by membrane-bound enzymes and the ER is the corresponding compartment. The smooth ER is also probably the site for the synthesis of other lipophilic compounds. The various steps in a biosynthesis can proceed in a channelled array in one compartment; in other instances different plant organs, cell types or organelles are involved. Extensive intra- and intercellular translocation of SM or intermediates would be a consequence.

The biosynthesis of the major groups of SM has been reviewed in more detail in this volume: alkaloids (including betalains) by M. Roberts, D. Strack

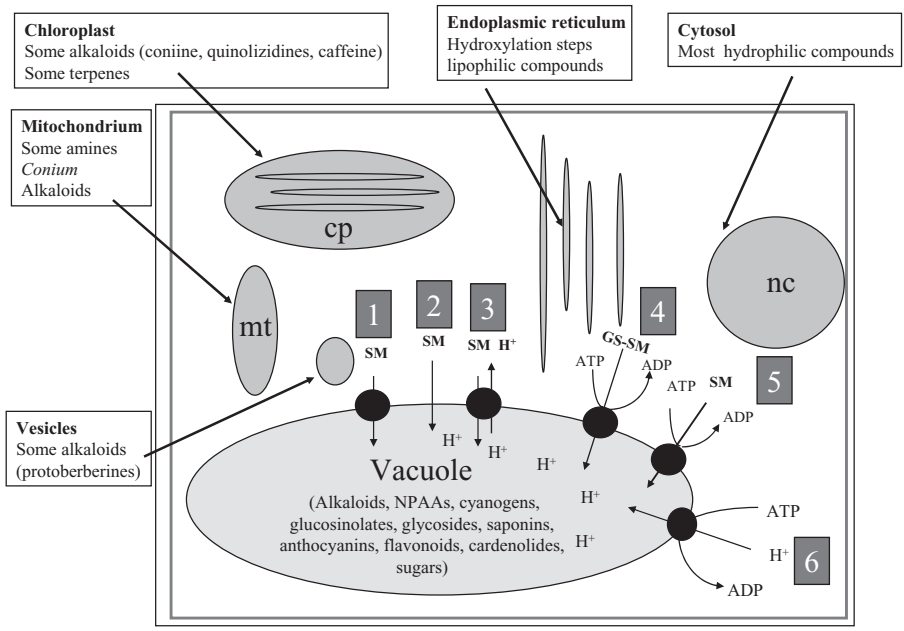


Figure 1.4 Compartmentation of biosynthesis and sequestration. Abbreviations: SM, secondary metabolites; GS-SM, conjugate of SM with glutathione; NPAAs, non-protein amino acids; ATP, adenosine triphosphate; ADP, adenosine diphosphate; mt, mitochondrion; cp, chloroplast; nc, nucleus; 1, passive transport; 2, free diffusion; 3, H^+/SM antiporter; 4, ABC transporter for SM conjugated with glutathione; 5, ABC transporter for free SM; 6, H^+-ATPase. (See Plate 3 in colour plate section.)

and M. Wink in Chapter 2; cyanogenic glycosides, glucosinolates and NPAAs by D. Selmar in Chapter 3; phenylpropanoids, lignin, lignans, coumarins, furocoumarins, tannins, flavonoids, isoflavonoids and anthocyanins by M. Petersen, J. Hans and U. Matern in Chapter 4; mono-, sesqui- and diterpenes by M. Ashour, M. Wink and J. Gershenzon in Chapter 5; and sterols, cardiac glycosides and steroid saponins by W. Kreis in Chapter 6.

1.3 Transport, storage and turnover

Water soluble compounds are usually stored in the vacuole (Matile, 1978, 1984; Boller and Wiemken, 1986; Wink, 1993, 1997; Terasaka *et al.*, 2003; Kutchan, 2005; Yazaki, 2005, 2006) (Table 1.2), whereas lipophilic substances are sequestered in resin ducts, laticifers, glandular hairs, trichomes, thylakoid membranes or on the cuticle (Wiermann, 1981; Kutchan, 2005) (Fig. 1.5).

As mentioned previously, most substances are synthesized in the cytoplasm, the ER or in organelles, and, if hydrophilic, they are exported to the vacuole. They have to pass the tonoplast, which is impermeable to many of the polar SM. For some alkaloids and flavonoids, a specific transporter

Table 1.2 Examples for vacuolar sequestration of secondary metabolites (Wink, 1997)

Phenolics
Anthocyanins
Bergenin
Coumaroyl-glycosides (esculin)
Flavonol-glycosides
Gallic acid
7-Glucosyl-pleurostimin
Isoflavanone malonyl glycosides
Sinapylglycosides
Isoflavone malonyl glycosides
Kaempherol 3,7-O-glycoside
Orientin-C-glycosides
Pterocarpan malonyl glycosides
Quercetin-3-triglucoside
7-Rhamnosyl-6-hydroxyluteolin
Shikimic acid
Tricin 5-glucoside
Terpenoids
Convallatoxin and other cardenolides
Gentiopicroside
Oleanolic acid (3-O-glucoside)
Oleanolic acid (3-O-glucuronide)
Cardiac glycosides (lanatoside A, C; purpureaglycoside A)
Saponins (avenacosides)
Oligosaccharides
Gentianose
Gentiobiose
Stachyose
Nitrogen-containing compounds (excluding alkaloids)
Cyanogenic glycosides (linamarin)
Glucosinolates
Alkaloids
Ajmalicine
Atropine
Nicotine
Berberine
Betaine
Betalains
Capsaicin
Catharanthine
Codeine
Dopamine
Lupanine
Morphine
Noscapine
Papaverine
Polyamines
(*S*)-Reticuline
Sanguinarine
Scopolamine
(*S*)-Scoulerine
Senecionine-N-oxide
Serpentine
Solanidine
Thebaine
Vindoline

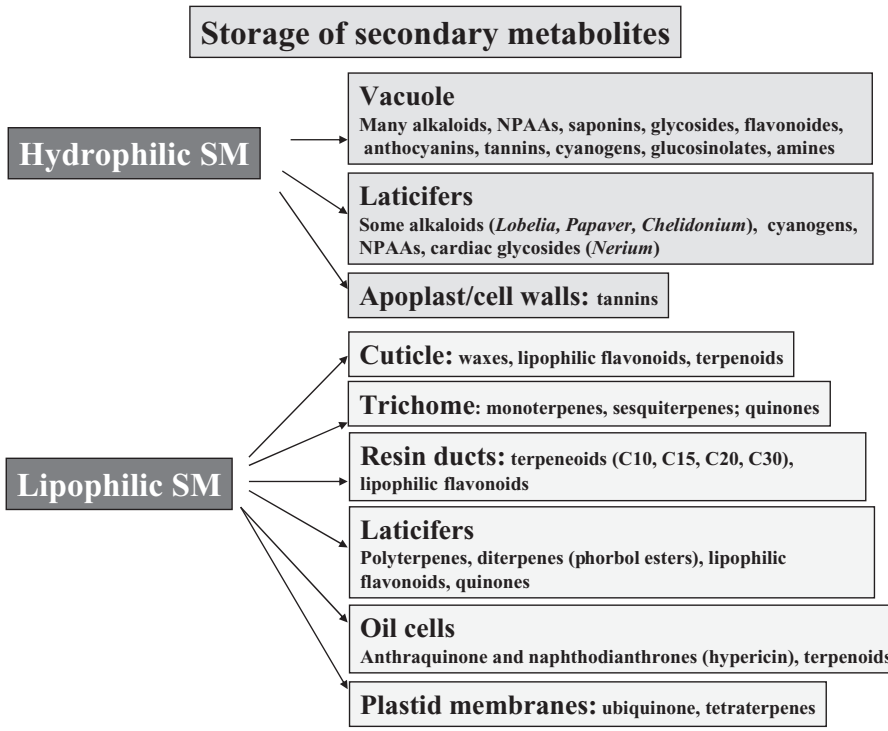


Figure 1.5 Storage compartments for hydrophilic and lipophilic compounds. Abbreviation: NPAAs, non-protein amino acids. (See Plate 4 in colour plate section.)

has been described, which pumps the compounds into the vacuole (Fig. 1.4). The proton gradient, which is built up by the tonoplast-residing adenosine triphosphatase (ATPase), is used as a driving force (by a so-called proton antiport mechanism) (Deus-Neumann and Zenk, 1984; Mende and Wink, 1987). Alternatively, diverse trapping mechanisms (e.g. isoquinoline alkaloids by chelidonic acid or meconic acid in the latex vesicles of *Chelidonium* or *Papaver*, respectively) can also help to concentrate a particular compound in the vacuole. Moreover, conjugation of SM with glutathione in the cytoplasm (Martinoia *et al.*, 1993; Li *et al.*, 1995) and subsequent transportation by an adenosine triphosphate (ATP)-dependent transporter into the vacuole have been proposed for xenobiotics and some SM that can be conjugated (for reviews, see Wink, 1993, 1997).

During the past 10 years, it became obvious that plants also contain a high diversity of ABC transporters (Martinoia *et al.*, 2002; Rea, 2007). These membrane proteins, which can pump lipophilic compounds across biomembranes, are driven by ATP. They are common in animal cells and important for multidrug resistance observed in patients undergoing chemotherapy (Dean *et al.*, 2001; Linton, 2006). Two types of efflux pumps, which belong to the ABC

transporter family, have been described in humans: 1. P-glycoprotein (P-gp) (molecular weight 170 kD) or MDR protein (multiple drug resistance protein) that is encoded by the MDR1 gene (P-gp is an efflux pump directed to the gut lumen) and 2. MRP 1 and 2 (multiple resistance-associated protein; 190 kD) that are encoded by the MRP1 and MRP2 genes. MRP transports drugs conjugated to glutathione (GSH), and also unmodified cytostatics, usually into the blood system. Several of the pathogenic human parasites (*Plasmodium, Leishmania, Trypanosoma*) often develop resistance against prophylactic and therapeutic compounds, such as quinolines, naphthoquinones and sesquiterpene lactones. The underlying bases are membrane glycoproteins that are orthologous to the human P-gp, which can be induced and activated (for a review, see Wink, 2007). It became apparent that the intracellular transport of some alkaloids in plants, such as berberine, also appears to be catalysed by plant ABC transporters (Terasaka *et al.*, 2003; Yazaki, 2005, 2006; Rea, 2007). It was shown earlier that many alkaloids are transported by alkaloid/H^+ antiporters (review in Wink, 1993). At that time, ABC transporters were unknown. Since these antiporters were ATP dependent, it might be worthwhile to revisit alkaloid transport mechanisms in plants (Martinoia *et al.*, 2002; Yazaki, 2005, 2006).

Lipophilic compounds will interfere not only with the biomembranes of microbes and herbivores, but also with those of the producing plant. In order to avoid autotoxicity, plants cannot store these compounds in the vacuole but usually sequester them on the cuticle, in dead resin ducts or cells, which are lined not by a biomembrane but by an impermeable solid barrier (Fig. 1.5). In some cases, the compounds are combined with a polar molecule, so that they can be stored as more hydrophilic chemicals in the vacuole.

In many instances, the site of biosynthesis is restricted to a single organ, such as roots, leaves or fruits, but an accumulation of the corresponding products can be detected in several other plant tissues. Long-distance transport must take place in these instances. The xylem or phloem are likely transport routes, but an apoplastic transport can also be involved.

Table 1.3 summarizes the evidence for xylem and phloem transport of some SM.

Storage can also be tissue and cell specific (Guern *et al.*, 1987). In a number of plants, specific idioblasts have been detected that contain tannins, alkaloids or glucosinolates. More often, SM are concentrated in trichomes or glandular hairs (many terpenoids in Lamiaceae, Asteraceae), stinging hairs (many amines with neurotransmitter activity in Urticaceae) or the epidermis itself (many alkaloids, flavonoids, anthocyanins, cyanogenic glycosides, coumarins, etc.) (Wiermann, 1981; Wink, 1993, 1997; Wink and Roberts, 1998). Flowers, fruits and seeds are usually rich in SM, especially in annual plants. In perennial species, high amounts of SM are found in bulbs, roots, rhizomes and the bark of roots and stems.

Several SM are not end products of metabolism, but are turned over at a regular rate (Barz and Köster, 1981). During germination, in particular,

Table 1.3 Examples of xylem and phloem transport of secondary metabolites (SM)

Compounds	Xylem	Phloem
Quinolizidine alkaloids	−	+
Pyrrolizidine alkaloids	−	+
Aconitine	−	+
Polyhydroxy alkaloids (swainsonine)	−	+
Glucosinolates	−	+
Cardiac glycosides	−	+
Cyanogenic glycosides	−	+
Nicotine	+	−
Tropane alkaloids	+	−

N-containing SM, such as alkaloids, NPAAs, cyanogenic glycosides and protease inhibitors, are metabolized and serve as a nitrogen source for the growing seedling (Wink and Witte, 1985). Carbohydrates (e.g. oligosaccharides and lipids) are also turned over during germination. Concentrations of some SM, such as quinolizidine alkaloids, nicotine, atropine, monoterpenes and phenylpropanoids, vary diurnally; an active interplay between synthesis and turnover is involved in these instances. Turnover of SM is readily seen in cell suspension cultures (for reviews, see Barz and Köster, 1981; Wink, 1997).

It is well established that profiles of SM vary with time, space and developmental stage. Since related plant species often show similarities in the profiles of their SM, these profiles have been used as a taxonomic tool in plant systematics (Harborne and Turner, 1984). However, profiles of closely related plants or even between organs (such as seeds versus leaves or roots) quite often differ substantially or those of unrelated plant groups show strong similarities; this clearly shows that SM patterns are not unambiguous systematic markers but that convergent evolution and selective gene expression are common themes. In this volume, Chapter 7 by Kreis and Müller-Uri summarizes the evidence for and against the use of SM in chemotaxonomy.

1.4 Costs of secondary metabolism

Analogous with other proteins in cells, the enzymes involved in the biosynthesis and transport of SM show a regular turnover. This means that messenger ribonucleic acid (mRNA) must be regularly transcribed and translated into proteins, even for constitutive compounds. Both transcription and translation require a substantial input of energy in terms of ATP. Furthermore, the biosynthesis itself is often costly, demanding ATP or reduction equivalents, i.e. nicotinamide adenine dinucleotide phosphate (reduced formed) ($NADPH_2$). In order to exhibit their function as defence or signal compounds, allelochemicals need to be present in relatively high concentrations at the right

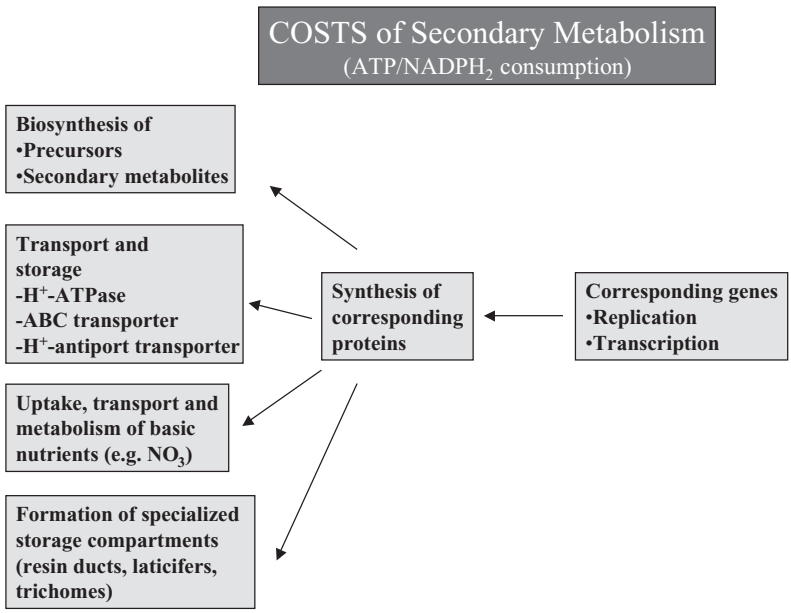


Figure 1.6 Costs of chemical defence and signal compounds. Abbreviations: ATP, adenosine triphosphate; $NADPH_2$, nicotinamide adenine dinucleotide phosphate (reduced form). (See Plate 5 in colour plate section.)

place and time. Many SM are synthesized in the cytoplasm or in cell organelles (Fig. 1.4), but are stored in the vacuole. Energy for the uphill transport across the tonoplast and/or for trapping the metabolite in the vacuole is provided by a H^+-ATPase or ABC transporters. If special anatomical differentiations (ducts, gland cells, trichomes) are needed, the formation and maintenance of these structures are also costly. As a consequence, both biosynthesis and sequestration (and the corresponding transcription and translation of related genes and mRNAs) are processes which require substantial amounts of ATP; in other words, it must be costly for plants to produce defence and signal compounds (a schematic overview is presented in Fig. 1.6).

1.5 Ecological role of secondary metabolites

The biosynthesis of SM exhibits a remarkable complexity. Enzymes are specific for each pathway and are highly regulated in terms of compartmentation, time and space. The same is true for the mechanisms of accumulation or the site and time of storage. In general, we find that tissues and organs which are important for survival and multiplication, such as epidermal and bark tissues, flowers, fruits and seeds, have distinctive profiles of SM, and secondary compounds are stored in high amounts in them. As an example, the complex pattern of alkaloid synthesis, transport and storage is illustrated in Fig. 1.7.

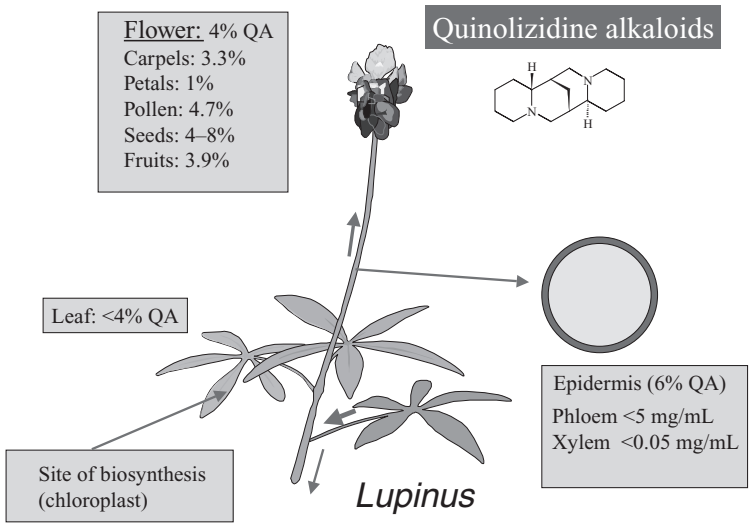


Figure 1.7 Example of the complicated biochemistry and physiology of alkaloid formation: quinolizidine alkaloids (QAs) in lupins (genus *Lupinus*, Fabaceae). QAs are formed in leaf chloroplasts and exported via the phloem all over the plant. QAs predominantly accumulate in vacuoles of epidermal tissue. Organs important for survival and reproduction, such as flowers and seeds, store especially high amounts of defence alkaloids. (See Plate 6 in colour plate section.)

All these processes and the corresponding means and structures necessary to express these traits are costly in terms of ATP and NAD(P)H, so it would be highly unlikely that SM were waste products or had no function at all, as has been suggested in the older literature. Costly traits without a function or advantage usually do not survive in evolution, as plants expressing these traits should perform less well than plants without them. Because these metabolites are maintained and diversified in an astounding fashion, it must be assumed that these traits are indeed important, even if their functions are not directly evident.

During the past few decades, experimental and circumstantial evidence has made it clear that SM do indeed have functions that are vital for the fitness of a plant producing them (Fig. 1.8). Their main roles are

(a) Defence against herbivores (insects, vertebrates)
(b) Defence against fungi and bacteria
(c) Defence against viruses
(d) Defence against other plants competing for light, water and nutrients
(e) Signal compounds to attract pollinating and seed-dispersing animals
(f) Signals for communication between plants and symbiotic microorganisms (N-fixing *Rhizobia* or mycorrhizal fungi)
(g) Protection against UV light or other physical stress
(h) Selected physiological functions

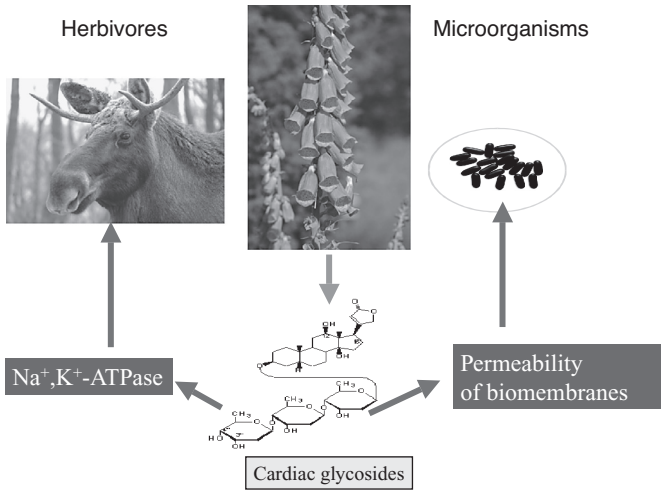


Figure 1.8 Schematic view of the ecological roles of plant SM. Foxglove (*Digitalis purpurea*) produces cardiac glycosides, which are very toxic to animals (vertebrates, insects) because they inhibit Na^+, K^+-ATPase, one of the most important transporters in animal cells. Cardiac glycosides are additionally toxic to microbes because the molecules have detergent properties and disturb membrane fluidity. (See Plate 7 in colour plate section.)

In order to fulfil these functions, the structures of SM have been shaped during evolution, so that they can closely interact with molecular targets in cells and tissues or other physiological features in animals or microorganisms. Quite often structures of SM resemble endogenous substrates, hormones or neurotransmitters and can thus mimic a response at the corresponding molecular targets. The process leading to these structure similarities could be termed 'evolutionary molecular modelling'.

There is hardly a target in animals or microorganisms for which a natural product does not exist. Thus, plants provide a wide array of bioactive substances. This is the reason so many natural products can be used in so many ways in biotechnology, pharmacy, medicine and agriculture. Using substances that are already known or looking for new ones, hitherto undiscovered compounds or the corresponding genes encoding the enzymes for their biosynthesis can be discovered in plants living in deserts or rain forests (a strategy called bioprospection or gene prospection).

SM often interfere with more than a single molecular target (multi-target substances), which is advantageous for the producer, as a toxin might be more efficient if it knocks out two targets instead of one. Furthermore, SM are always produced as mixtures of several substances, often from different classes; e.g. polyphenolics are often accompanied by terpenoids. As a consequence, it will be more difficult for a herbivore or microbe to develop resistance to such a cocktail, as concomitant resistance at several targets would be required. In addition, the activity of individual metabolites in the mixtures may be

additive or even synergistic. It can be postulated that mixtures contain substances which might facilitate the uptake of polar SM across biomembranes, for which biomembranes normally constitute a permeation barrier. These properties make these mixtures even more powerful as means of defence and protection than mono-target substances (Wink, 2008a,b).

Because of this evolutionary logic, most plants are able to withstand various threats from herbivores, microbes and the physical environment. Exceptions are many agricultural crops which have been optimized for yield and, quite often, their original lines of defence have been selected away, as these metabolites were unpalatable or toxic for humans or their lifestock.

The role and function of SM as well as their potential biotechnological applications are the topic of Volume 39 of Annual Plant Reviews, *Functions of Plant Secondary Metabolites and Biotechnology*.

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Annual Plant Reviews (2010) **40**, 20–91 www.interscience.wiley.com
doi: 10.1002/9781444320503.ch2

Chapter 2

BIOSYNTHESIS OF ALKALOIDS AND BETALAINS

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Abstract: Alkaloids represent a structurally diverse group of nitrogen-containing secondary metabolites. Many of them have pronounced pharmacological activities and are therefore important for medicine and biotechnology. Most alkaloids derive from an amino acid as a precursor, such as ornithine, arginine, lysine, phenylalanine, tyrosine or tryptophan. The biosynthetic pathways of the main groups of alkaloids have already been elucidated at the enzyme and gene levels. In a few cases, it was already possible to produce alkaloids (e.g. benzylisoquinoline alkaloids) in transgenic microorganisms which were transformed with the respective genes of alkaloid biosynthesis. Details are given for nicotine and tropane alkaloids, pyrrolizidine alkaloids, benzylisoquinoline alkaloids, monoterpene indole alkaloids, ergot alkaloids, acridone alkaloids, purine alkaloids and taxol. Betalains (the red–violet betacyanins and the yellow betaxanthins) are structurally related to alkaloids ('chromoalkaloids') and are typical for plants in the order Caryophyllales. Their biosynthesis and function are discussed in this chapter.

Keywords: alkaloid biosynthesis; alkaloid genes; nicotine; tropane alkaloids; pyrrolizidine alkaloids; benzylisoquinoline alkaloids; monoterpene indole alkaloids; ergot alkaloids; acridone alkaloids; purine alkaloids; taxol; betalains

2.1 Introduction

The biogenesis of alkaloids has been studied from the beginning of the past century, first to determine their structures and subsequently to study their biosynthesis in plants (Mothes *et al.*, 1985). Detailed hypotheses of alkaloid

 ISBN: 978-1-405-18397-0

biosyntheses have been advanced following radio-labelled studies; however, we are still a long way from understanding how most alkaloids are synthesized in plants and how such biosynthesis is regulated. Moreover, there is much to be learned about the chemical ecology of alkaloids, so that we can better understand their roles within the plant (Roberts and Wink, 1998; Wink, 2008).

Alkaloids are an integral part of many medicinal plants and have enjoyed a long and important history in traditional medicine. Our first drugs originated from plant extracts and some important contemporary pharmaceuticals are still either isolated from plants or structurally derived from natural products (Seigler, 1998; Wink, 2000, 2007; Dewick, 2002; van Wyk and Wink, 2004).

The majority of alkaloids have been found to be derived from amino acids, such as tyrosine, phenylalanine, anthranilic acid, tryptophan/tryptamine, ornithine/arginine, lysine, histidine and nicotinic acid (Fig. 2.1). However, alkaloids may be derived from other precursors such as purines in case of caffeine, terpenoids, which become 'aminated' after the main skeleton has been synthesized; i.e. aconitine or the steroidal alkaloids, are found in the Solanaceae and Liliaceae. Alkaloids may also be formed from acetate-derived polyketides, where the amino nitrogen is introduced as in the hemlock alkaloid, coniine.

Originally, alkaloids were thought to be essentially plant products; however, these basic compounds also occur in microorganisms and animals. Although, at present, the majority of known alkaloids are amino acid-derived, increasing numbers of alkaloids from insects and marine organisms are being discovered that are either terpenoid or polyketide in origin.

Interest in growing and manipulating microorganisms and plants in cell culture for commercial purposes (Verpoorte *et al.*, 2007) has given impetus to the study of alkaloid biosynthesis and, in particular, to the elucidation of the enzymes involved. It has also brought about a renewed interest in the regulation of alkaloid synthesis and in the location and means of sequestration of these substances within the plant. In recent years, attempts have been made to express the genes of alkaloid biosynthesis in microorganisms (Marasco and Schmidt-Dannert, 2007; Minami *et al.*, 2008; Wu and Chappell, 2008; Ziegler and Facchini, 2008; Schäfer and Wink, 2009). Ultimately, it might be possible to produce valuable alkaloids, be it recombinant bacteria or yeast.

It was not until the early 1970s that the enzymes associated with alkaloid formation were isolated. Now, however, the enzymes of every step of entire pathways, for instance from tyrosine to berberine and protopine, are known. The relatively few pathways isolated so far clearly indicate that most of the enzymes required are highly specific for a given biosynthetic step. The results of research over the past 20 years have helped to revise routes to alkaloid synthesis that were previously hypothesized as a result of feeding radio-labelled precursors to plants. The investigation of enzymes and, more recently, the genes of alkaloid biosynthesis has also helped to answer some of the questions regarding where and at what time during the plant growth cycle the alkaloids are actively made, and has provided an insight into the location of

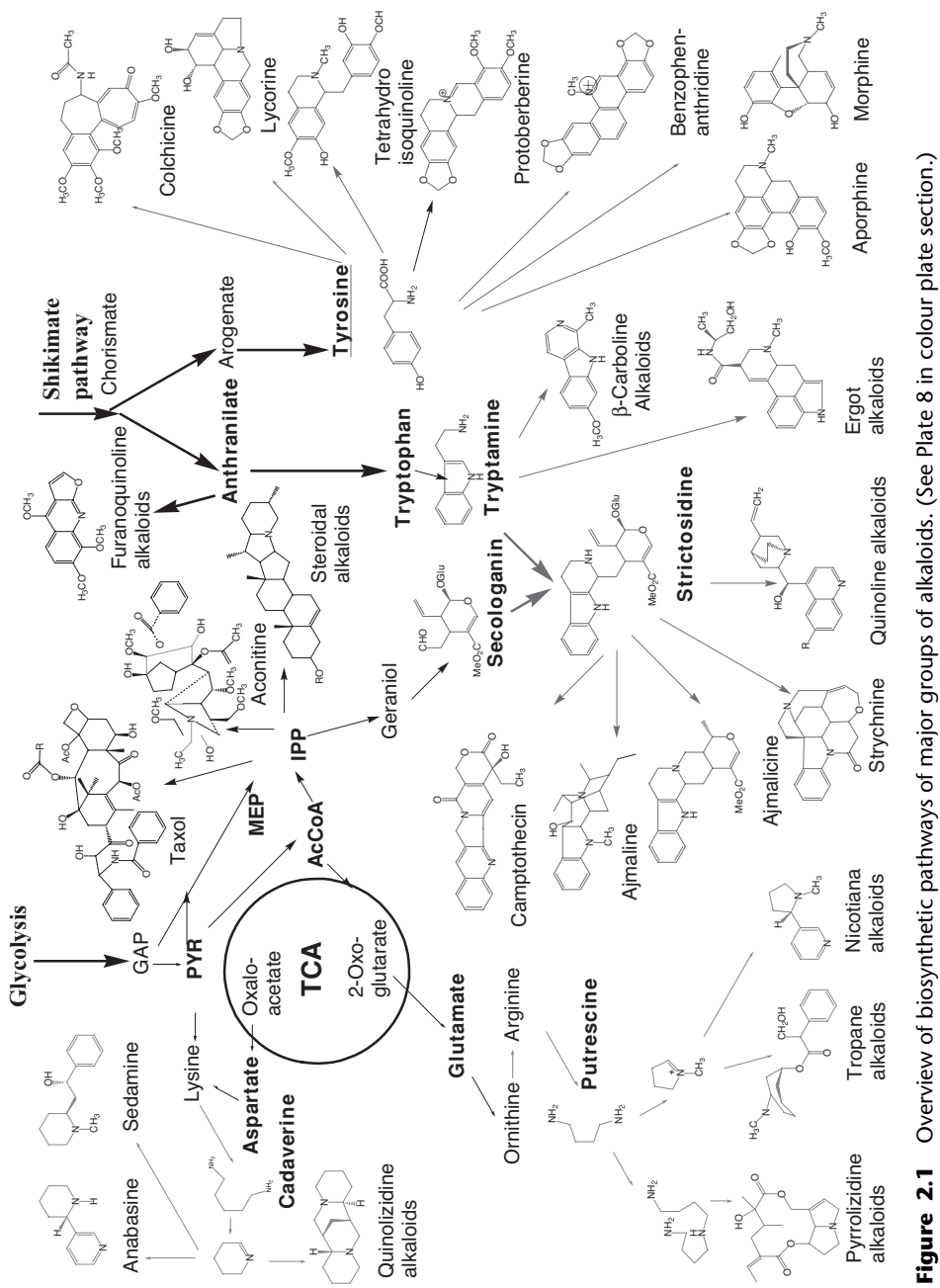


Figure 2.1 Overview of biosynthetic pathways of major groups of alkaloids. (See Plate 8 in colour plate section.)

enzymes and alkaloids within the plant and the cell. Technical breakthroughs, such as expressed sequence tags (EST) and EST databases, DNA microarrays and proteome analysis by MALDI–MS and MS–MS have contributed to a substantial progress in alkaloid research (Ziegler and Facchini, 2008).

This chapter focuses on recent data in areas where the enzymes of whole pathways and the genes for key enzymes for alkaloids have been isolated. We are aware that the field of alkaloids is much larger and comprises more structural groups. More information is found in Chapter 7 (this volume) and Chapter 2 in Volume 39 of this series (Wink, 2010). These studies have improved our understanding of the formation, mobilization and sequestration of alkaloids, and their role in plant defence mechanisms (Hashimoto and Yamada, 1994; Facchini, 2001; Zenk and Juenger, 2007; Liscombe and Facchini, 2008; Ziegler and Facchini, 2008).

The Alkaloids (1950–2008), (Academic Press, New York) *Alkaloids: Chemical and Biological Perspectives*, (Volumes 1–8, Pergamon Press, Oxford); and Roberts and Wink (1998) *Alkaloids: Biochemistry, Ecology and Medical Applications*, Plenum Press, New York.

2.2 Nicotine and tropane alkaloids

In the early 1980s, root cultures of *Nicotiana*, *Hyoscyamus*, *Datura* and *Duboisia* species were found to give high yields of nicotine and tropane alkaloids and have proved useful tools for recent studies of the biosynthetic pathways to these alkaloids. Genetically transformed and untransformed root cultures have been generated and used as models for biosynthetic studies (Rhodes *et al.*, 1990; Robins *et al.*, 1994a,b; Wildi and Wink, 2002).

2.2.1 *Nicotiana* alkaloids

Nicotiana rustica and *N. tabacum* root cultures principally contain nicotine, which is made from putrescine and nicotinic acid (Fig. 2.2). Putrescine is produced by the decarboxylation of either ornithine or arginine, as a result of the activities of either ornithine (ODC) or arginine decarboxylase (ADC), and is used for the biosynthesis of the polyamines, spermine and spermidine. The conversion of putrescine to *N*-methylputrescine by putrescine *N*-methyltransferase (PMT) is, therefore, the first committed step of the alkaloidal pathway. *N*-Methylpyrrolinium, formed spontaneously after the oxidative deamination of *N*-methylputrescine to *N*-methylamino butanal, is then condensed with an intermediate derived by the decarboxylation of nicotinic acid (such as 3,6-dihydronicotinic acid). Three specific enzymes, namely, putrescine *N*-methyltransferase (PMT), *N*-methylputrescine oxidase (MPO) and nicotine synthase (conclusive findings concerning the final step have not been obtained, yet), are involved. A certain NADPH-dependent reductase, called A622, which is related to isoflavone reductase, might be a candidate for

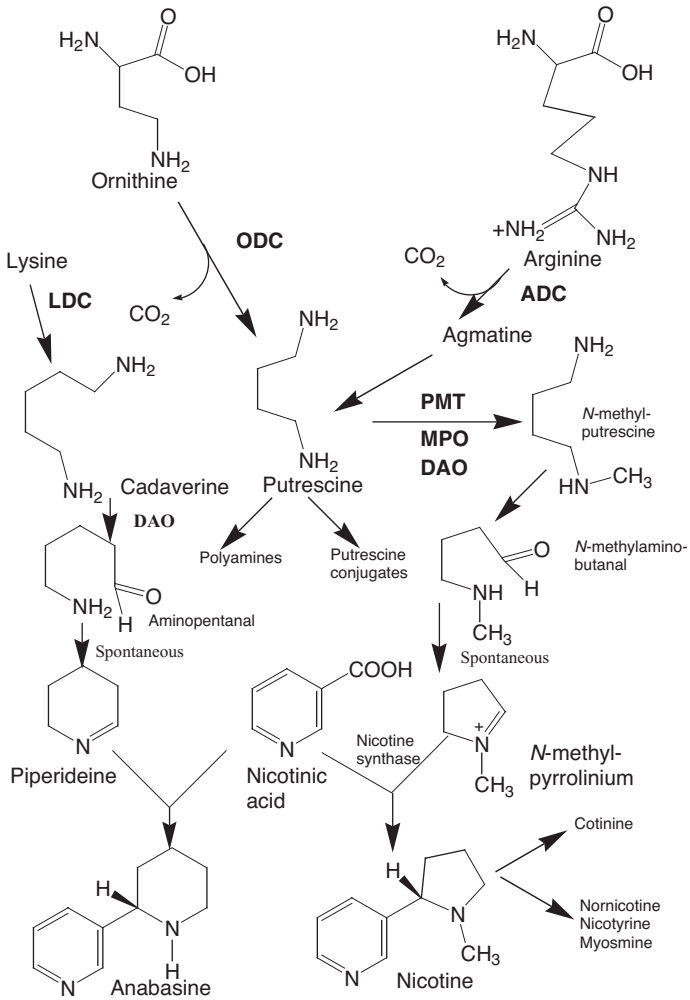


Figure 2.2 Biosynthesis of nicotine and anabasine. ODC, ornithine decarboxylase; ADC, arginine decraboxylase; PMT, putrescine *N*-methyltransferase; DAO, diamine oxidase; MPO, *N*-methylputrescine oxidase.

nicotine synthase (Shoji *et al.*, 2002). The regulation of these enzymes and the control of flux into the pathway have been the subject of particular study over the past 20 years (Friesen and Leete, 1990; Leete, 1990; Oksman-Caldentey *et al.*, 2007). PMT has been characterized by X-ray crystallography (Teuber *et al.*, 2007).

Nicotine is demethylated to nornicotine by CYP82E4 (a nicotine demethylase) (Siminszky *et al.*, 2005). Nornicotine can be converted into nicotyrine and myosmine.

Nicotiana alkaloids, which serve as chemical defence compounds, are synthesized in the roots and are transported to other plant organs, such as aerial parts, via the xylem. These alkaloids accumulate in vacuoles. PMT and A622 oxidoreductase are strongly expressed in the endodermis and outer cortex cells of tobacco root tips and to a lesser degree in other parts of the cortex and parenchyma cells surrounding the xylem (Shoji *et al*., 2002). The localization of nicotine biosynthesis in the parenchyma cells surrounding the xylem may aid the loading of the xylem with nicotine.

The correlation between nicotine accumulation and its defensive role in *N. sylvestris* has been convincingly demonstrated. Increased alkaloid production may also be demonstrated by true herbivory. Tobacco plants subjected to leaf damage showed a fourfold increase in the alkaloid content of their undamaged leaves. This resulted from increased alkaloid synthesis and, as a result, a tenfold increase in alkaloids in the xylem. Experimental evidence has indicated that alkaloid induction may be triggered by a phloem-translocated signal (Hartmann, 1991 and references therein).

2.2.1.1 Regulation of the pyrrolidine alkaloid pathway

Precursor feeding experiments in root cultures of *N. rustica* have indicated that a major limitation in accumulation occurs subsequent to *N*-methylpyrrolinium formation. However, small enhancements in alkaloid production are seen with putrescine or agmatine but not with ornithine or arginine, indicating a possible limitation in the supply of putrescine, which may be regulatory (Walton *et al*., 1988; Robins and Walton, 1993). The use of 'suicide' inhibitors of ODC and ADC, namely, α-difluoromethylornithine (DFMO) and α-difluoromethylarginine (DFMA) (Robins and Walton, 1993), indicate that arginine is probably the preferred origin of the putrescine incorporated into nicotine. In root cultures, nicotine production and PMT activity are lost if roots are subcultured into media containing phytohormones (Rhodes *et al*., 1989). This effect is reversible; roots competent in nicotine production are obtained when cells are passaged into a phytohormone-free medium. Therefore PMT, rather than ADC or ODC, has been targeted for genetic engineering. PMT is an important key enzyme as it drives the flow of nitrogen away from polyamine biosynthesis to nicotine biosynthesis (Robins *et al*., 1997).

Two enzymes of pyrrolidine alkaloid formation responsible for the conversion of putrescine to the *N*-methylpyrrolinium ion have been investigated in some detail. PMT, partially purified from cultures of *Hyoscyamus niger* and fully characterized from *Datura stramonium*, has been cloned by differential screening of complementary deoxyribonucleic acid (cDNA) libraries from high- and low-nicotine-yielding *N. tabacum* plants (Hibi *et al*., 1994). The enzyme shows considerable sequence homology to spermidine synthase but is distinct from this enzyme as it only shows PMT activity when expressed in *Escherichia coli*. MPO has been isolated in pure form from *N. tabacum* transformed root cultures (McLauchlan *et al*., 1993). It is quite widely spread in

the Solanaceae, as shown by Western blotting, and is apparently both immunologically (McLauchlan *et al.*, 1993) and kinetically (Robins and Walton, 1993; Hashimoto and Yamada, 1994) related to a wide range of diamine oxidases (DAO) found in plants. The MPO gene has been characterized recently (Heim *et al.*, 2007; Katoh *et al.*, 2007). MPO can also convert cadaverine into 5-aminopentanal, which cyclisizes to piperideine (Fig. 2.2). Whereas DAO from pea and pigs have a low affinity for *N*-methylputrescine, MPO from alkaloid-producing species prefer this substrate over putrescine. While PMT is important in determining the overall extent to which cultures can make pyrrolidine alkaloids, the level of activity normally found in transformed root cultures of *N. rustica* does not limit the ability of the cultures to accumulate nicotine. Feeding putrescine had some effect on nicotine levels and, therefore, experiments were conducted to try to enhance nicotine formation by engineering the supply of this metabolite (Robins and Walton, 1993).

The *odc* gene obtained from *Saccharomyces cerevisiae* was expressed with the enhanced cauliflower mosaic virus 35S protein promoter in transgenic roots of *N. rustica*. The level of ODC was enhanced in several root clones. The level of ODC remained elevated even in the late stationary phase of these cultures, in contrast to control lines. Other enzymes (ADC, PMT and MPO) were not enhanced. The introduced gene appeared to be expressed in a deregulated manner; this was confirmed by showing that ODC messenger ribonucleic acid (mRNA) was also present at a high level throughout the growth cycle. Some of the *odc*-expressing clones had increased levels of putrescine, in particular *N*-methylputrescine. In addition, the mean nicotine content of the cultures at 14-day-old was increased from 2.28 ± 0.22 to 4.04 ± 0.48 μmol/g fresh mass.

Once the supply of putrescine was enhanced, no larger increases in nicotine were found, presumably because other enzymes contributed, more than previously, to limiting nicotine accumulation. MPO is present at, typically, two- to fivefold higher levels than PMT, and therefore PMT may become limiting. Now that the *pmt* gene has been cloned (Hibi *et al.*, 1994), this possibility can be tested directly.

Nicotine biosynthesis also involves the incorporation of nicotinic acid (Fig. 2.2) (Robins *et al.*, 1987), and the availability of this moiety can be as important in nicotine accumulation as that of the putrescine-derived portion. However, the enzyme responsible for the condensation of *N*-methylpyrrolinium with decarboxylated nicotinic acid, nicotine synthase (Friesen and Leete, 1990), was measured at only a very low level of activity, quite inadequate to account for the rates of nicotine accumulation observed in cultures. The molecular analysis of low-nicotine mutants of *N. tabacum* suggested the presence of regulatory genes (*Nic 1* and *Nic 2*) governing the expression of nicotine biosynthesis (Hibi *et al.*, 1994).

Several genes of nicotine biosynthesis appear to be regulated by methyljasmonate (MJM); among 20 000 gene tags, 591 were modulated by MJM (Goossens *et al.*, 2003). A total of 58% of the genes showed homology

with known genes and 26% were completely unknown. In this approach, several genes were detected with a putative function in nicotine biosynthesis (Häkkinen *et al*., 2007; Oksman-Caldentey *et al*., 2007). About 34 candidate genes were selected and overexpressed in tobacco cell suspension cultures and hairy roots (BY-2). This approach identified a lysine decarboxylase (LDC) gene and a GH3-like protein gene. Overexpressing the GH3-like enzyme gene in hairy roots increased the nicotine production significantly (Häkkinen *et al*., 2007).

Genetic engineering makes in vivo manipulation of the alkaloid mixture possible. Anabasine, a minor alkaloid in some *Nicotiana* species, is derived from lysine via cadaverine, in a pathway parallel to that for the biosynthesis of nicotine (Fig. 2.2). Root cultures of *N. rustica* (Walton *et al*., 1988) and *N. hesperis* (Walton and Belshaw, 1988) accumulated anabasine when fed cadaverine; the nicotine:anabasine ratio in the former changing from 10:1 to 1:5. Thus, the enhanced anabasine formation was at the expense of nicotine, indicating that the two pathways may be competing for nicotinic acid. However, some steps in each pathway may be catalysed by the same enzyme. It has been shown that MPO from *N. tabacum* catalyses the oxidation of both *N*-methylputrescine and cadaverine (Robins and Walton, 1993), with a 34-fold higher affinity for *N*-methylputrescine but a capacity to oxidize cadaverine, which is threefold greater. Hence, the occurrence of excess cadaverine might be expected to dominate the reaction, leading to formation of more anabasine and less nicotine.

Walton and co-workers (1988) found that feeding lysine hardly affected the alkaloid ratio, suggesting a deficiency in LDC. In order to test this, Berlin and co-workers inserted the *ldc* gene from *Hafnia alvei* into transgenic root cultures of *Nicotiana glauca* under the control of the cauliflower mosaic virus 35S promoter (Fecker *et al*., 1992). *Nicotiana glauca* root cultures contain only low LDC activity, even though anabasine is accumulated as a major product. Two clones were isolated showing about a sixfold increase of LDC activity. This was accompanied by a tenfold rise in cadaverine, a twofold rise in anabasine and a change in the nicotine:anabasine ratio from 75:25 in controls to 60:40 in *ldc* transgenic roots. The experiment clearly demonstrated that anabasine production is limited, in part, by cadaverine supply and provided further evidence for at least one common step in the pathways of nicotine and anabasine production. Anabasine can be converted into anatalline, especially after MJM induction (Goossens *et al*., 2003).

2.2.2 Tropane alkaloids

Both untransformed (Hashimoto and Yamada, 1994) and transformed root cultures of *Datura*, *Hyoscyamus*, *Atropa* and *Duboisia* species (Robins and Walton, 1993) accumulate high levels of the tropane alkaloids, hyoscyamine and scopolamine (Fig. 2.3). These medically important tropane alkaloids present not only an interesting biochemical problem but also a realistic

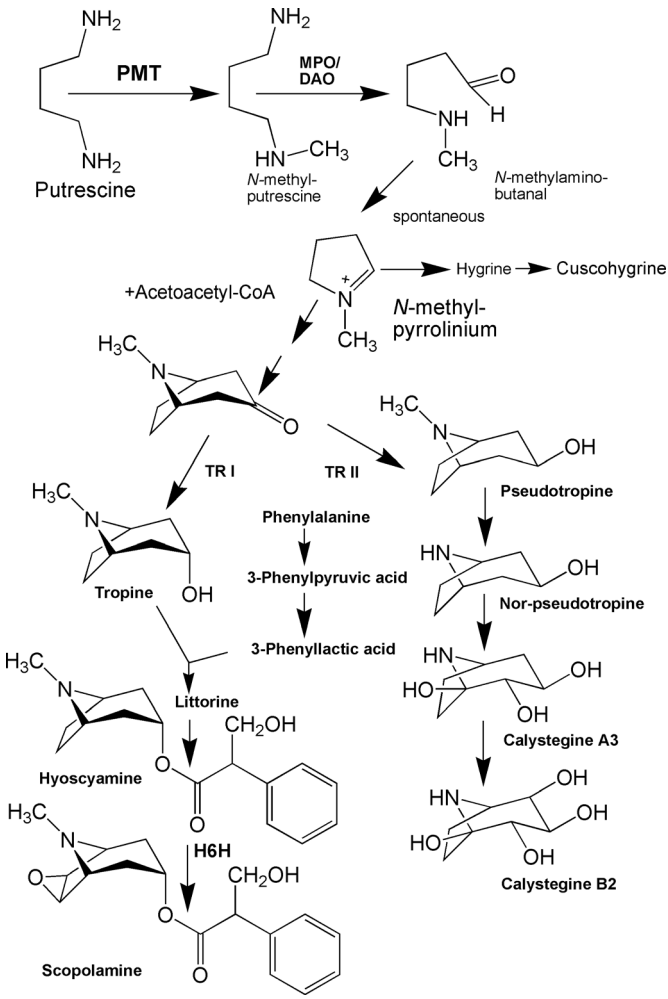


Figure 2.3 Biosynthesis of the tropane alkaloids. PMT, putrescine *N*-methyltransferase; DAO, diamine oxidase; MPO, *N*-methylputrescine oxidase; TR I and II, tropinone reductase; H6H, hyoascyamine 6-hydroxylase.

target for genetic manipulation. The biosynthetic route to hyoscyamine and scopolamine is now well documented. Tropane and pyrrolidine alkaloids have a common biosynthetic pathway to *N*-methylpyrrolinium (Fig. 2.3), the first unique step towards the tropanes being the condensation of *N*-methylpyrrolinium with a C-3 unit to form tropinone (Fig. 2.3). This is stereospecifically reduced to form tropine (tropan-3α-ol) by tropinone reductase I. Tropine is esterified with a moiety of phenyllactic acid to form littorine (Robins and Walton, 1993). Recent experiments (Chesters *et al.*, 1996) have shown that D-phenyllactate is converted to tropate by a rearrangement

in which, during carboxylate migration, an inversion of configuration occurs at both migration termini to produce hyoscyamine. Further metabolism of hyoscyamine, involving the introduction of a 7β-hydroxyl group followed by oxidation to the 6β,7β-epoxide by hyoscyamine-6-hydroxylase, results in formation of scopolamine (Robins and Walton, 1993). The reduction of tropinone by tropinone reductase II (TR II) leads to pseudotropine, which is a precursor for calystegine, a group of hydroxylated nortropane alkaloids (Fig. 2.3). Calystegines occur in Convolvulaceae but also in several Solanaceae, Brassicaceae, Erythroxylaceae and Moraceae (Dräger, 2004; Biastoff and Dräger, 2007).

2.2.2.1 Regulation of tropane alkaloid production

Recent investigations of the regulation of the tropane alkaloid pathway in *Datura*, *Hyoscyamus* and *Atropa* species have focused on understanding the enzymes involved at the branch points and in investigating the role these play in regulating the flux into the different groups of products.

The enzymes of hyoscyamine and scopolamine biosynthesis are present throughout much of the growth cycle of both *Datura* (Robins and Walton, 1993) and *Hyoscyamus* (Hashimoto and Yamada, 1994) root cultures. The level of activity present is maximal in rapidly growing tissue, but levels of the enzymes ODC, ADC and PMT in *D. stramonium* roots do not greatly exceed the minimum required to synthesize the amounts of alkaloid accumulated in vivo (Rhodes *et al.*, 1989). However, levels of the tropinone reductases I and II are much higher than required (Portsteffen *et al.*, 1992, 1994; Dräger and Schaal, 1994). Experiments in feeding various precursors have suggested that, in these root cultures, the esterification of tropine may be crucial in limiting hyoscyamine accumulation (Robins and Walton, 1993).

Tropinone reductases, which catalyse the stereospecific reduction of the keto group of tropinone to 3α- and 3β-hydroxy groups (Dräger, 2005), were analysed in detail by dissection of the peptides and construction of chimeric enzymes. The opposite stereospecificity of the two reductases was ascribed to the carboxy-half of the proteins, to which the substrate tropinone is assumed to bind with the reverse orientation in the two enzymes (Nakajima *et al.*, 1993, 1994; Hashimoto and Yamada, 1994). Only tropinone with the 3α-hydroxy group is used to produce hyoscyamine (Leete, 1990). Tropinone with the 3β-hydroxy group forms esters with other acids, but these occur only as minor alkaloids. When *trII* was overexpressed in *A. belladonna* root cultures, a substantial amount of pseudotropine and related alkaloids was observed (Richter *et al.*, 2005).

Some *Hyoscyamus* and *Duboisia* root cultures accumulate scopolamine as a major product (Robins and Walton, 1993). In contrast, only traces of scopolamine were found in *D. stramonium* roots. This implies that the expression of hyoscyamine 6β-hydroxylase (H6H) that forms the 6,7-epoxide is variable and, hence, this enzyme has also been targeted for genetic engineering.

Experiments using radio-labelled precursors have been performed in intact plants, aimed at delineating the tropane pathway (Leete, 1990). Nevertheless, a number of steps remain to be clarified and some inconsistencies in the proposed pathway to the tropanes require resolving.

Robins and Walton (1993), for example, were able to show that 4-*N*-methylornithine was not an intermediate and that the presence of ODC and PMT in excess strongly suggested the route via *N*-methylputrescine, with PMT the first enzyme of the pathway, as in *Nicotiana*.

As in *Nicotiana*, it was debatable whether ADC or ODC might provide the putrescine incorporated. This possibility was tested by growing roots in the presence of DFMO and DFMA (Robins and Walton, 1993) in experiments analogous to those performed in *Nicotiana*. Inhibition of ADC specifically depressed hyoscyamine accumulation and the pools of intermediates, indicating that ADC might be more important for the tropane alkaloid pathway.

Another area of uncertainty concerned the route by which the tropic acid moiety is incorporated. Although early reports claimed to synthesize hyoscyamine from tropine and tropic acid or tropoyl-CoA, these findings were not readily substantiated (Robins and Walton, 1993). A series of experiments in which labelled phenyllactic acids were fed to plants of *D. inoxia* or root cultures of *D. stramonium* confirmed unequivocally that this compound was an intermediate of the pathway (Ansarin and Woolley, 1993, 1994; Chesters *et al.*, 1994, 1995a,b; Robins *et al.*, 1994a). A recent reappraisal (Chesters *et al.*, 1996) suggested that it is the *S*-isomer that is incorporated.

Other experiments have demonstrated that littorine (the phenyllactoyl ester of tropine) rearranges in vivo to hyoscyamine (Robins *et al.*, 1994b) with the aid of CYP80F1 (Ziegler and Facchini, 2008). Direct rearrangement was demonstrated unequivocally by incorporating three ^{2}H nuclei in the *N*-methyl of the tropinyl portion and two ^{13}C nuclei in the phenyllactoyl moiety.

It was proposed that the rearrangement of littorine to hyoscyamine might occur by a cytochrome P450-catalysed reaction (Fig. 2.3), and that the minor alkaloids 3α-phenylacetoxytropane and 3α-(2′-hydroxyacetoxy)tropane, which are also formed, are side-products of the mechanism (Robins *et al.*, 1995). This particular area of tropane biosynthesis requires further clarification.

The calystegines are also formed from ornithine and, therefore, belong biosynthetically to the tropane alkaloids (Goldmann *et al.*, 1992; Dräger, 2004; Biastoff and Dräger, 2007). This has been confirmed by Dräger and coworkers (1994), who fed root cultures of *Atropa belladonna* with ^{15}N-labelled tropinone and obtained good incorporation of isotope. These experiments have cast doubt on the intermediacy of hygrine in the direct pathway to hyoscyamine (Goldmann *et al.*, 1992; Robins and Walton, 1993; Dräger *et al.*, 1994). When ^{13}C-labelled hygrine was fed, no incorporation into hyoscyamine or scopolamine could be detected. Feeding other labelled precursors

suggested a pathway in which acetoacetate reacts via its C-4 position with *N*-methylpyrrolinium salt to give 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate. This intermediate favours cyclization to give 2-carboxytropinone, tropinone being formed by decarboxylation (Robins *et al.*, 1997).

As in *Nicotiana*, PMT appears to be regulatory in the pathway. Treating *D. stramonium* root cultures with phytohormones causes dispersion of the cultures, degradative metabolism of tropine and hyoscyamine and a loss of PMT activity. PMT is completely absent in dispersed cultures. Differentiated roots, alkaloid production and PMT activity are fully restored following the removal of phytohormones. Now that a clone for PMT is available, it will be interesting to examine this phenomenon at the molecular level. Robins and Walton (1993) and Hibi and co-workers (1994) have clearly demonstrated that *pmt* expression in *N. tabacum* plants is downregulated by auxin, in agreement with the observed effect of auxins on PMT activity in root cultures (Rhodes *et al.*, 1989; Robins and Walton, 1993). Roots treated with DFMA demonstrated a decreased PMT activity (Robins and Walton, 1993), but normal levels were restored by adding agmatine to the cultures. As DFMA treatment results in a loss of agmatine from the system, this effect was interpreted as a possible stimulation of PMT expression by agmatine. PMT has been characterized by X-ray crystallography (Teuber *et al.*, 2007).

Several enzymes of tropane alkaloid biosynthesis have been purified and characterized from root cultures: namely, putrescine *N*-methyltransferase from *D. stramonium* (Walton *et al.*, 1994) and *H. niger* (Hibi *et al.*, 1992) and tropinone reductases I and II from *Atropa belladonna* (Dräger and Schaal, 1994), *D. stramonium* (Portsteffen *et al.*, 1992, 1994) and *H. niger* (Hashimoto and Yamada, 1994). TR I and TR II have been characterized by X-ray crystallography (Nakajima *et al.*, 1998).

Another enzyme involved in the production of minor alkaloids, tigloyl-CoA: pseudotropine acyltransferase, has been purified from roots of *D. stramonium* (Rabot *et al.*, 1995). It catalyses the transfer to pseudotropine of an acyl group from a range of acyl-CoA thioesters. Esters of pseudotropine do not accumulate significantly in *D. stramonium* roots, although they do appear under abnormal metabolic conditions (Dräger *et al.*, 1992).

Important for the production of scopolamine, hyoscyamine 6β-hydroxylase was the first enzyme of tropane alkaloid metabolism to be purified and remains the most rigorously studied. It was obtained in pure form from *H. niger* root cultures and the preparation showed that it is a bifunctional enzyme with activity both as the 7β-hydroxylase and as the 6,7β-epoxidase. A clone for H6H was obtained following the purification of enzyme activity. The gene shows some similarity to other hydroxylases, including those involved in oxidative reactions in the formation of ethylene and anthocyanins (Hashimoto and Yamada, 1994).

A detailed study of H6H has allowed the genetic manipulation of scopolamine formation. The alkaloid spectrum of transformed root cultures of *A. belladonna* contains hyoscyamine and scopolamine in a ratio between 10:1

and 5:1 (Robins and Walton, 1993). Following the isolation and introduction of the *h6h* gene into cultures of transformed roots of *A. belladonna*, an engineered root-line was isolated, which showed an increased H6H activity and about a twofold higher accumulation of 7β-hydroxyhyoscyamine and scopolamine. This experiment effectively demonstrated that the ability of these cultures to accumulate hyoscyamine was limited by H6H activity and that, by increasing expression of *h6h*, *A. belladonna* plants almost exclusively contained scopolamine, in contrast to controls (Hashimoto and Yamada, 1994). Simultaneous overexpression of *pmt* and *h6h* in *H. niger* hairy root cultures resulted in a high yield of scopolamine (411 mg/L) (Zhang *et al*., 2004).

Since the 6β-hydroxylase is a bifunctional enzyme catalysing two consecutive reactions from hyoscyamine to scopolamine, expression of this single gene could change the alkaloid pattern of the host *A. belladonna* plants and could be of commercial benefit. Yun and co-workers (1993) showed that only the single polypeptide is required to carry out both reactions. The *h6h* was inserted into transgenic *N. tabacum* plants. As a result of this single insertion, the plants acquired the ability to biotransform hyoscyamine into scopolamine, showing unequivocally that a single gene product was responsible both for the hydroxylation and epoxidation steps. Species-dependent expression controlled by the promoter of the hyoscyamine 6β-hydroxylase gene was observed in experiments on transgenic plants, using the β-glucuronidase gene as a visible reporter gene (Kanegae *et al*., 1994). Pericycle-specific accumulation of hyoscyamine 6β-hydroxylase (Hashimoto and Yamada, 1994) was attributed to the 0.8 kb length 5′-flanking region of the gene from *H. niger*. Expression in *E. coli*, in which plant genes were overexpressed, allowed for biotransformation and biosynthesis of alkaloids when feeding appropriate precursors of the tropane alkaloids. Reaction products were accumulated in the medium, suggesting free permeability of the bacterial cell membrane to the products (Hashimoto and Yamada, 1994, 2003). The consequences of overexpression of genes involved in nicotine and tropane biosynthesis and the prospects of metabolic engineering have been discussed in Häkkinen *et al*. (2007), Oksman-Caldentey *et al*. (2007), Sato *et al*. (2007) and Verpoorte *et al*. (2007).

Tropane alkaloids are being synthesized in roots and translocated via the xylem to aerial parts, where they accumulate in the vacuole. In *Atropa*, PMT and other enzymes of tropane alkaloid biosynthesis such as H6H are expressed in the pericycle of the differentiation region of the root facing the xylem (Kanegae *et al*. 1994; Suzuki *et al*., 1999). The localization of H6H next to the xylem is strategically important, since scopolamine is transported in the xylem. TR I, however, is localized in the endodermis and nearby cortical cells, but not in the pericycle of *H. niger* (Nakajima and Hashimoto, 1999). Tropane alkaloids strongly affect the muscarinic acetylcholine receptor as antagonists and are therefore powerful neurotoxins; they apparently serve as defence compounds against herbivores. A few specialized herbivores (e.g. thrushes) exist that can inactivate atropine by expressing an esterase; for

them atropine is less toxic. Calystegines can be regarded as sugar-mimics; they possess strong glycosidase inhibitory activity (Asano *et al.*, 2000). TR-II is localized in companion cells of sieve elements in the phloem (Kaiser *et al.*, 2006).

2.3 Pyrrolizidine alkaloids (PAs)

This group of alkaloids is found in a wide range of families, centred around the Asteraceae, Boraginaceae and Fabaceae (Hartmann and Witte, 1995; Hartmann, 2007). PAs are metabolically activated in the liver of herbivores and can then alkylate DNA and proteins, leading to mutations and even cancer. The occurrence of PAs in many *Senecio* species accounts for the high toxicity of these plants. PAs function as defence compounds against many herbivores; however, a number of specialized insects are known which store and utilize the dietary defence chemicals (for a review, see Wink, 1993; Hartmann and Witte, 1995).

The biosynthesis of pyrrolizidine alkaloids has been studied mainly in *Senecio* species (Hartmann, 1991, 2007). These alkaloids are esters between a necine base, derived from arginine or ornithine via homospermidine (Fig. 2.4) and a necic acid moiety, frequently derived from isoleucine. The formation of homospermidine from one molecule of putrescine and one molecule of spermidine is the first committed step in the pathway (Böttcher *et al.*, 1993; Graser and Hartmann, 1997, 2000). This step is catalysed by homospermidine synthase (HHS). HHS apparently evolved by duplication of a gene encoding desoxyhypusine synthase (Ober and Hartmann, 1999). The formation of homospermidine is a side activity in desoxyhypusine synthase, but became a main activity in HHS (Ober, 2005). Thus, there is a close parallel between this pathway and that described for the tropane alkaloids, with two routes starting from amino acids that provide acidic and alkamine moieties, which are condensed by esterification later in the pathway.

The major product accumulated is senecionine-*N*-oxide (Fig. 2.4) and, since neither suspension cultures nor shoot cultures of *Senecio* form these alkaloids, this suggests that the root is the sole site of biosynthesis (Hartmann, 1994). In root cultures of *Senecio vulgaris*, feeding experiments with a range of ^{14}C-labelled precursors and inhibitors of metabolism showed both ornithine and arginine to be incorporated into senecionine-*N*-oxide (Hartmann, 1991). Experiments with DFMA and DFMO gave results suggesting that, in contrast to *Nicotiana* and *Datura*, label from ornithine is incorporated via arginine. The mechanism for this is not clear. Spermidine and putrescine were found to be rapidly interconverted and both spermine and spermidine reduced the incorporation of label from arginine, suggesting that there is feedback control of agmatine biosynthesis that leads to a depression of alkaloid formation. This interaction between alkaloid and polyamine formations was not apparent in *D. stramonium* roots (Robins and Walton, 1993). A higher degree of regulation

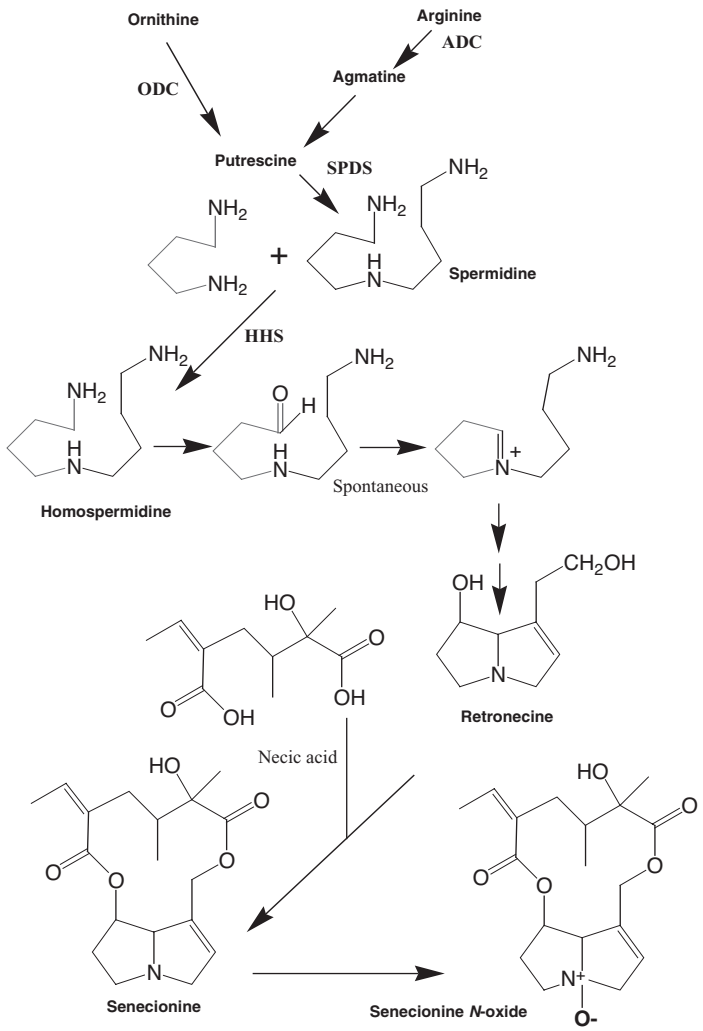


Figure 2.4 Biosynthesis of the pyrrolizidine alkaloid, senecionine-*N*-oxide. ODC, ornithine decarboxylase; ADC, arginine decarboxylase; SPDS, spermidine synthase; HHS, homospermidine synthase. (See Plate 9 in colour plate section.)

may be required in this pathway due to the greater demand for putrescine-homospermidine requiring two moles per mole of alkaloid. Senecionine-*N*-oxide is synthesized only in the actively growing parts of root cultures and is not significantly turned over, but is slowly transported throughout the root mass. Some limited metabolism occurs during this process, primarily oxidation and acetylation (Hartmann, 1991).

The biosynthesis of the necic acid moiety has, in contrast, received relatively little attention. Label from ^{14}C-isoleucine is effectively

incorporated into senecionine-*N*-oxide by root cultures of *S. vulgaris* (Hartmann, 1991).

So far, only one enzyme, HHS, has been partially purified and characterized using root cultures of *Eupatorium cannabinum* (Böttcher *et al.*, 1993). Walton and co-workers (1994) found PMT activity in all pyrrolizidine-alkaloid-forming species so far examined; its presence confirms that the biosynthetic route for this group of alkaloids is not via free pyrroline. The enzyme carries out two sequential steps, the first of which is a deaminative oxidation that generates nicotinamide adenine dinucleotide (reduced form) (NADH) and the second of which is a reduction, utilizing NADH. This reaction sequence was clearly demonstrated by using chirally labelled, C1–^{2}H, putrescines (Böttcher *et al.*, 1994). These authors also suggested that spermidine may act, at least in part, as a co-substrate with putrescine in homospermidine formation. The apparent rapid interconversion of putrescine and spermidine in these cultures makes this a difficult problem to solve.

However, it has been shown that more than half the aminobutyl moiety of homospermidine comes directly from spermine, and the aminobutyl moiety of spermine is also incorporated directly into the necine base of pyrrolizidine alkaloids (Graser and Hartmann, 1997).

2.3.1 Translocation, accumulation and ecology

The roots have been shown to be the major, if not exclusive, sites of pyrrolizidine alkaloid synthesis, where it occurs preferentially at the root apex, thus coinciding with the sites of active growth. HSS could be localized in defined groups of root cells comprising endodermis and cortex cells (Moll *et al.*, 2002). Senecionine-*N*-oxide is produced as a stable product without significant turnover. Pyrrolizidine alkaloids are mobile, being, at least in part, translocated via the phloem into newly growing aerial tissues, with the highest concentration in the inflorescences, where alkaloid concentrations are 30-fold higher than in the leaves. As soon as root growth stops, synthesis of pyrrolizidine alkaloids ceases. Transport occurs via the phloem, and subsequent vacuolization of the pyrrolizidine alkaloids as salts has been demonstrated. Recent experiments have shown a role for pyrrolizidine alkaloids as part of the plant defence against predation (Wink, 1993; Hartmann and Witte, 1995; Hartmann, 2007 and references therein).

2.4 Benzylisoquinoline alkaloids

Benzylisoquinolines, of which more than 2500 structures are known, are found as defence chemicals within the families of the superorder Magnoliids (comprising Piperales, Laurales and Magnoliales with the families Annonaceae, Eupomatiaceae, Aristolochiaceae, Magnoliaceae, Lauraceae, Monimiaceae), Nelumbonaceae and Ranunculales (i.e. Berberidaceae,

Ranunculaceae, Menispermaceae and Papaveraceae). This highly clustered distribution in two main groups is of interest from a chemotaxonomic point of view (but see Chapter 6), as there are few exceptions, the most notable being the Erythrina alkaloids that occur throughout the genus *Erythrina* (Fabaceae). This group of families contains such alkaloids as colchicine (a microtubule disrupter and gout suppressant), berberine (an antimicrobial against eye and intestinal infections), morphine (a narcotic analgesic), codeine (a narcotic analgesic and antitussive) and sanguinarine (an antimicrobial used in oral hygiene).

The benzylisoquinolines are formed from two molecules of the aromatic amino acid, tyrosine. In the past ten years, this pathway has been probed at the enzyme and gene level. The recent linking of the phloem-specific expression of tyrosine/Dopa decarboxylase (TYDC) genes with the biosynthesis of the isoquinoline alkaloids in the opium poppy, *Papaver somniferum* (Facchini and De Luca, 1994, 1995, 2008; Liscombe and Facchini, 2008), and the association with alkaloid accumulation as part of the plant defence mechanism (Wink, 1993; Facchini *et al.*, 1996) are of particular interest in furthering our knowledge of the location of alkaloid biosynthesis.

As a result of research over the past 20 years (Facchini, 2001; Ziegler *et al.*, 2006; Sato *et al.*, 2007; Zenk and Juenger, 2007; Liscombe and Facchini, 2008; Ziegler and Facchini, 2008), it is now clear that the first committed step in the biosynthesis of isoquinoline is the formation of (*S*)-norcoclaurine (Fig. 2.5). This alkaloid is an important precursor of a variety of pathways that lead to a series of diverse structures within this alkaloid group.

Plant cell cultures established from various isoquinoline-bearing plants have provided useful systems for the study of biosynthetic pathways at the enzyme level. Excellent progress has been made in unravelling the route to (*S*)-norcoclaurine and the sequences leading to some of the more important groups of isoquinolines. Only recently, as a result of investigations into the enzymes of the biosynthetic pathways to morphine, berberine and sanguinarine, have the early steps of the pathway been fully elucidated. These studies have also helped to improve our understanding of the localization at the subcellular level of both enzymes and products (Zenk, 1990; Kutchan and Zenk, 1993; Kutchan, 1995, 1996; Facchini, 2001; Ziegler *et al.*, 2006; Sato *et al.*, 2007; Zenk and Juenger, 2007; Liscombe and Facchini, 2008; Ziegler and Facchini, 2008).

2.4.1 Formation of (*S*)-norcoclaurine

Investigations of a number of enzymes involved in tyrosine conversion have suggested that the first committed step in the biosynthesis of benzylisoquinolines involves a Pictet–Spengler-type condensation of dopamine with 4-hydroxyphenylacetaldehyde (which derived from tyrosine) to give (*S*)-norcoclaurine, a compound that has proved to be pivotal in the formation of all benzylisoquinoline alkaloids (Fig. 2.5). The condensation step is catalysed

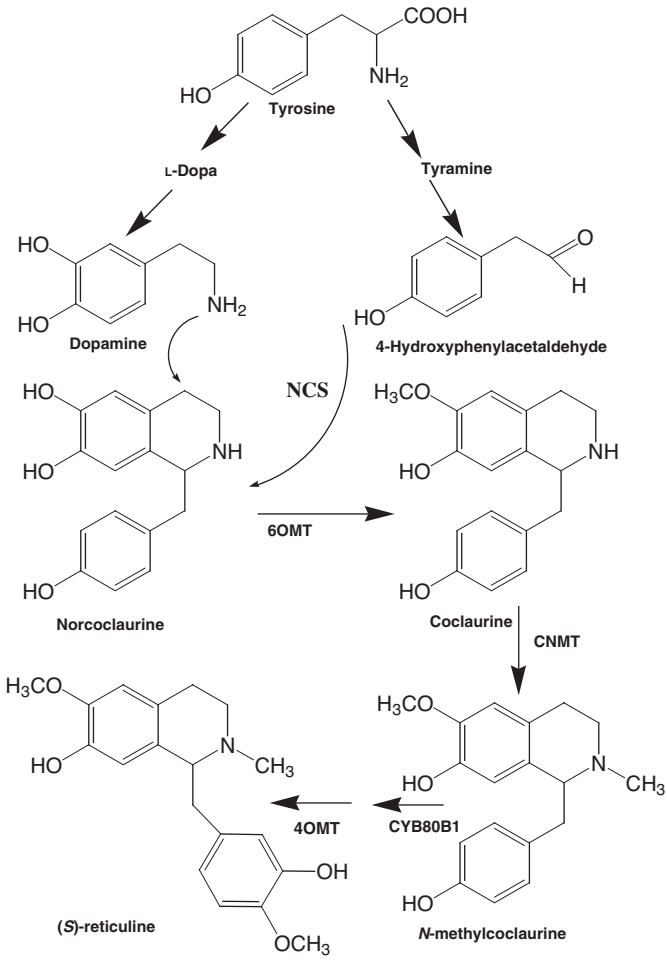


Figure 2.5 Formation of (*S*)-reticuline. NCS, norcoclaurine synthase; 6OMT, 6-hydroxy-*O*-methyltransferase; CNMT, coclaurine *N*-methyltransferase; 4OMT, 4 hydroxy-*O*-methyltransferase.

by (*S*)-norcoclaurine synthase (NCS), which has been isolated and characterized from *Thalictrum flavum* (Samanani *et al.*, 2004) (TfNCS) and *Coptis japonica* (Minani *et al.*, 2007) (CjNCS). Only TYDC have been purified and characterized so far. Interestingly, homologue genes for TYDC and NCS have been detected in *Arabidopsis* or rice, which do not produce isoquinoline alkaloids, suggesting a much wider occurrence of alkaloid genes than hitherto assumed (see Chapter 7 for more examples).

(*S*)-Reticuline is readily formed from (*S*)-norcoclaurine as a result of a series of hydroxylations and methylations. From intermediates observed in vivo and enzyme studies, it may be concluded that (*S*)-norcoclaurine is

stereospecifically metabolized to (*S*)-reticuline via (*S*)-coclaurine (by 6-hydroxy-O-methyltransferase, 6OMT; Morishige *et al*., 2000), (*S*)-*N*-Methylcoclaurine (by coclaurine *N*-methyltransferase, CNMT; Choi *et al*., 2002) and (*S*)-3′-hydroxy-*N*-methylcoclaurine (by a P450 hydroxylase). The final step to (*S*)-reticuline is catalysed by 4-*O*-methyltransferase (Morishige *et al*., 2000). The order in which the various hydroxylations and methylations occur is substantiated by the distribution of radioactivity in the benzylisoquinoline alkaloids of *Berberis stolonifera* cell cultures after feeding [U-^{14}C]tyrosine (Zenk, 1990; Kutchan and Zenk, 1993). The sequence of three methylation and one hydroxylation steps has been determined at the protein and gene levels (Stadler and Zenk, 1990; Pauli and Kutchan, 1998; Sato *et al*., 2007; Liscombe and Facchini, 2008). The methyltransferases involved show relaxed substrate specificity (Sato *et al*., 2007). Except for CYT80B1, which is membrane bound, all other enzymes are cytosolic. Minami *et al*. (2008) succeeded to express the genes of reticuline biosynthesis (NCS, CNMT, 4OMT) (Fig. 2.5) to produce reticuline in recombinant *E. coli* and *S. cerevisiae*.

When coclaurine and *N*-methylcoclaurine are combined by CYP80A1 (berbamunine synthase), dimeric bisbenzoylisoquinoline alkaloids are generated (Kraus and Kutchan, 1995), among the several with pronounced biological activities, such as berbamine and tubocurarine.

(*S*)-Reticuline is the precursor for aporphine alkaloids (Fig. 2.1). Minami *et al*. (2008) succeeded to express the genes of magnoflorine biosynthesis (NCS, CNMT, 4OMT, CYP80G2) to produce corytuberine and magnoflorine in recombinant *E. coli* and *S. cerevisiae*.

(*S*)-Reticuline is also the precursor for the biosynthesis of benzophenanthridine (e.g. sanguinarine, marcarpine), protoberberine, berberine, palmatine) and morphinan alkaloids (morphine, codeine) (see next few paragraphs).

2.4.2 Biosynthesis of tetrahydroberberine alkaloids

The enzymatic route to berberine was one of the first to be completely elucidated, with all (four) of the participating enzymes isolated and characterized (Dittrich and Kutchan, 1991; Ikezawa *et al*., 2003). The conversion of (*S*)-reticuline to (*S*)-scoulerine by the berberine bridge enzyme may be considered as the first committed step in the production of the tetrahydroprotoberberines and the whole range of alkaloidal types that are derived from this basic skeleton (Fig. 2.6). The berberine bridge enzyme ([S]-reticuline:oxygen oxidoreductase [methylene bridge-forming]; E.C. 1.5.3.9.) catalyses the stereospecific conversion of the *N*-methyl group of (*S*)-reticuline into the berberine bridge carbon, C-8 of scoulerine (Dittrich and Kutchan, 1991). In *Eschscholzia californica*, this enzyme is found to be elicitor-inducible, which implies that regulation of transcription of this enzyme may regulate benzophenanthridine alkaloid accumulation. Complementary deoxyribonucleic acid encoding the berberine bridge enzyme, overexpressed in insect cell culture, contained covalently attached flavin adenine dinucleotide (FAD) in

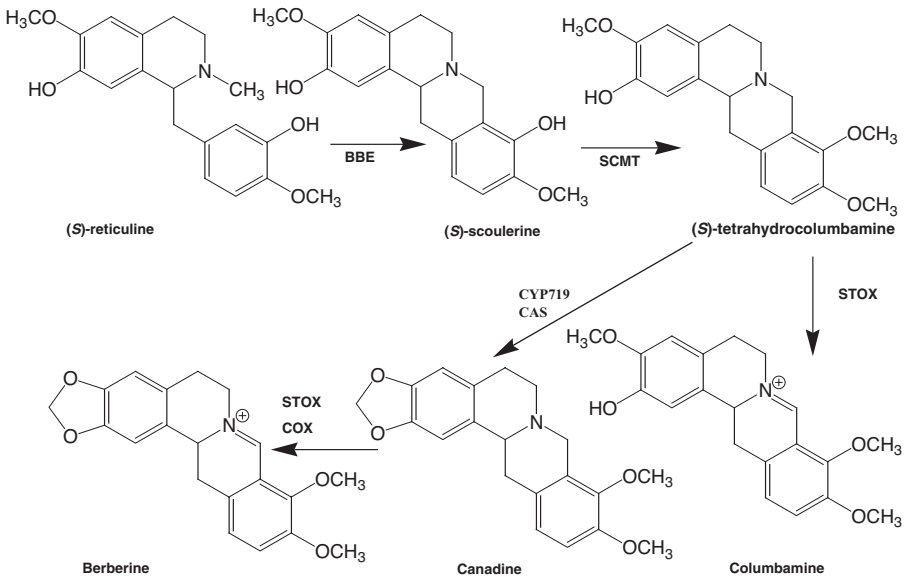


Figure 2.6 Biosynthesis of berberine from (*S*)-reticuline in *Berberis* species and *Coptis japonica*. BBE, berberine bridge enzyme; SCMT, (*S*)-scoulerine-9-*O*-methyltransferase; CAS, (*S*)-canadine synthase; STOX, tetrahydroberberine oxidase; COX, enzyme found in *Coptis japonica*.

the molecular cofactor to protein ratio of 1:1.03 (Kutchan and Dittrich, 1995). Translation of the nucleotide sequence of *bbe1* confirmed the presence of a signal peptide that directs the enzyme into the endoplasmic reticulum and then into the smooth vesicles, in which it accumulates. Elicitor-induced transcription of *bbe1* and other inducible genes along the benzophenanthridine alkaloid pathway should help to elucidate the complex defence response signal transduction chain that exists in plants. Minami *et al*. (2008) succeeded to express the genes of scoulerine biosynthesis (NCS, CNMT, 4OMT and BBE) (Figs. 2.5 and 2.6) to produce scoulerine in recombinant *E. coli* and *S. cerevisiae*.

The next enzyme in the sequence has been shown to be (*S*)-scoulerine-9-*O*-methyltransferase (SCMT), which catalyses the conversion of (*S*)-scoulerine to (*S*)-tetrahydrocolumbamine (Fujiwara *et al.*, 1993). Subsequently, a methylene bridge is formed to yield (*S*)-canadine utilizing the enzyme (*S*)-canadine synthase (CAS), a specific methylenedioxy bridge-forming enzyme (Rueffer and Zenk, 1994). By molecular cloning and characterization, the methylene-bridge-forming enzyme from cultured *Coptis japonica* cells was shown to be CYP719 that belongs to a novel P450 family which is not present in *Arabidopsis* (Ikezawa *et al*., 2003).

(*S*)-canadine can act as a substrate for the tetrahydroberberine oxidase (STOX) enzyme isolated from *Berberis* and may be converted by this enzyme to berberine (Zenk, 1995); however, the oxidase found in *Coptis japonica* (COX)

is specific for (*S*)-canadine (Okada *et al.*, 1988; Rueffer and Zenk, 1994). These two oxidases differ, in that STOX contains a flavin and produces 1 mole each of H_2O_2 and water per mole of substrate consumed, whereas COX has a cofactor requirement for iron and produces 2 moles of H_2O_2 per mole of substrate utilized (Okada *et al.*, 1988) (Fig. 2.6). It would appear that either enzyme may be used to oxidize canadine, the type of oxidase being species-dependent. Hence, there is reason not to generalize metabolic pathways, unless enzymatic steps have been elucidated for each species.

The formation of the methylenedioxy bridge in *Berberis* has been found to be caused by the demethylating activity of a peroxidase (POD) found within the vesicle. It was also found that the cytochrome P450-requiring enzyme (canadine synthase) from microsomes of *Berberis*, *Thalictrum* and *Coptis* species formed the methylene bridge in (*S*)-tetrahydrocolumbamine (Ikezawa *et al.*, 2003), but not in the quaternary alkaloid columbamine (Galneder *et al.*, 1988; Zenk, 1995). Because of the substrate specificity of canadine synthase, the berberine pathway is considered to be that presented in Fig. 2.5 (Rueffer and Zenk, 1994). Columbamine, once proposed as an alternative route to berberine, is however converted to palmatine by a specific methyltransferase first isolated from *Berberis wilsoniae* cell cultures (Rueffer and Zenk, 1985; Ikezawa *et al.*, 2003).

A unique C–O phenolic coupling cytochrome P450 enzyme CYP80A1 (berbamunine synthase), isolated from *Berberis stolonifera* cell cultures, catalyses the oxidation of three different chiral benzyltetrahydroisoquinolines, namely, (*S*)-coclaurine, (*R*)-*N*-methylcoclaurine and (*S*)-*N*-methylcoclaurine, leading to the formation of three distinct dimeric products, namely, (*R*,*S*)berbamunine, (*R*,*S*)-2′-norberbamunine and (*R*,*R*)-guattegaumerine (Stadler and Zenk, 1993). Molecular cloning of the cDNA encoding for berbamunine synthase, utilizing cell suspension cultures of *Berberis stolonifera*, has allowed heterologous expression in a functional form in insect cell cultures. This oxidase was accumulated in an active form in insect cell microsomes and accepted electrons from the endogenous NADPH-cytochrome P450 reductase (Kraus and Kutchan, 1995).

Important to our understanding of the mechanisms of secondary metabolism was the discovery that all of these enzymes from (*S*)-scoulerine to the production of berberine (especially BBE and STOX) are firmly associated with vesicles that are thought to be derived from the endoplasmic reticulum. These vesicles appear to be specific sites for the formation of quaternary protoberberine alkaloids. Because of their positive charge, the alkaloids are prevented from leaving the vesicles, and there is some evidence to suggest that they end up in the vacuole when the vesicle membrane fuses with the tonoplast (Bock *et al.*, 2002). Tertiary tetrahydrobenzylisoquinolines, such as (*S*)-scoulerine, are able to diffuse freely out of the vesicle to undergo further modifications (Zenk, 1989). BBE was seen in idioblasts that were not connected to the laticifer system, indicating that protobererine and morphinan pathways are strictly separated in plants (Bock *et al.*, 2002).

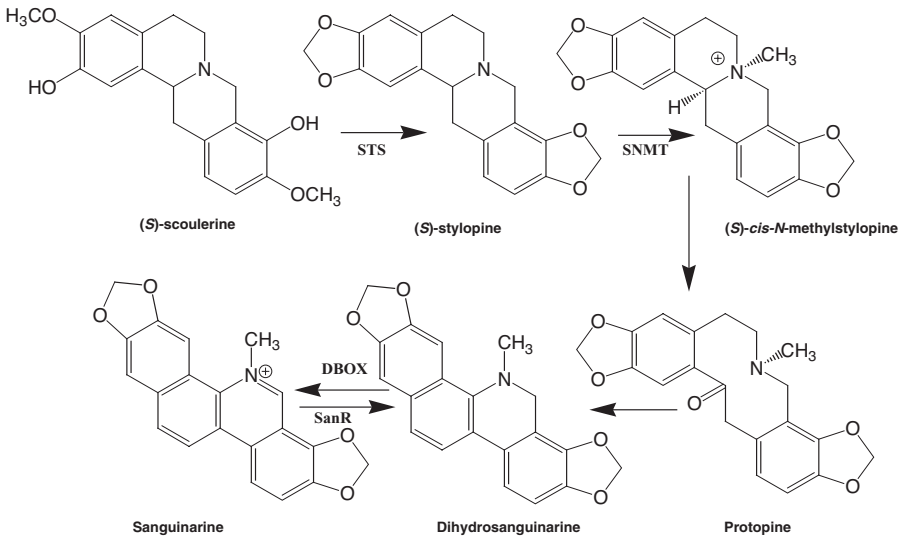


Figure 2.7 Biosynthesis of protopine and the benzophenanthridine alkaloids. STS, stylopine synthase; SNMT, stylopine *N*-methyltransferase; DBOX, dihydrosanguinarine oxidase; SanR, sanguinarine reductase.

Genes of protoberberine biosynthesis are abundantly expressed in rhizomes of *Thalictrum flavum*, but were also active in roots and other organs (Samanani *et al*. 2005). In roots, transcripts were localized in the immature endodermis and root pericycle. In rhizomes transcripts were found in the protoderm of leaf primordial. As known from other plants, these data show that the sites of synthesis are not identical with the sites of accumulation. In many instances, a long-distance transport must occur. If this is the case, alkaloids have to pass several biomembranes. ABC-transporters and H^+-alkaloid antiporters can be involved (see Chapter 1).

2.4.3 Route to the protopine and benzophenanthridine alkaloids

Another important route stems from the formation of the *N*-methylated moieties of the (*S*)-tetrahydroprotoberberines, which serve as precursors for the protopine, benzophenanthridine, tetrahydrobenzazepines (rhoeadines) and spirobenzylisoquinoline alkaloids (Kutchan and Zenk, 1993; Liscombe and Facchini, 2008; Ziegler and Facchini, 2008) (Fig. 2.7).

Microsomal, cytochrome P450-dependent enzymes isolated from the cells of *E. californica* convert (*S*)-scoulerine to (*S*)-stylopine by the introduction of methylenedioxy bridges (Bauer and Zenk, 1991). This conversion is catalysed by P450-dependent synthases (chalanthifoline and stylopine synthase) (Facchini, 2001; Ikezawa *et al*., 2007). The subsequent *N*-methylation requires

S-adenosyl-L-methionine:(*S*)-tetrahydro-*cis*-*N*-methyltransferase, and this enzyme has been isolated from the cell cultures of a variety of plants found within the Berberidaceae, Fumariaceae, Menispermaceae, Papaveraceae and Ranunculaceae (Rueffer *et al.*, 1990).

The route to protopine requires oxidation at C-14 of the tetrahydroprotoberberine molecule (Rueffer and Zenk, 1987b; Kutchan and Zenk, 1993). The enzyme responsible for this oxidation is a microsomal cytochrome P450-NADPH-dependent enzyme that hydroxylates (stereo- and regiospecifically) C-14 of (*S*)-*cis*-*N*-methyltetrahydroprotoberberines, and has been found in a number of cell cultures developed from plants of the Fumariaceae and Papaveraceae. Some of the best activity was observed using cell cultures of *Fumaria officinalis* and *F. cordata*. The protopines may be further metabolized to produce benzazepine and benzophenanthridine alkaloids. Protopine has been found to be a central intermediate in the biosynthesis of the benzophenanthridine, sanguinarine and also the more highly oxidized alkaloids, such as macarpine (Schumacher and Zenk, 1988). Essential to this conversion is hydroxylation of the tetrahydroprotoberberine skeleton at C-6, and it is this that leads to C-6/N bond fission followed by intramolecular cyclization. Important to these events is the fact that, as acid salts, protopines are not simple *N*-protonated structures. The absence of carbonyl absorption indicates the closure of the ten-membered ring (as shown in Fig. 2.6).

The microsomal enzyme that catalyses the hydroxylation of protopine (PRH, protopine hydroxylase) has been isolated from *E. californica* and is strictly dependent on NADPH as a reducing factor and on molecular oxygen. Studies with inhibitors have suggested that the enzyme is a cytochrome P450-linked monooxygenase. The enzyme was also found to be specifically present only in plant species that produce benzophenanthridine alkaloids in culture (Kutchan and Zenk, 1993). The dihydro moieties are readily converted to benzophenanthridine alkaloids by an oxidase (Arakawa *et al.*, 1992). This latter enzyme, together with a 12-*O*-methyltransferase (Kammerer *et al.*, 1994), converts dihydrosanguinarine, dihydrochelirubine and dihydromacarpine to sanguinarine, chelirubine and macarpine, respectively.

Dihydrobenzophenanthridine oxidase (DBOX) responds to elicitors implicated in signal transducer mechanisms leading to acquired resistance to pathogens in plants (Ignatov *et al.*, 1996). Sanguinarine can be converted back to dihydrosanguinarine by sanguinarine reductase (SanR). The route to the benzophenanthridine alkaloids is now clearly defined at the enzyme level (Liscombe and Facchini, 2008). In contrast to the berberine pathway, the enzymes of benzophenanthridine biosynthesis are located in the cytosol.

2.4.4 Biosynthesis of the morphinan alkaloids

About ten chemical steps are required in the pathway leading from tyrosine to morphine. Almost all steps have been characterized at the enzyme level and

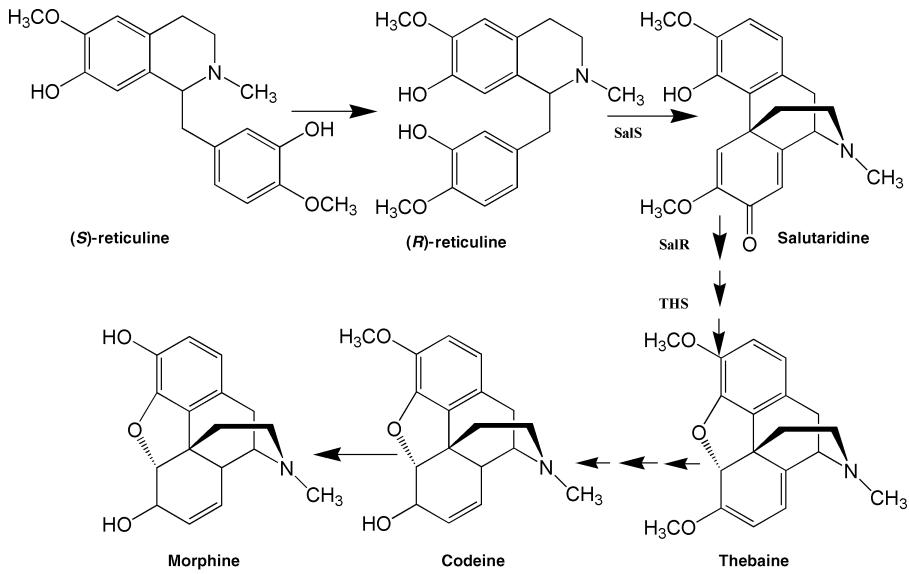


Figure 2.8 Biosynthesis of morphine via the conversion of (*S*)-reticuline to (*R*)-reticuline, salutaridine and thebaine. SalS, salutaridine synthase; SalR, salutaridine reductase; THS, thebaine synthase; COR, codeinone reductase.

nine genes have been cloned by now (Ziegler *et al*., 2006; Zenk and Juenger, 2007; Liscombe and Facchini, 2008).

The role of reticuline as an intermediate in the biosynthesis of the morphinan alkaloids (Fig. 2.8) was demonstrated by the isolation both of (*S*)- and (*R*)-reticuline from the opium poppy. An excess of the (*S*)-reticuline over the (*R*)-isomer was found in opium (poppy latex) obtained from the mature plant, in contrast to the roughly equal amounts of these two isomers that occur in poppy seedlings. Both isomers were found to be incorporated into morphine, the major alkaloid isolated from opium, although incorporation of the (*R*)-isomer was slightly more efficient. (*R*)-Reticuline is firmly established in *P. somniferum* as the precursor of the morphinan-type alkaloids (Loefer and Zenk, 1990). (*S*)-Reticuline, however, is the central intermediate in isoquinoline alkaloid biosynthesis. It has been postulated that (*R*)-reticuline is formed from (*S*)-reticuline by isomerization. This inversion of configuration can be explained by the intermediate formation of the 1,2-dehydroreticulinium ion originating from (*S*)-reticuline, followed by stereospecific reduction to yield the (*R*) counterpart. The 1,2-dehydroreticulinium ion is efficiently incorporated into opium alkaloids and its role as a precursor of the morphinan-type alkaloids has been unequivocally established (De-Eknamkul and Zenk, 1990, 1992).

The conversion of (*S*)-reticuline to 1,2-dehydroreticuline has been accomplished using a novel oxidase isolated from cell cultures of plants of the

Berberidaceae. This enzyme, (*S*)-tetrahydroprotoberberine oxidase, has previously been shown to catalyse, in the presence of oxygen, the dehydrogenation of (*S*)-tetrahydroprotoberberine (Zenk, 1995). This flavoprotein is compartmentalized in a specific vesicle and can stereospecifically oxidize (*S*)-benzylisoquinolines to their corresponding 1,2-dehydro analogues. Although this enzyme more efficiently oxidizes the tetrahydroprotoberberines, it has been shown to occur in *P. somniferum* roots and leaves (Zenk, 1995). The question to be answered is whether, in vivo, this is the enzyme primarily responsible for the conversion of (*S*)-reticuline to its iminium ion. The conversion of 1,2-dehydroreticuline to (*R*)-reticuline was brought about by crude cell preparations from young seedlings of *P. somniferum* in the presence of NADPH at pH 8.5. The purified enzyme stereospecifically transfers the pro-S-hydride from NADPH to C-1 of the 1,2-dehydroreticuline. The reaction is highly substrate-specific, with no evidence for the reverse reaction. No activity was found either in plants that do not normally synthesize the morphinans or in cell cultures of the genus *Papaver*, i.e. *P. somniferum*, *P. rhoeas*, *P. bracteatum*, *P. feddei* and *P. dubium*, in which the plants do normally synthesize morphinans. The formation of (*R*)-reticuline in this manner enables a narrow range of *Papaver* species to form the morphinandienone alkaloids, morphine, codeine and thebaine, which also possess the (*R*) configuration at the chiral centre.

The next step in the pathway to morphine is the intramolecular condensation of (*R*)-reticuline in a regio- and stereoselective manner to salutaridine, a morphinandienone (De-Eknamkul and Zenk, 1990, 1992). The natural occurrence of salutaridine was confirmed by the isolation of the compound from extracts of opium. The enzyme responsible for this reaction has recently been found to be a highly selective microsomal-bound cytochrome P450-dependent enzyme (salutaridine synthase, SAS) isolated from young poppy capsules (Gerardy and Zenk, 1993; Zenk *et al.*, 1995). The conversion of salutaridine to salutaridinol with the (7S) configuration (Lotter *et al.*, 1992) (Fig. 2.7) by a salutaridine NADPH-7-oxidoreductase (SalR) isolated from *P. somniferum* has taken the elucidation of the morphinan pathway a step further (Gerardy and Zenk, 1992, 1993). Salutaridinol possesses the correct configuration for an allylic *syn*-displacement of the activated C-7 hydroxyl by the phenolic C-4 hydroxyl to produce thebaine. A highly substrate-specific enzyme that transfers the acetyl moiety from acetyl coenzyme A (AcCoA) to the 7-OH group of salutaridinol has been discovered and purified to homogeneity (acetylcoenzyme A: salutaridinol-7-O-acetyltransferase, SalAT) (Grothe *et al.*, 2001). Subsequently, the salutaridine-7-O-acetate that is formed spontaneously closes, at a cellular pH of 8–9, to produce the oxide bridge between C-4 and C-5 and thus produce thebaine (Lenz and Zenk, 1994, 1995a); this step is also catalysed by thebaine synthase (Liscombe and Facchini, 2008).

The sequences from thebaine via various intermediates to morphine, although known from ^{14}C-labelling studies, have recently been explored at

the enzyme level (Fig. 2.8). Cell cultures of *P. somniferum* and *Mahonia nervosa* will convert thebaine to codeine, thus proving that these cells have the enzymes necessary for enolether cleavage (Wilhelm and Zenk, 1997). An NADPH-requiring codeinone-reducing enzyme (COR) has now been isolated and characterized from cell cultures of *P. somniferum*. Using capsule tissue of differentiated *P. somniferum* plants and applying similar isolation procedures, two isoenzymes were isolated. These cytosolic codeinone reductases (NADPH/$NADP^+$) convert codeinone to codeine. Finally, codeine is demethylated to morphine (Lenz and Zenk, 1995b; Unterlinner *et al*., 1999) (Fig. 2.8). These recent findings mean that most of the enzymes of the metabolic route to morphine have now been isolated. Thebaine can also be demethylated in two steps via oripavine and morphinone to morphine (Liscombe and Facchini, 2008).

The genes of morphine biosynthesis are expressed in all organs of *P. somniferum*. Highest expression occurs in stems and flower buds (Unterlinner *et al*., 1999; Grothe *et al*., 2001; Facchini and De Luca, 2008). Most of the genes, except COR, can be induced by elicitor treatment and wounding, indicating that these alkaloids are part of the defence system. Seven genes (6OMT, CNMT, CYP80B, 4OMT, BBE, SalAT, COR) are localized in sieve elements in supporting companion cells of *P. somniferum* (Bird *et al*., 2003; Facchini and St. Pierre, 2005; Facchini and De Luca, 2008; Liscombe and Facchini, 2008). Another study demonstrated 4OMT and SalAT in phloem parenchyma cells and COR in laticifers, which are the site of opium accumulation (Weid *et al*., 2004). According to recent studies, alkaloid biosynthetic enzymes are assembled in companion cells and subsequently transported to sieve elements (Facchini and De Luca, 2008; Liscombe and Facchini, 2008). A summary of the intricate cellular compartmentation and cellular distribution of the enzymes involved in benzoisoquinoline alkaoids is provided by Facchini and De Luca (2008) and Ziegler and Facchini (2008).

2.4.5 Alkaloid production in transgenic organisms

When the first genes of secondary metabolism became isolated, one could start to dream of assembling complete pathways and to express them in vitro, in appropriate culture systems (Wink, 1989). This dream might become true in the near future, because by now genes for complete pathways have been cloned and characterized. In the pathway from norcoclaurine to berberine and even to morphine, most of the genes were successfully and functionally expressed in microbial systems (Dittrich and Kutchan, 1991; Pauli and Kutchan, 1998; Morishige *et al*., 2000; Facchini, 2001; Ikezawa *et al*., 2003; Samanani *et al*., 2004, Minami *et al*., 2007, 2008). In the next steps, the corresponding genes need to be organized in a sort of minichromosome, which is regulated by a common promotor, similar to the situation in *Streptomyces*, where the genes of biosynthesis are clustered. Also the use of transgenes for individual biotransformation steps might be biotechnologically interesting.

Minami *et al*. (2008) succeeded to express the genes of scoulerine and magnoflorine biosynthesis (NCS, CNMT, 4OMT, BBE) to produce scoulerine and the aporphine alkaloid magnoflorine in recombinant *E. coli* and *S. cerevisiae*.

A few of the enzymes (SalR, 6OMT) have been purified and characterized by X-ray crystallography (Ziegler and Facchini, 2008).

The heterologous expression of biosynthetic genes in alkaloid-producing and non-producing plants provides an interesting research platform. Also, the specific inactivation of defined steps in alkaloid biosynthesis by RNAi offers a fantastic tool to better understand the formation, transport and storage, and function of alkaloids. Some of these approaches have been discussed in Marasco and Schmidt-Dannert (2007), Oksman-Caldentey *et al.* (2007), Sato *et al*. (2007), Verpoorte *et al*. (2007) and Wu and Chappell (2008).

2.5 Monoterpene indole alkaloids (MIA)

The monoterpene indole alkaloids, of which more than 3000 structures have been described, have been mainly isolated from three tropical plant families, Loganiaceae, Apocynaceae and Rubiaceae, all of the Gentianales. The indole alkaloids are rich in biologically active constituents, some of which are used as therapeutic agents in medicine, for example, vinblastine and vincristine. These dimeric alkaloids, used in the treatment of leukaemia and Hodgkin's disease and present in small amounts in *Catharanthus roseus* (Apocynaceae), have led to extensive investigation of this plant and cell cultures derived from it. However, the formation of neither vincristine or vinblastine nor vindoline, the major alkaloid of *C. roseus*, was unequivocally found in cell cultures (De Luca, 1993). Cell cultures of *C. roseus*, however, do produce many other indole alkaloids and have proved to be very useful for biochemical studies at the enzyme and gene levels (Meijer *et al.*, 1993b). Reviews discussing the biosynthesis of monoterpene indole alkaloids include Facchini (2001), Ruppert *et al*. (2005), Rischer *et al*. (2006), Oksman-Caldentey *et al.* (2007), Stöckigt *et al*. (2007), Zenk and Juenger (2007), Facchini and De Luca (2008) and Murata *et al*. (2008).

2.5.1 Biosynthesis of indole alkaloid precursors

Indole alkaloids are derived from tryptophan, which is formed in the shikimate pathway. In the case of the terpenoid indoles, tryptophan is usually first converted to tryptamine by the enzyme tryptophan decarboxylase (TDC) (Fig. 2.9). This enzyme occurs in the cytosol and has been detected in all parts of the developing seedling and in cell cultures of *C. roseus* (De Luca, 1993). It appears to be a pyridoxoquinoprotein, as two molecules of pyridoxal phosphate and two molecules of covalently bound pyrroloquinoline quinone were found per enzyme molecule (Pennings *et al.*, 1989). A *tdc* cDNA clone has been isolated by immunoscreening of a *C. roseus* cDNA expression library (De

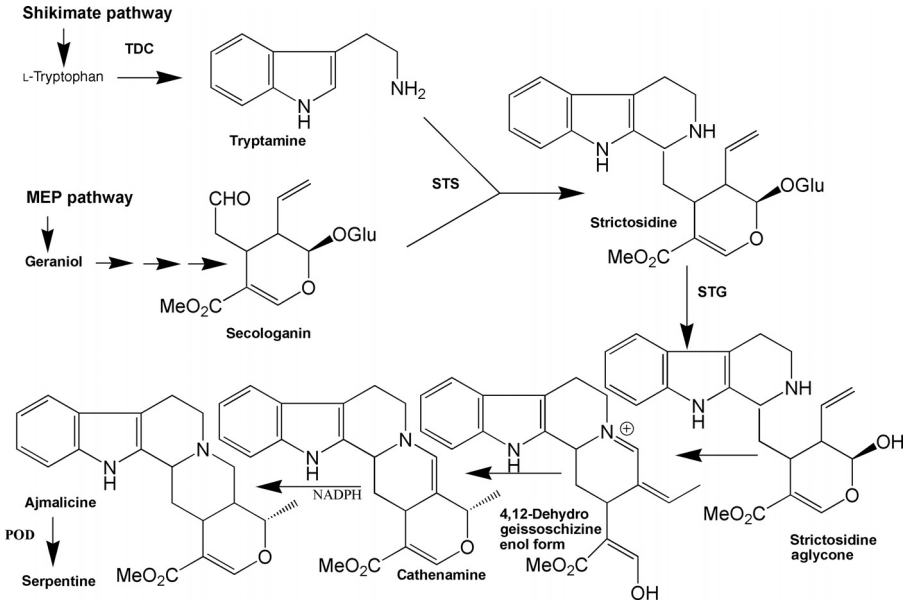


Figure 2.9 Enzymic formation of ajmalicine. TDC, tryptophan decarboxylase; STS, strictosidine synthase; STG, strictosidine glucosidase; POD, peroxidase.

Luca, 1993). Its identity was confirmed by expression in *E. coli* and *N. tabacum* (De Luca, 1993; Songstad *et al.*, 1990). TDC is capable of decarboxylating both L-tryptophan and L-tyrosine in vivo. The *tdc* occurred as a single copy in *C. roseus* and the protein, when isolated, was found to be similar to that found in parsley and the fruitfly, except that it was found to lack 13 *N*-terminal amino acids compared with TDC from these sources. This suggested a processing of TDC protein in *C. roseus* and *Camptotheca acuminata*, from which it has also been isolated (Goddijn, 1992). However, this cleaved form acts as a functional enzyme and confirms that TDC is a cytosolic enzyme (De Luca, 1993; Stevens *et al.*, 1993). The 13 *N*-terminal amino acids present in other TDC probably function as a signal peptide for membrane insertion or translocation.

Expression of *tdc* appears to be highly regulated at the transcriptional level. In plants, the highest steady-state *tdc* mRNA levels were observed in roots (Pasquali *et al.*, 1992). In seedlings, the appearance of the *tdc* mRNA was shown to be under developmental control (Roewer *et al.*, 1992), since the gene was UV-inducible, downregulated by auxin and induced by fungal elicitors (Goddijn *et al.*, 1992; Pasquali *et al.*, 1992). The short half-life of *tdc* mRNA (1 h) is another indication that the gene may represent an important regulatory point in alkaloid biosynthesis. TDC protein also has a short half-life (21 h) in vivo, and in developing seedlings protein degradation and transcriptional regulation seem to be important controlling factors (Fernandez *et al.*, 1989). Finally, feedback regulation by tyramine could be another

mechanism of regulatory control at the level of TDC (Eilert *et al.*, 1987). The *tdc* cDNA driven by the strong cauliflower mosaic virus 35S promoter was introduced into *C. roseus* using *Agrobacterium tumefaciens*. Overexpression did not appear to result in an increase in alkaloid accumulation but enhanced the TDC protein level, TDC activity and tyramine content. Therefore, TDC is not the only rate-limiting step in alkaloid biosynthesis.

Tryptamine condenses with the monoterpene, *seco*-loganin, which is derived from geraniol or nerol in the MEP pathway (starting with glyceraldehydes-3-phosphate and pyruvate; see Chapter 5) by hydroxylation at C-10 with retention of configuration (Fretz and Woggon, 1986; Fretz *et al.*, 1989; Facchini and De Luca, 2008). The enzyme responsible for this latter reaction is a membrane-bound cytochrome P450-requiring hydroxylase, which was first characterized from *C. roseus* and found with low activity in cell cultures of that plant. Plant cell cultures have been used for further investigation of this enzyme, which appears to have a regulatory effect on alkaloid production; its activity pattern being more closely related to the pattern of indole alkaloid accumulation than that of TDC. Meijer and co-workers (1993a) found that the NADPH:cytochrome P450 reductase is probably encoded by a single copy gene in the *C. roseus* genome, indicating that all cytochrome P450 enzyme activity in this plant is dependent on the same reductase enzyme. Steady-state mRNA levels for this reductase observed in *C. roseus* were highest in the flowers, much lower in leaves and stems and intermediate in roots.

In cell cultures, the expression of the reductase mRNA, like the *tdc* and strictosidine synthase (*sts*) genes, was found to be induced by elicitors and downregulated by auxins. G10H was found to be localized in provacuolar membranes and not in the endoplasmic reticulum like many other cytochrome P450 enzymes. Interestingly, this enzyme is inhibited by the end product, alkaloid catharanthine, but not by vindoline and vinblastine. Therefore, feedback regulation may also operate in vivo, provided that the catharanthine and G10H are within the same cellular compartment (Facchini and De Luca, 2008).

A regulatory role for geraniol-10-hydroxylase (G10H; CYP76B6) was first proposed by Schiel and co-workers (1987), who observed an increase in the activity of this enzyme when cells were placed in an alkaloid-producing medium. The distribution of G10H in *Catharanthus* was reported by Burlat *et al*. (2004). The intermediate accumulation of tryptamine and its later incorporation into indole alkaloids, such as ajmalicine, indicated that the coordination of the two precursor pathways for monoterpene indole alkaloid formation are not synchronized (Schiel *et al.*, 1987). The most recent studies have suggested that loganic acid is synthesized from 10-hydroxynerol via 7-deoxyloganic acid by a route involving 10-oxogeraniol, 7-deoxyloganetic acid (Ziegler and Facchini, 2008). The methyltransferase (LAMT) required for the formation of *seco*-loganic acid from loganic acid has been partially purified from young *C. roseus* seedlings (Meijer *et al.*, 1993b and references therein; Facchini and De Luca, 2008).

2.5.2 Formation of (*S*)-strictosidine

Stereospecific condensation between tryptamine and *seco*-loganin in a Mannich-like reaction is carried out by the enzyme (*S*)-strictosidine synthase and results in the formation of the glucoalkaloid, (*S*)-strictosidine, from which most monoterpene indole alkaloids are derived (Figs. 2.1 and 2.9).

Isolation of the stereospecific strictosidine synthase (STS) and formation of strictosidine with the 3α-(*S*) configuration proved conclusively that this was the natural precursor of the terpenoid indole alkaloids. Strictosidine occurs naturally in *Rhazya stricta* and the synthase has been isolated from a number of other species: *Amsonia salicifolia*, *A. tabernaemontana*, *Catharanthus pusillus*, *C. roseus*, *Rauvolfia verticillata*, *R. vomitoria*, *R. serpentina*, *Rhazya orientalis* and *Voacanga africana*. The enzyme has been purified to homogeneity from *R. serpentina* (Hampp and Zenk, 1988). A comparison of the activity of STS from *C. roseus* roots, the only portion of the plant to contain ajmalicine, with that present in plant cell cultures producing the same alkaloid demonstrated that the plant cell cultures are far more metabolically active (Ziegler and Facchini, 2008).

STS has a number of isoforms, but the physiological significance of this is not yet obvious (Pfitzner and Zenk, 1989). However, it has been demonstrated that *sts* occurs as a single copy gene in *C. roseus*, indicating that the reported isoforms of STS result from posttranslational modification of a single precursor (Pasquali *et al.*, 1992).

The cDNA for STS has now been expressed in an enzymatically active form in *E. coli*, *Saccharomyces cereviseae* and cell cultures of the insect *Spodoptera frugiperda* (Kutchan, 1989; Kutchan *et al.*, 1991). Modified cDNA encoded STS from *C. roseus* has been introduced into tobacco plants. Transgenic tobacco plants expressing this construct had 3–22 times greater STS activity than *C. roseus* plants. Ultrastructural immunolocalization demonstrated that STS is a vacuolar protein in *C. roseus* and is correctly targeted to the vacuole in transgenic tobacco (McKnight *et al.*, 1991). Comparison of the terminal amino acid sequence of purified STS with the protein sequence deduced from *sts* mRNA indicated that the primary translation product contained a signal peptide of 31 amino acids, which appeared to be essential for vacuolar targeting (Pasquali *et al.*, 1992; McKnight *et al.*, 1991).

2.5.3 Deglucosylation of strictosidine

Deglucosylation of strictosidine, a key reaction in the formation of the many types of indole alkaloids, is carried out by a specific glucosidase, strictosidine-β-D-glucosidase (SGD) (Fig. 2.9). The protein and cDNA have been isolated from *C. roseus* and a number of other indole alkaloid-containing plants of the Apocynaceae. The specific glucosidase is involved in an essential initial reaction that leads to a complex sequence of events and a series of highly reactive intermediates. When glucose is split off, the hemiacetal opens and exposes

an aldehyde group, which can react with the secondary amine function to form a Schiff base. Allylic isomerization, i.e. moving the double bond of the vinyl side chain into conjugation with the iminium, leads to dehydrogeissischizine and cathenamine. Overexpression of STS and SGD in yeast leads to the formation of cathenamine (Geerlings *et al*., 2001).

Geissoschizine, which is formed from these reactive intermediates, is converted via geissoschizine dehydrogenase to 4,21-dehydrogeissoschizine. The enzyme that removes the 21α-hydrogen of geissoschizine in an $NADP^+$-dependent reaction has been partially purified from *C. roseus* cell suspension cultures. This enzyme is not thought to be directly involved in ajmalicine production, rather it ensures that geissoschizine is fed back into the pathway. However, geissoschizine and 4,21-dehydrogeissoschizine are key substances in the formation of the Corynanthean (ajmalicine), Sarpagan (ajmaline), Ibogan (catharanthine) and Aspidosperma (vindoline) alkaloids (Stöckigt *et al.*, 1992; Meijer *et al.*, 1993b; Zenk and Juenger, 2007; Facchini and De Luca, 2008).

2.5.4 Formation of corynanthe-type alkaloids

Ajmalicine, 19-epi-ajmalicine and tetrahydroalstonine are formed from 4,21-dehydrogeissoschizine via cathenamine (Fig. 2.9). The enzymatic synthesis of these corynanthe-type alkaloids has been investigated using *C. roseus* cell suspension cultures, and the enzymes involved have been reviewed by De Luca (1993) and Ziegler and Facchini (2008). Ajmalicine can be oxidized by POD to serpentine. This reaction may take in the vacuole.

2.5.5 Formation of sarpagan-type alkaloids

Ajmaline is an antiarrhythmic alkaloid from *Rauvolfia serpentina*. Vinorine and ajmaline and the related alkaloid glucoside, raucaffricine, are also formed via a series of enzymatic steps from 4,21-dehydrogeissoschizine (Fig. 2.10). The complete pathway leading to ajmaline has been characterized by J. Stöckigt and co-workers at the enzymic and partly gene levels (review: Ruppert *et al*., 2005). Four enzymes, among them STS, could be crystallized and the X-ray structures could be described in detail (Stöckigt *et al*., 2007). The knowledge of the active centre in the key enzymes may allow a rational enzyme design, leading to enzymes with new affinities (McCoy and O'Connor, 2006; Zenk and Juenger, 2007).

The step from 4,21-dehydrogeissoschizine (keto form) to the sarpagan structure has been verified at the enzyme level; recent studies have shown that the sarpagine bridge in polyneuridine aldehyde (PNA) (Fig. 2.10) is formed by a microsomal enzyme that requires NADPH and oxygen. Inhibition studies have indicated a cytochrome P450-dependent monooxygenase (Schmidt and Stöckigt, 1995). From structural similarities and the next enzyme in the sequence, it has been proven that PNA is one of the stable intermediates at the beginning of this route. The enzyme that acts on the aldehyde has been well

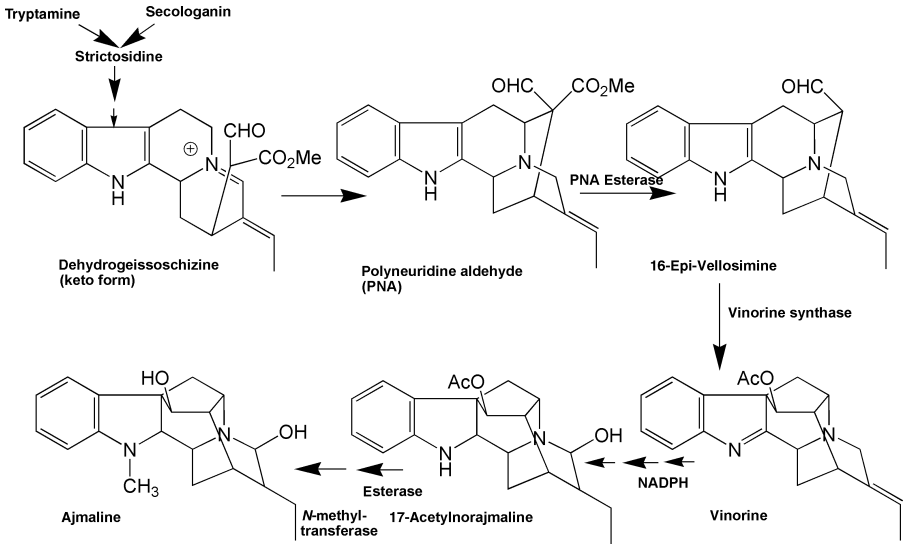


Figure 2.10 Formation of ajmaline. NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); CoA, coenzyme A.

characterized from *R. serpentina* cells and is the specific methylesterase, PNA esterase; the product of the reaction, polyneuridine acid, is highly unstable and decarboxylates to give 16-epi-vellosimine (Stöckigt *et al.*, 1992; Ruppert *et al*., 2005), which has the correct stereo-requirement for the formation of the ajmaline-type bond (Schmidt and Stöckigt, 1995).

The next step in the sequence to ajmaline is catalysed by the enzyme, vinorine synthase. Vinorine, a constituent of *Rauvolfia* cell cultures, is an acetylated indolenine alkaloid. Vinorine synthase has a requirement for AcCoA as cosubstrate. The acetyl unit has a stabilizing effect on the indolenine structure. Vinorine is hydroxylated to vomilenine (21-OH-vinorine) by a cytochrome P450-dependent hydroxylase (Falkenhagen and Stöckigt, 1995). This intermediate product is then converted to ajmaline via a series of enzymatic reactions. First of the sequence is the reduction by an NADPH-requiring reductase to 1,2-dihydrovomilenine, followed by further reduction, also NADPH requiring, to 17-acetylnorajmaline. Deacetylation proceeds with the aid of acetylesterase (specific for the 2β(*R*) configuration) to give norajmaline (Polz *et al.*, 1986; Facchini and De Luca, 2008). It has high substrate selectivity and exclusively accepts acetylated ajmaline derivatives with the naturally occurring 2β(*R*) configuration. The highest enzyme activities were observed in leaves and cell suspension cultures of the tribe Rauvolfieae, which are known to synthesize ajmaline and its congeners. Finally, *N*-methylation occurs to complete the sequence with the production of ajmaline (Fig. 2.10).

In *R. serpentina* cell cultures, vomilenine is converted to its glycoside, raucaffricine, and this has a very significant effect on ajmaline production. In

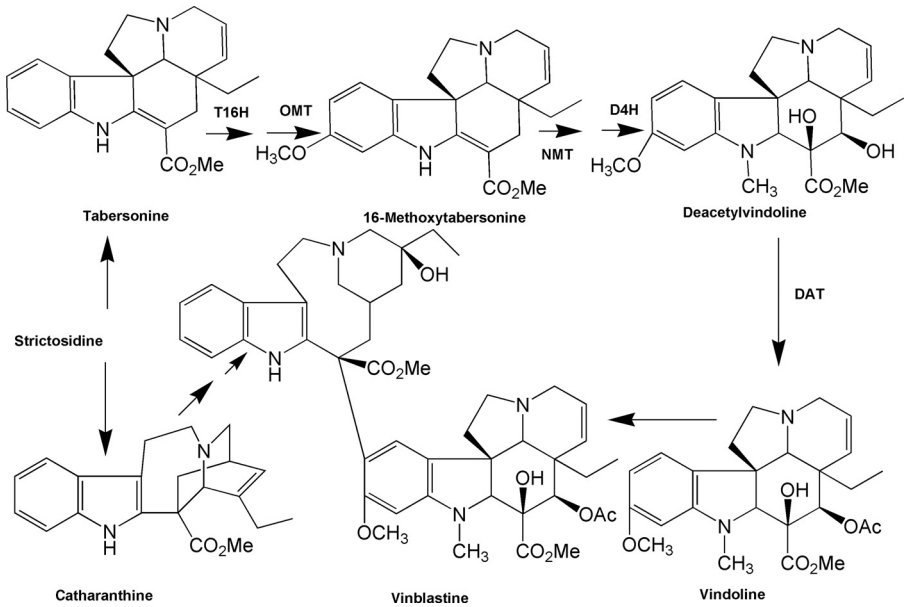


Figure 2.11 Biosynthesis of vindoline, catharanthine and the dimeric alkaloids vinblastine and vincristine. T16H, tabersonine-16-hydroxylase; OMT, *S*-adenosylmethionine:16-hydroxy-tabersonine-*O*-methyltransferase; NMT, *S*-adenosylmethionine:16-methoxy-2,3-dihydro-3-hydroxymethyltabersonine-*N*-methyltransferase; D4H, desacetoxy-vindoline-4-dioxygenase; DAT, acetylcoenzyme A:4-*O*-deacetylvindoline-4-*O*-acetyltransferase.

R. serpentina cell cultures, raucaffricine levels amounted to 1.2 g/L medium, whereas ajmaline levels reached only 0.3 g/L medium (Schübel *et al.*, 1986). It is interesting to note that raucaffricine is a typical constituent of *R. caffra*, but has not been isolated from other *Rauvolfia* species. However, in *Rauvolfia* cell cultures the compound is found in all species tested, with a maximum yield in *R. serpentina*. In other words, under these growth conditions, the pathway to ajmaline appears to have become deregulated.

2.5.6 Formation of aspidosperma-type alkaloids

Studies performed with *C. roseus* seedlings have suggested that the route from tabersonine to vindoline proceeds by the sequence shown in Fig. 2.11. More than 30 enzymes appear to be involved (van der Heijden *et al.*, 2004; Facchini and De Luca, 2008; Ziegler and Facchini, 2008).

Tabersonine is hydroxylated at C-16, followed by methylation and hydration of the 2,3 double bond, *N*(1)-methylation, hydroxylation at C-4 and 4-O acetylation. Hydroxlation of tabersonine at C-16 requires a cytochrome P450-mediated monooxygenase (T16H with CYP71D12). This enzyme was found to be located in the endoplasmic reticulum, was at maximal activity

in seedlings at day 9 postimbibition and was induced by light. The leaf-specific distribution of this enzyme in the mature plant is consistent with the localization of the other enzymes (St-Pierre and De Luca, 1995). The methyltransferase (OMT) required for the formation of 16-methoxytabersonine from tabersonine and desacetoxyvindoline from 16-methoxy-2,3-dihydro-3-hydroxytabersonine (16OMT) have now been isolated and partially purified (Fahn and Stöckigt, 1990; Dethier and De Luca, 1993; Facchini and De Luca, 2008). These first two steps in vindoline biosynthesis appear to comprise the only enzymes also found in plant cell cultures. Substrate-specificity studies confirm that hydroxylation at C-3 and *N*-methylation is required prior to hydroxylation at position 4 to convert desacetoxyvindoline to deacetylvindoline (De Luca, 1993). The C-4 hydroxylation of 2,3-dihydro-3-hydroxy-*N*(1)-methyltabersonine (desacetoxyvindoline) to the 3,4-dihydroxy derivative, deacetylvindoline by desacetoxyvindoline-4-hydroxylase (D4H), utilizes an enzyme that has an absolute requirement for 2-oxyglutarate. Enzymatic activity was enhanced by ascorbate, establishing that the enzyme involved is a 2-oxyglutarate-dependent dioxygenase. This enzyme is specific for position 4 of various alkaloid substrates and has recently been cloned and characterized (Vazquez-Flota *et al.*, 1997). The appearance of 4-hydroxylase activity was shown to be developmentally regulated and is inducible by light treatment of seedlings.

The final step in the formation of vindoline is the acetylation of 4-*O*-deacetylvindoline by a 4-*O*-acetyltransferase (DAT). This enzyme has been purified to homogeneity from *C. roseus* leaves (De Luca, 1993; Facchini and De Luca, 2008).

A summary of studies with heterologuous expression of MIA genes is given in Marasco and Schmidt-Dannert (2007).

2.5.6.1 Developmental control and tissue specificity

Seedlings grown in the dark produced an early accumulation of tabersonine as a major alkaloid. Transfer of 5-day-old seedlings to the light resulted in the rapid loss of vindoline precursors followed by a more gradual disappearance of tabersonine and the subsequent enhancement of vindoline accumulation. Although light enhanced vindoline biosynthesis, it was not essential (Aerts and De Luca, 1992). The time course of induction indicated that an increase of TDC coincided with tabersonine accumulation, whereas increase of AcCoA:deacetylvindoline-*O*-acetyltransferase activity coincided with vindoline accumulation. Results with young seedlings suggested that the enzymes of the tabersonine biosynthetic pathway occur in all plant parts, whereas the last five steps in vindoline biosynthesis are restricted to aerial parts of the plant, and that the whole pathway to vindoline biosynthesis is developmentally regulated (De Luca, 1993). Vindoline accumulation is light dependent and influenced by jasmonate (Vazquez-Flota and De Luca, 1998).

Further investigations of some of the enzymes involved in vindoline production, using young seedlings of *C. roseus*, showed that while TDC, STSSTS,

N-methyltransferase (NMT) and *O*-acetyltransferase (DAT) activities appeared early in seedling development, TDC activity was highly regulated and peaked over a 48-h period, achieving a maximum by day 5 postimbibition. Both TDC and STS were present in all tissues of the seedlings. NMT and DAT enzyme activities were induced after TDC and STS had peaked, and these activities could only be found in hypocotyls and cotyledons. TDC, STS and NMT did not require light for induction and DAT enzyme activity increased approximately tenfold after light treatment of dark-grown seedlings (De Luca, 1993). TDC, STS and DAT were found to be cytoplasmic enzymes, but NMT was found in the chloroplasts associated with the thylakoid. The participation of the chloroplast in this pathway suggests that the indole alkaloid intermediates enter and exit the compartment during vindoline synthesis (De Luca, 1993). Furthermore, both enzymes and substrates require substantial intra- and intercellular translocations (Burlat *et al*., 2004). A hypothesis for the compartmentation of terpenoid indole alkaloid biosynthesis in *C. roseus* was given in a paper by Meijer and co-workers (1993b) (Fig. 2.12). More elaborate models of the complex interactions of different tissues were presented by Murata *et al*. (2008) and Facchini and De Luca (2008) (Fig. 2.13).

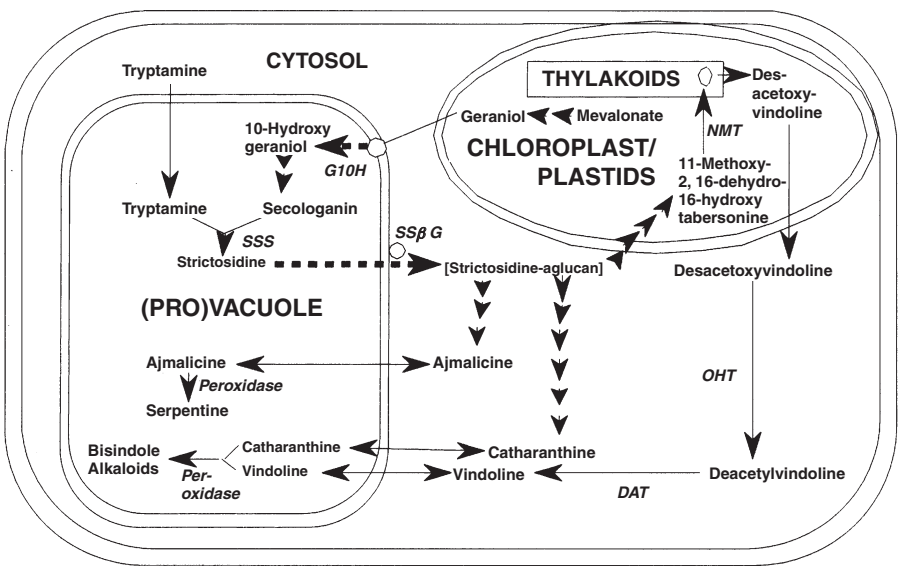


Figure 2.12 A hypothetical view of compartmentation of indole alkaloid biosynthesis in *Catharanthus roseus*. Enzymes located with dashed arrows are hypothetical and circles indicate membrane associated enzymes (after Meijer *et al.*, 1993b). G10H, geraniol-10-hydroxylase; NMT, *S*-adenosyl-L-methionine:11-methoxy 2,16-dihydro-16-hydroxytabersonine *N*-methyltransferase; DAT, acetylcoenzyme A: deacetylvindoline 17-*O*-acetyltransferase; OHT, 2-oxyglutarate-dependent dioxygenase; SSβG, strictosidine-(β)-glucosidase; SSS, strictosidine synthase.

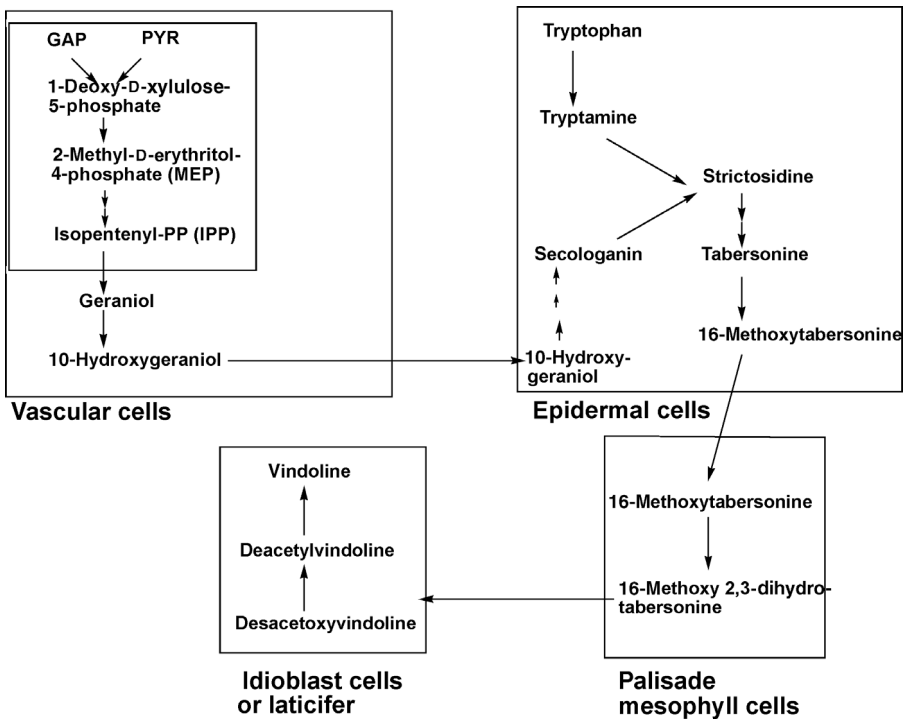


Figure 2.13 Putative localization and intercellular trafficking of monoterpene indole alkaloids in *Catharanthus roseus*. (After Facchini and De Luca, 2008; Murata *et al*., 2008.)

Using cDNA–AFLP, transcript profiling of most known genes involved in TIA biosynthesis were studied in *C. roseus* in response to elicitation (Rischer *et al*., 2006). A total of 417 differentially expressed transcript tags were discovered; the majority represented new sequence information and 37% were not similar to any known plant gene. In addition, a metabolic network was established linking metabolites and gene expression profiles (Rischer *et al*., 2006; Oksman-Caldentey *et al*., 2007).

2.5.7 Dimeric indole alkaloids

Catharanthine and vindoline condense to form the dimeric alkaloids, vincristine and vinblastine (Fig. 2.11) (for a discussion of this earlier work see Meijer *et al*., 1993b). Catharanthine has to undergo ring opening and ring closure and hydroxylation during this procedure which is thought to be catalysed by a POD (Dewick, 2002). Whilst these dimeric alkaloids are not produced in unorganized cell cultures, they have been found to occur in multiple shoot cultures (Miura *et al*., 1988). Cell-free extracts from *C. roseus* will convert [2–^{14}C]tryptophan and *seco*-loganin to vindoline (Kutney, 1987).

Furthermore, the same cell-free extracts will also couple vindoline and catharanthine to yield the dimeric 3′,4′-anhydrovinblastine, which forms the natural dimeric alkaloids, leurosine, catharine and vinblastine (Fig. 2.11) (Kutney, 1987). The enzyme, which apparently brings about the coupling appears to be a POD (Endo *et al.*, 1986; Goodbody *et al.*, 1988). A commercial method for production of vincristine depends on the efficient conversion of 3′,4′-anhydrovinblastine to vinblastine, which is yet to be achieved.

2.6 Ergot alkaloids

The fungus, *Claviceps purpurea*, normally lives on rye and other cereals, contains alkaloids of a type that is derived, like other indole alkaloids, from tryptophan and, because it is important as a medicinal agent, it has been extensively studied. The interaction between a fungus and host plants appears to be symbiotic, as the plants take advantage from the neurotoxic alkaloids to defend themselves against herbivores and, as a sort of payment, feed the fungus with nutrients (Markert *et al.*, 2008 and references therein).

The naturally occurring ergot alkaloids can be divided into two classes on the basis of their chemical structure: the lysergic acid derivatives and the clavine alkaloids. They all possess the tetracyclic ergoline system. In addition to the sclerotia of *Claviceps*, other fungi and several higher plants can contain ergot alkaloids; some examples of fungi are *Aspergillus fumigatus*, *Rhizopus arrizus*, *Penicillium roqueforti* and *Sclerotium dephinii*, and some examples of higher plants are *Rivea corymbosa* and *Ipomoea tricolor*. The alkaloids from these sources are restricted to low yields and, therefore, for practical purposes *Claviceps* remains the only commercial source. *C. purpurea* in submerged culture is now used to obtain ergotamine and ergocryptine commercially. The ergot alkaloids found in plants are apparently produced by an endophytic fungus that infects the plants (Steiner *et al.*, 2006) (for a wider discussion, see Chapter 7).

The formation of ergot alkaloids from L-tryptophan is well-known (Herbert, 1989; Groeger and Floss, 1998; Markert *et al.*, 2008). L-Tryptophan condenses with dimethylallylpyrophosphate (DMAPP) to give γ,γ-dimethylallyltryptophan (DMAT), which is modified via chanoclavine I to give agroclavine and finally elymoclavine (Fig. 2.14). Lysergic acid may be formed from this last alkaloid.

The amide portion of the alkaloid may be a smaller peptide or simple alkylamide, the basic skeleton being called an ergopeptide. Peptidic ergot bases contain lysergic acid and an amide portion is reduced to a tricyclic ring system. In the clavine alkaloids, the carboxyl group at C-17 is converted to a group with a lower oxygen state (Fig. 2.14).

The enzyme responsible for the first step in the biosynthesis of these alkaloids is DMAT synthase. This enzyme, which brings about the condensation of L-tryptophan with DMAPP, has been isolated from *C. purpurea* cultures,

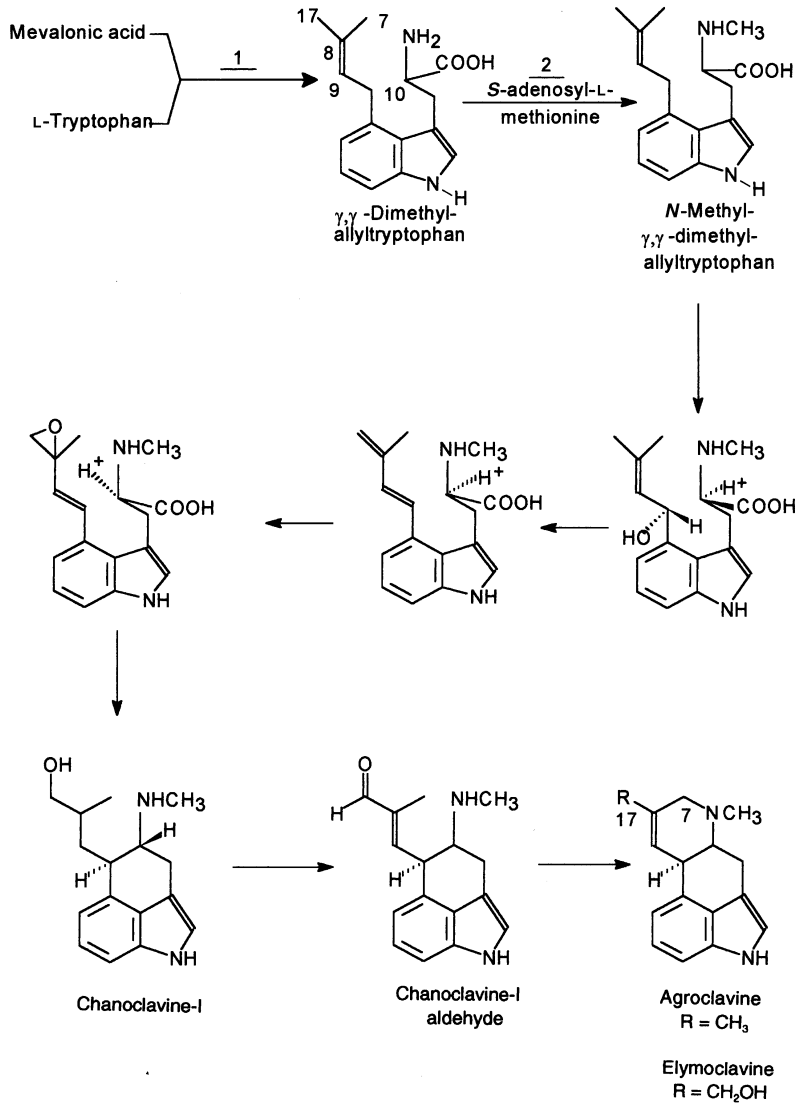


Figure 2.14 Biosynthesis of agroclavine and elymoclavine in *Claviceps purpurea* cultures.

purified and characterized. The mechanism by which the enzyme works has been probed using a set of analogues of DMAPP and L-tryptophan; it was concluded that the reaction was an electrophilic aromatic substitution similar to that catalysed by farnesylpyrophosphate synthase. There is a feedback mechanism operative, with inhibition of the enzyme by elymoclavine (Shibuya *et al.*, 1990; Gebler and Poulter, 1992; Gebler *et al.*, 1992). The DMAT

formed is methylated using the methyl group of *S*-adenosylmethionine; the activity of the enzyme in cultures roughly parallels that of other ergoline enzymes and has been isolated, purified and characterized. This is, therefore, the second pathway-specific step; however, further reactions are required in the isoprenoid side chain before C-ring formation can take place. DMAT synthase has been cloned from *Claviceps*, *Neotyphodium* and *Aspergillus*; the gene appears to be clustered with other genes of ergot alkaloid biosynthesis in the genome of Claviceps (Tydzynski *et al*., 2001; Haarmann *et al*., 2005; Fleetwood *et al*., 2007).

In the conversion of *N*-methyl–DMAT to give chanoclavine I, there is a potential gap in existing knowledge. The oxygen atoms of both elymoclavine and chanoclavine I are derived from molecular oxygen (Kobayashi and Floss, 1987). Chanoclavine aldehyde was found as a natural constituent of a blocked mutant strain of *C. purpurea*, which strongly suggested that it was an intermediate on the route to the tetracyclic ergolines. Conformation at the enzyme level is required to validate this hypothesis. Elegant experiments with radio-labelled precursors suggested that two *cis–trans* isomerizations occur during the conversion of *N*-methyl–DMAT, by ring closure, to agroclavine. The most recent experiments on the formation of the C-ring utilizing deuterated intermediates suggest that the incorporation of *N*-methyl–DMAT into chanoclavine I is via a mechanism that involves C-10 hydroxylation, followed by 1,4-dehydration and epoxidation at C-7, the terminal double bond of the resulting diene (Fig. 2.14). The epoxide can then cyclize with simultaneous decarboxylation and attack of the resulting C-5 anion on C-10 followed by epoxide ring opening to give chanoclavine I. Whether the decarboxylation occurs in concert with ring closure, as seems most plausible, or as a separate step remains to be determined (Kozikowski *et al.*, 1993).

Chanoclavine I cyclase catalyses the conversion of chanoclavine I and/or chanoclavine I aldehyde to agroclavine and/or elymoclavine. A requirement for NAD or NADP has been observed. The enzyme's appearance and decline in cultures resembled that of DMAT synthase. The conversion of agroclavine to elymoclavine has been achieved with a cell-free preparation. This enzyme, a microsomal hydroxylase, is NADPH requiring and the lack of inhibition by ethylenediamine tetra-acetic acid (EDTA) and cyanide suggests a cytochrome P450 monooxygenase. The enzyme had great activity during maximum alkaloid production (Kim *et al.*, 1981).

Elymoclavine is the precursor of lysergic acid, although the exact mechanism of formation is still unclear. It is assumed that a double-bond shift from $\Delta^{8,9}$ to $\Delta^{9,10}$ occurs at the aldehyde stage and this was confirmed with feeding experiments with the enol acetate of lysergic aldehyde (Fig. 2.15). To form the amide alkaloids, it is suggested that activation of lysergic acid as lysergyl-CoA is required, but this remains controversial. A particulate fraction isolated from an ergotamine-producing strain of *C. purpurea* converted elymoclavine to paspalic acid. NADPH was required as was cytochrome P450. A particulate system has also been isolated that converts elymoclavine directly to

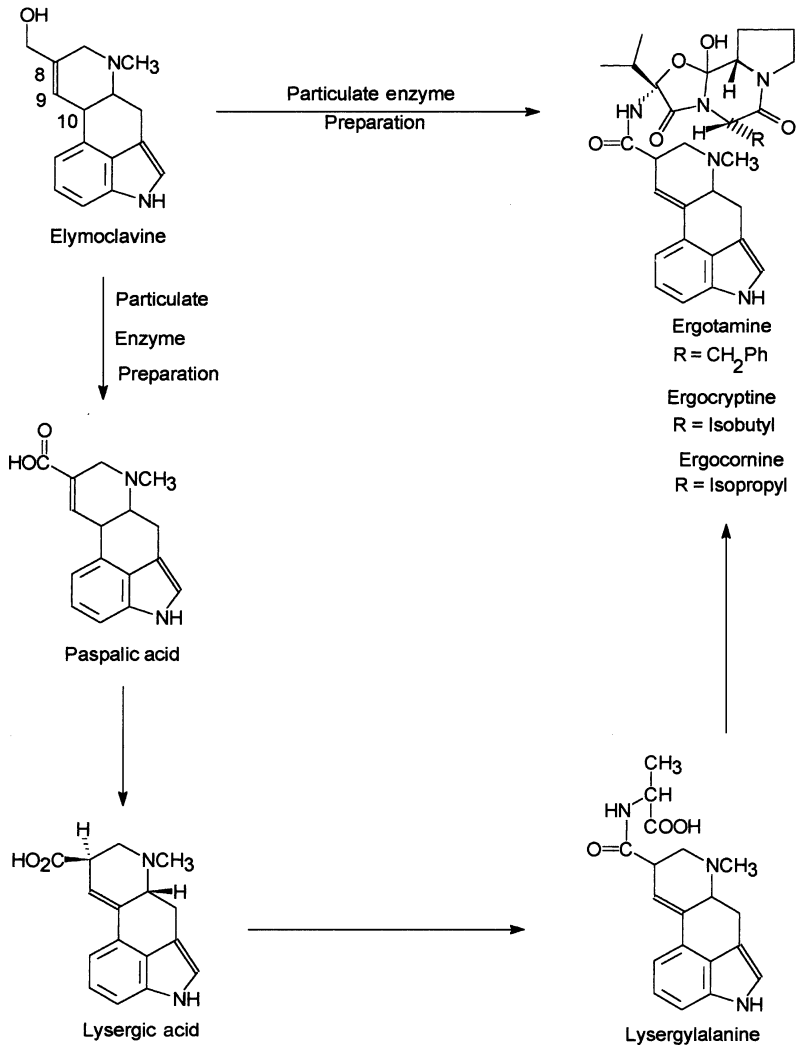


Figure 2.15 Formation of ergotamine alkaloids from elymoclavine.

ergotamine (Maier *et al.*, 1988), suggesting that under normal circumstances paspalic acid is not a free intermediate (Fig. 2.15). This is also borne out by the fact that $^{18}O_2$ is incorporated equally into the carbonyl oxygen of lysergic acid and the oxygen attached to the α-carbon of the alanine of ergotamine. The peptide ergot alkaloids have rather complex structures (Fig. 2.15). The formation of the modified peptide portion of ergotamine involves the conversion of the α-amino acid alanine into the corresponding α-hydroxy-α-amino acid moiety, which then reacts with the carboxyl group of proline to give the unique cyclol structure (Fig. 2.15). This transformation is thought to occur

after the formation of the entire lysergyl-tripeptide, i.e. ergotamine (lysergyl-alanyl-phenylalanyl-proline), ergocornine (lysergyl-valyl-valyl-proline) and ergocryptine (lysergylalanyl-aminobutyryl-proline).

There are many gaps in our knowledge of the biosynthesis of these alkaloids, despite elegant research using radio-labelled precursors. There is now a real need for more of the enzymes of these sequences to be isolated, so that improvements to commercial production may be effected. Real progress came to the field by cloning of DMAT synthase and other pathway genes (Tydzynski *et al*., 2001; Haarman *et al*., 2005; Fleetwood *et al*., 2007).

2.7 Acridone alkaloid biosynthesis

Acridone alkaloids comprise a relatively small group of alkaloids that are found solely in some Rutaceae genera. Some 100 examples of this alkaloid group have been isolated and these include the monomeric acridones and the acridone–coumarin dimers (acrimarines) isolated from *Citrus* plants; some binary alkaloids have recently been isolated and described (Takamura *et al.*, 1995).

The monomeric acridone alkaloids are derived from anthranilic acid and acetate via a polyketide. First studies, in which [^{13}C]-acetate was utilized by cell cultures of *Ruta graveolens*, indicated that the C-ring of the acridone nucleus was acetate derived. Further research revealed that anthranilic acid is specifically incorporated into the A-ring of rutacridone (Baumert *et al.*, 1982).

Cell-free extracts of *R. graveolens* convert anthranilic acid into *N*-methylanthranilate utilizing *S*-adenosyl-L-methionine and a methyltransferase, which has recently been isolated and partially purified from *R. graveolens* cell cultures (Maier *et al.*, 1995). This is the first committed step in the biosynthesis of the rutacridones. The formation of *N*-methylanthraniloyl-CoA from anthranilate utilizing a CoA-ligase (Baumert *et al*., 1985, 1992) made it possible to study the enzyme that catalyses the condensation of *N*-methylanthraniloyl-CoA with malonyl-CoA. The product of this reaction, 1,3-dihydroxy-*N*-methylacridone, leads directly to the more complex acridones, such as rutacridone (Fig. 2.16) (Baumert *et al.*, 1994). This enzyme has been purified to homogeneity from *R. graveolens*. Complementary deoxyribonucleic acid has been isolated from clones harbouring acridone synthase and is introduced into *E. coli*, where high acridone synthase activity was expressed. An insert of roughly 1.4 kb encoded the complete acridone synthase and, although this enzyme expressed no chalcone synthase activity, alignments at both DNA and protein levels corroborated a high degree of homology to chalcone synthase (Junghanns *et al.*, 1995).

Synthesized 1,3-dihydroxy-*N*-methylacridone is readily incorporated into rutacridone by cell-free extracts of *Ruta graveolens* (Maier *et al.*, 1993). It has been hypothesized that the final step in the biosynthesis of these alkaloids

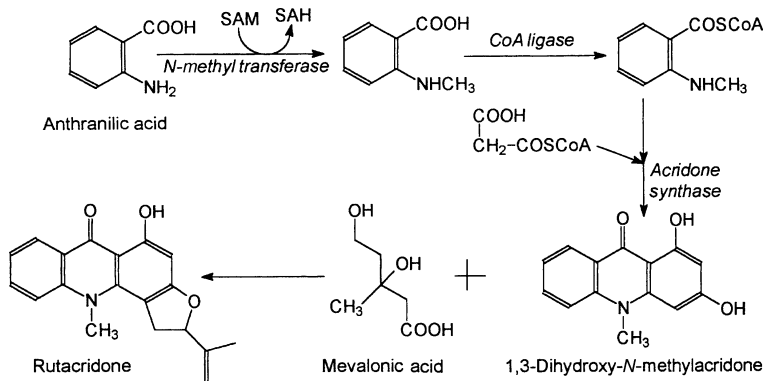


Figure 2.16 Biosynthesis of rutacridone in *Ruta graveolens*. SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine.

requires mevalonic acid (Baumert *et al.*, 1982). The enzyme involved in this step is yet to be elucidated.

The *Ruta* alkaloids are usually found in idioblasts and early experiments using fluorescent microscopy, and *Ruta graveolens* showed heavy deposits of acridone alkaloids in the xylem (Wink and Roberts, 1998).

2.8 Purine alkaloids

The biosynthetic pathway from primary metabolism to caffeine is considered to start with the methylation of xanthosine, yielding 7-methylxanthosine. After deribosylation, the resulting 7-methylxanthine is further methylated to theobromine and finally to caffeine (Schulthess and Baumann, 1995; Ashihara and Suzuki, 2004; Ziegler and Facchini, 2008). The *N*-7-methyltransferase required for the methylation of xanthosine, the key enzyme in caffeine biosynthesis, has recently been isolated (Waldhauser *et al.*, 1997a). The enzymes responsible for the *N*-3 and *N*-1 methylations of 7-methylxanthosine to yield theobromine and caffeine, respectively, have also been isolated. The changes in levels of these enzymes, as well as of theobromine and caffeine, during leaf expansion indicated that each methylation in the sequence required a separate enzyme and these have now been partially separated (Waldhauser *et al.*, 1997b) (Fig. 2.17). The results suggest a role for these purine alkaloids in defence mechanisms that are strongly correlated with leaf emergence and expansion. In tea (*Camellia sinensis*) the final two methyltransferase steps are carried out by a bifunctional enzyme 'caffeine synthase' (Ashihara and Crozier, 1999). The three methyltransferases (i.e. 7-methylxanthosine synthase, theobromine synthase and caffeine syntahse) have been cloned and recombinantly expressed in *E. coli* (for a review see Marasco and Schmidt-Dannert, 2007). The genes of the three synthases were combined and expressed in *E. coli*

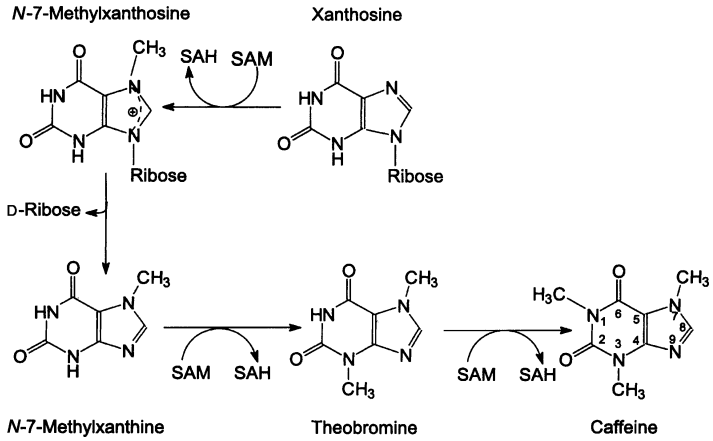


Figure 2.17 Biosynthesis of theobromine and caffeine in *Coffea arabica*. SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine.

and in tobacco in order to produce caffeine (Uefuji *et al*., 2003). RNAi has been used to downregulate caffeine production in coffee plants (Ogita *et al*., 2004). XMT and DXMT have been characterized by X-ray crystallography (McCarthy and McCarthy, 2007).

Caffeine and related purines are uncharged under physiological conditions and, due to their dual hydrophilic and lipophilic character, easily penetrate cell-, tissue- and organ-related barriers. In *Coffea arabica*, compartmentation of purine alkaloids, e.g. caffeine, depends exclusively on the physical chemistry of their vacuolar complexation with chlorogenic acid (Waldhauser and Baumann, 1996).

The purine is synthesized and stored in large quantities in the seed. Directly after germination, caffeine remains in the cotyledons surrounding the endosperm and does not migrate to the hypocotyl or root. In older seedlings, caffeine accumulation continues during leaf expansion, and in the mature plant the fruits actively synthesize purine alkaloids as they mature (Aerts and Baumann, 1994).

2.9 Taxol

The novel diterpenoid, taxol (Fig. 2.18), is now well established as a potent chemotherapeutic agent, showing excellent activity against a range of cancers, including ovarian and breast cancers. The limited supply of the drug from the original source, the bark of the Pacific yew (*Taxus brevifolia*), prompted intensive efforts to develop alternative means of production from constituents in needles and plant cell culture. Total synthesis is not yet commercially viable and semi-synthesis of taxol and its analogue, taxotere, based on the availability of baccatin III (Fig. 2.18) and other taxane metabolites available from

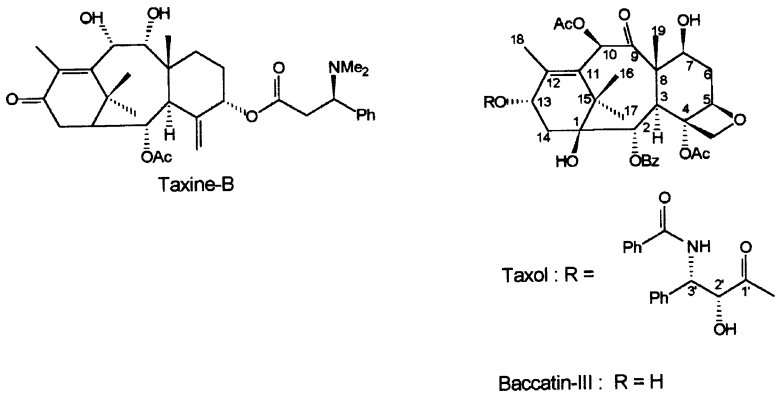


Figure 2.18 Taxine-B, taxol and baccatin III.

renewable natural sources (such as needles of *T. baccata*), has been developed as an interim measure. In considering future routes to these constituents through biotechnology, it is important to understand the pathway for taxol biosynthesis, the enzymes catalysing the sequence of reactions, especially the slow steps, and the genes encoding the proteins.

2.9.1 Biosynthesis of taxanes

Early work on taxanes has shown that there are several natural taxanes in which the structures analogous to the taxol C-13 side chain are esterified to

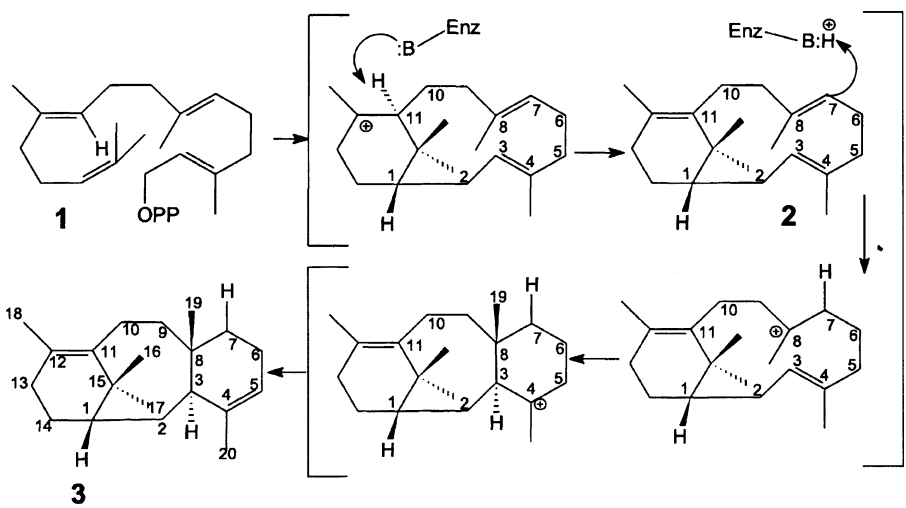


Figure 2.19 Stereochemical mechanism for the cyclization of geranylgeranyl diphosphate (**1**) via 1*S*-verticilline (**2**), as a transient intermediate, to taxa-4(5),11(12)-diene (**3**).

the 5-hydroxyl group of the diterpene moiety. This, together with the fact that the curvature of the molecule brings the C-13 hydroxyl group into close proximity with the C-5 position, led to the hypothesis that the side chain is first attached to the 5(4) position and is then transferred to the C-13 oxygen by intramolecular transesterification (Gueritte-Voegelein *et al.*, 1987). The side chain at C-13 has been found to be derived from phenylalanine by way of β-phenylalanine and phenylisoserine. Recent experiments with tritium and carbon-14-labelled baccatin III and the side-chain precursors showed baccatin III to be a precursor of taxol but cast serious doubt on Potier's transesterification theory (Fleming *et al.*, 1994).

The first committed step in the formation of taxol has been shown to involve the cyclization of geranylgeranyl diphosphate to taxa-4(5),11(12)-diene (Fig. 2.19). The formation of this endocyclic diterpene olefin isomer as a precursor of taxol was unexpected, since the exocyclic isomer, taxa-4(20),11(12)-diene, had been predicted as the initial product of the taxol pathway on the basis of metabolite concurrence. The cyclization of geranylgeranyl diphosphate variously labelled with tritium was accomplished using a partially purified taxadiene synthase from *T. brevifolia* stems. From this reaction involving the taxadiene synthase, a stereochemical mechanism has been proposed involving the initial cyclization of geranylgeranyl diphosphate to a transient veticillyl cation intermediate, with the transfer of the C11 α-proton to C7 to initiate transannular B/C-ring closure to the taxenyl cation, followed by deprotonation at C5 to yield the taxa-4(5),11(12)-diene product directly (Fig. 2.19) (Lin *et al.*, 1996). The taxane skeleton is further functionalized in a series of eight hydroxylations by CYP P450 enzymes, three CoA-mediated acylations (side chains) by specific transferases (Jennewein and Croteau, 2001; Walker and Croteau, 2001) (Fig. 2.20).

Concurrently, Eisnreich and co-workers (1996), using cell cultures of *Taxus chinensis* that produce the diterpene, 2α,5α,10β,14β-tetra-acetoxy4(20),11-taxadiene (taxuyunnanine C) (Fig. 2.19), in 2.6% (dry weight) yield, have suggested that the taxane carbon skeleton is not of mevalonoid origin. Experiments with ^{13}C-labelled glucose and acetate showed the following: (1) the four isopreneoid moieties of taxuyunnanine C have virtually identical labelling patterns, (2) a two-carbon unit and a three-carbon unit are diverted to the taxoid intermediate from glucose, (3) the connectivity of the three-carbon unit is disrupted by a skeletal rearrangement, but can still be diagnosed unequivocally by the analysis of long range ^{13}C–^{13}C coupling and (4) exogenous acetate contributes to the acetyl side chains of taxuyunnanine C, but not to the taxane ring system. Biosynthesis via the mevalonate pathway could explain neither the observed contribution of a three-carbon fragment from glucose to the diterpene nor the label distribution in the isoprenoid moieties.

The assembly of the isoprenoid moiety from a three-carbon fragment and a two-carbon fragment from glucose is reminiscent of the alternative isoprenoid pathway reported by Rohmer and co-workers (1993) in the

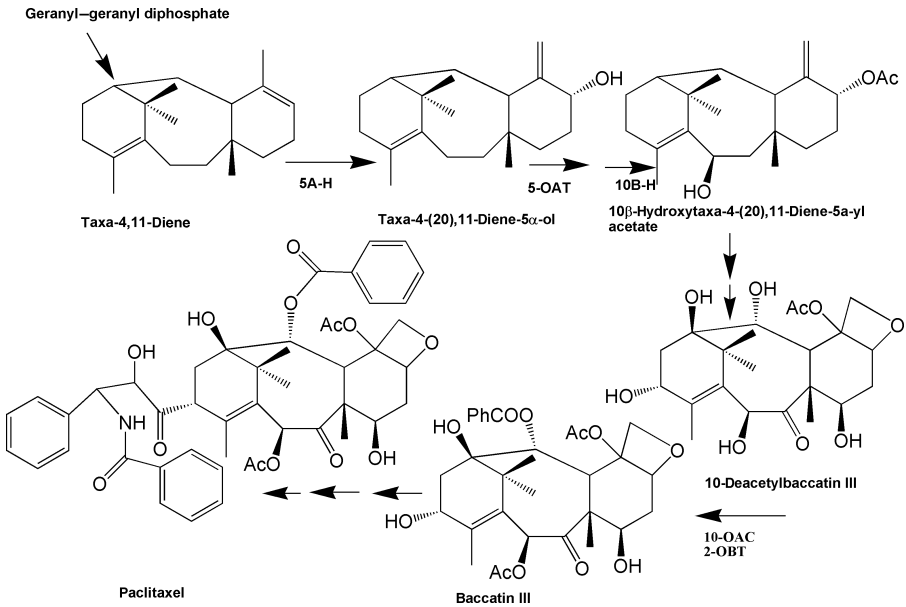
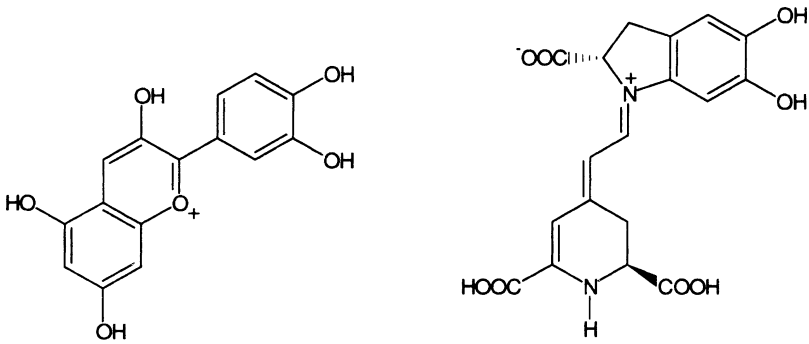


Figure 2.20 Biosynthetic pathway from taxa-4,11-dien to paclitaxel.

eubacterium, *Zymomonas mobilis*. These authors proposed that the isoprenoid moiety is assembled by condensation of a triose phosphate-type compound with activated acetaldehyde derived from the decarboxylation of pyruvate. A subsequent skeletal rearrangement has been proposed to disrupt the connectivity of the three-carbon unit. However, whilst the data on taxuyunnanine C from [U-$^{13}C_6$]-glucose yielded direct proof of the occurrence of an intramolecular rearrangement in the biosynthesis of isoprenoid precursors, it remains open as to whether the taxoid precursor is assembled from a triose phosphate-type compound and activated acetaldehyde. Thus, the ultimate precursor(s) of the isoprenoid unit in *T. chinensis* is as yet unknown. Hopefully, as the biosynthetic route to taxol is further clarified, this will allow new methods of production to become available.

The genes encoding several of the hydroxylation and acylation steps have been cloned and functionally expressed in *E. coli* (for a summary, see Marasco and Schmidt-Dannert, 2007). Whereas it was possible to produce the main skeleton, taxadiene, in recombinant bacteria and yeast in good yield, the later steps of taxol biosynthesis appear to be more difficult to achieve in recombinant systems at present (Jennewein *et al*., 2005; Dejong *et al*. 2006).

In 1993, Stierle *et al*. (1993) had isolated *Taxomyces andreanae*, an endophytic fungus of Pacific yew, and could show that it was able to produce taxol (for a discussion, see Chapter 7).



Cyanidin
λ_{max} 534 nm

Betanidin
λ_{max} 543 nm

Figure 2.21 Structures of betanidin and a typical anthocyanidin, cyanidin, accumulating as various glycosylated structures and their acylated forms. Both compounds exhibit similar λ_{max} values. Occurrence of these two classes of pigments mutually excludes each other. The betalains are exclusively found in most families of the plant order Caryophyllales, whereas the anthocyanins are ubiquitously distributed in the other families of the Angiosperms.

2.10 Betalains

Betalains constitute a class of taxonomically important water-soluble 'chromoalkaloids', the red–violet betacyanins and the yellow betaxanthins. They are characteristic of all families of the plant order, Caryophyllales, with the exception of the Caryophyllaceae and the Molluginaceae. Members of these two families accumulate anthocyanins, occurring ubiquitously in all other Angiosperms (Steglich and Strack, 1990). Fig. 2.21 presents structural schemes of betanidin and a typical anthocyanidin, cyanidin, exhibiting similar light absorption characteristics. Both are the aglycones of various glycosylated structures and their acylated forms.

A well-known example of the occurrence of betalains in higher plants is that of the roots of red beet (*Beta vulgaris* (L.) subsp. *vulgaris*). Unexpectedly, betalains were also detected in some higher fungi (Steglich and Strack, 1990), e.g. the fly agaric (*Amanita muscaria*). Whereas the anthocyanin-analogous functions of these pigments in plant flower and fruit coloration are obvious, their role in pigmentation of vegetative tissues and their occurrence in higher fungi are unknown. In a recent review, Clement and Mabry (1996) indicated the lack of knowledge about the possible importance of anthocyanins and betalains beyond their role in pollination and seed dispersal, but as yet there are no arguments for alternative functions of betalains. Gain and loss of the anthocyanin and betalain pathways remain a mystery (Clement *et al.*, 1994). Both pathways may have diverged prior to the origin of flower pigmentation (Mabry, 1973), or the ability to produce betalains may have evolved

subsequent to the loss of anthocyanin formation (Ehrendorfer, 1976). However, the possibility that both classes of pigments may have occurred concurrently in some ancestral plants cannot be excluded (Clement and Mabry, 1996, and references therein).

Betalains have received much attention from the food industry as natural colour additives (Adams *et al.*, 1976; Pourrat *et al.*, 1983). The betacyanins from red beet are used for colouring ice cream, jam and fruit conserves. Earlier interest in betacyanins came from their use in colouring red wine, although this was prohibited by law in 1892 due to the use of the apparently harmful pokeberry, *Phytolacca americana*, extract (Dreiding, 1961).

Research on betalains has received a significant impetus from recent developments in chromatography, spectroscopy, biochemistry and techniques of molecular biology. This has led to a rapid increase in our knowledge about new structures as well as key steps in their biosynthesis. Some new structural features of betalains from plants are reviewed below, resulting primarily from advances in work on their biosynthesis, whilst still being aware of the validity of earlier hypotheses.

2.10.1 Structures

In contrast to the rapid progress in clarifying the structure of anthocyanins early this century, it was only in the 1960s that the nature of betalains was elucidated, mainly by chemical methods. This led to the identification of betanidin by Wyler and co-workers (1963) and of indicaxanthin by Piattelli and co-workers (1964). Both groups of pigments were shown to be immonium derivatives of betalamic acid with *cyclo*-Dopa (betacyanins) and amino acids/amines (betaxanthins). Since then, rapid development of sophisticated techniques in chromatography and spectroscopy has led to the identification of the most complex betanidin conjugates (polyacylated oligoglycosides) known so far from higher plants, such as the betacyanins from *Bougainvillea* bracts (Heuer *et al.*, 1994). Nine betacyanins were identified from red–violet bracts as gomphrenin I (betanidin 6-*O*-glucoside) and derivatives of bougainvillein-v (betanidin 6-*O*-sophoroside), i.e. mono- and diglucosylsophorosides, which are acylated with 4-coumarate and caffeate (mono- and diesters). Fig. 2.22 shows the structure of the most complex betanidin conjugate isolated from *Bougainvillea* bracts (for recent reviews concerning betalain structures and methods of structural elucidation, see Steglich and Strack, 1990; Strack *et al.*, 1993; and Strack and Wray, 1994a).

It has only recently been discovered that acylated betacyanins exhibit intramolecular co-pigmentation that may also lead to stabilization of the chromophor, betanidin (Schliemann and Strack, 1998), a phenomenon which is well-known for anthocyanins (Brouillard and Dangles, 1993). Esterification of the sugar moieties of betanin (betanidin 5-*O*-glucoside), gomphrenin I and bougainvillein-v with hydroxycinnamates leads to bathochromic shifts

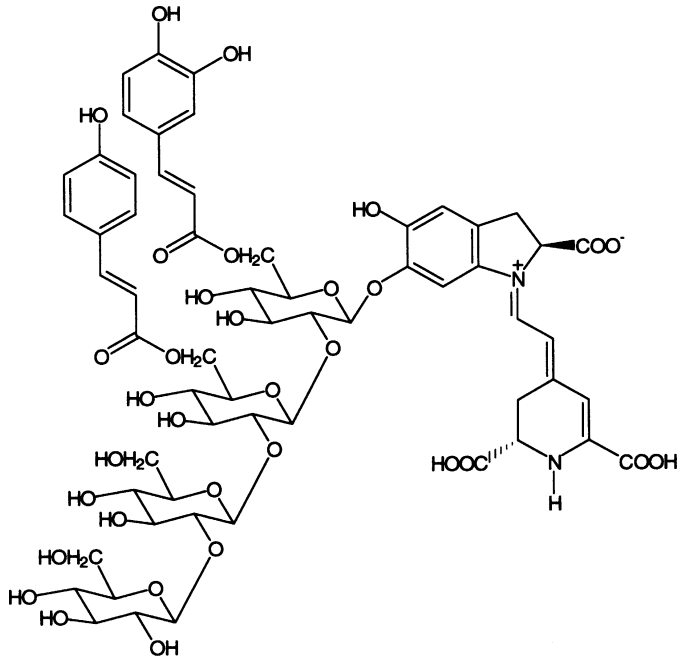


Figure 2.22 Structure of the most complex betanidin conjugate known so far from higher plants, isolated from *Bougainvillea* bracts.

of light absorption (Heuer *et al.*, 1992, 1994). Nuclear magnetic resonance (NMR) spectroscopic analyses showed ^{1}H chemical shift differences between gomphrenin I, bougainvillein-v and their respective acylated derivatives (Heuer *et al.*, 1992, 1994), as well as between betanin and lampranthin II (6′-*O*-*E*-feruloylbetanin) (Heuer *et al.*, 1992), indicating molecular association (stacking) of the aromatic acids to betanidin. The attachment of the acylglucosides at C-6 of the *cyclo*-Dopa moiety of betanidin enhances the observed bathochromic shift, which possibly results from a more rigid conformation. This is most interesting when considering the different colours of the red–violet acylated 5-*O*-glucosides of Bougainvillea 'Mrs Butt' (Piattelli and Imperato, 1970a) and the violet–red acylated 6-*O*-glucosides from *B. glabra* (Piattelli and Imperato, 1970b).

An important factor for intramolecular association and, in particular, for structural stabilization is the site of linkage of the hydroxycinnamates to the glycosyl moiety of betacyanins, which has to allow sufficient conformational flexibility on the betanidin skeleton. The binding of the feruloyl residue at the glucuronosyl moiety of a disaccharide in celosianin II apparently leads to a higher flexibility of the acylglycoside moiety, which may effectively protect the aldimine bond against hydrolytic attack (Schliemann and Strack, 1998).

2.10.2 Biosynthesis

Betalain-producing plants are unable to convert dihydroflavonols via flavan-3,4-*cis*-diols to anthocyanidins, whereas the dehydrogenation reactions of dihydroflavonols to flavonols still exist. On the other hand, the formation of flavan-3,4-*cis*-diols (leucoanthocyanidins) seems to be possible, as indicated by the occurrence of leucocyanidin in *Carpobrotus edulis* (Kimler *et al.*, 1970). The lack of the last enzymatic step in the formation of anthocyanidins is, therefore, characteristic of betalain-producing plants, i.e. a dioxygenase-type anthocyanidin synthase with a 2-hydroxylase activity towards leucoanthocyanidins, possibly including two dehydratase reactions. These plants instead express a different dioxygenase activity, catalysing a 4,5-extradiol ring cleavage of Dopa to 4,5-*seco*-Dopa, which subsequently cyclizes to betalamic acid in a spontaneous reaction.

Figure 2.23 presents a scheme of the betalain pathway. The initial key reactions were essentially deduced from feeding experiments with isotopically labelled tyrosine and Dopa (Hörhammer *et al.*, 1964; Minale *et al.*, 1965; Garay and Towers, 1966), and support the early suggestion of Wyler and co-workers (1963) that both the *cyclo*-Dopa and the betalamic acid moieties of betacyanins are derived from Dopa. By using [^{14}C,^{15}N]-labelled tyrosine, Liebisch and co-workers (1969) unambiguously proved that the entire C_6C_3N-skeleton of this amino acid was incorporated.

Based on genetic studies (cross-breeding) with *Portulaca grandiora*, Trezzini and Zryd (1990) postulated that only three loci are responsible for betalain biosynthesis. While two loci control the biosynthesis of *cyclo*-Dopa and betalamic acid, the third controls the transport of betalamic acid into the vacuole. They proposed that the formation of betanidin (condensation of *cyclo*-Dopa with betalamic acid) proceeds in the cytoplasm and the formation of betaxanthins (condensation of betalamic acid with an amino acid/amine) takes place spontaneously in the plant vacuole (Trezzini, 1990).

Some of the proposed biosynthetic reactions have only recently been proved by enzymatic studies. It has been suggested that the first enzyme in betalain biosynthesis was a phenol oxidase complex, catalysing both the conversion of tyrosine to Dopa and the dehydrogenation of the latter to a o-quinone (Constabel and Haala, 1968; Stobart and Kinsman, 1977; Endress, 1979; Elliott, 1983). This suggestion has been supported by Steiner and co-workers (1996, 1999). They showed that the formation of the *cyclo*-Dopa moiety of betanidin is catalysed by a tyrosinase in a two-step enzymatic reaction: hydroxylation of tyrosine followed by oxidation of the product, Dopa, yielding Dopaquinone (Steiner *et al.*, 1999). The final formation of *cyclo*-Dopa proceeds via a non-enzymatic ring closure. The enzyme involved in these reactions has been partially purified from betacyanin-producing callus cultures of *Portulaca grandiora*. It has been characterized as a tyrosinase (EC 1.14.18.1/EC 1.10.3.1) by inhibition experiments with copper-chelating agents (diethyldithiocarbamate and phenylthiocarbamide) and detection

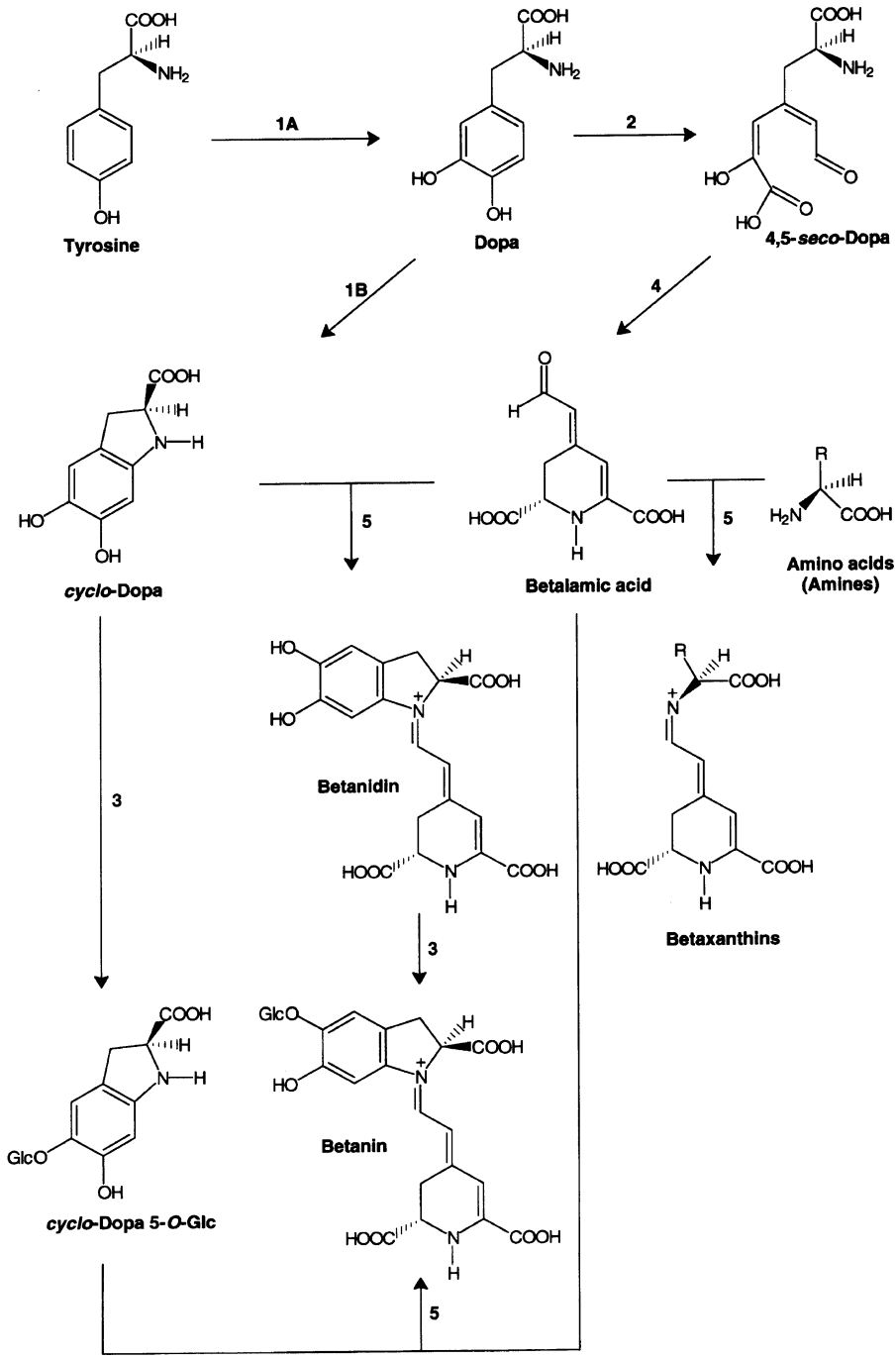


Figure 2.23 Biosynthesis of betalains, involving two 'early' enzymes, the tyrosinase (1A, hydroxylating activity; 1B, oxidizing activity) and the Dopa 4,5-dioxygenase (2), and one 'late' enzyme activity, glucosylating *cyclo*-Dopa and/or betanidin (3). Reactions 4 and 5 are considered to proceed spontaneously.

of concomitant *o*-diphenol oxidase activity (Steiner *et al.*, 1999). This is in agreement with Joy and co-workers (1995), who isolated two cDNA clones encoding polyphenol oxidases from a suspension culture of *Phytolacca americana* producing betalains. By northern analyses of RNA from various organs of *P. americana* plants, they demonstrated that spatial and temporal expressions correlated well with high rates of betalain accumulation in ripening fruits.

The hydroxylating activity of the *P. grandiora* tyrosinase showed an optimal pH of 5.7 and was specific for L-tyrosine, exhibiting reaction velocities with L-tyrosine and D-tyrosine in a ratio of 1:0.2. Other possible monophenolic substrates were not accepted. The enzyme appeared to be a monomer with a molecular mass of about 53 kDa. Mueller and co-workers (1996) characterized the respective enzyme from the fly agaric. This tyrosinase was apparently not specific for L-tyrosine, but also accepted tyramine, 4-hydroxyphenylpropionate and phenol. The enzyme exhibited maximum activity at approximately pH 6.0 and appeared to be a heterodimer of two subunits with molecular masses of 27 and 30 kDa, which is unusual for tyrosinases. These enzyme activities are unique examples of the involvement of a tyrosinase in the biosynthesis of low molecular weight natural products, such as betalains. The role of tyrosinase in the formation of Dopa as an end product, which accumulates in various plant tissues (Teramoto and Komamine, 1988), or as an intermediate metabolite, e.g. in the biosynthesis of benzylisoquinoline alkaloids (Rueffer and Zenk, 1987a), has been demonstrated. However, the most obvious function of tyrosinase in plants is to initiate polymerization of the oxidation product of *cyclo*-Dopa, Dopachrome, analogous to the formation of melanin in the skin of animals. A similar function might be ascribed to their involvement in plant defence reactions against insects and microbial pathogens, but this has yet to be demonstrated.

The second early enzymatic key reaction in the biosynthesis of betalains is the extradiol ring cleavage of Dopa, leading to betalamic acid. It has been established (Fischer and Dreiding, 1972; Impellizzeri and Piattelli, 1972) that there is a 4,5-extradiol cleavage of Dopa followed by closure of the dihydropyridine ring by a condensation between the amino and keto groups. An alternative 2,3-cleavage of Dopa could lead, through a dihydroazepine ring closure, to muscaflavin of the fly agaric (Fig. 2.24). Indeed, the postulated Dopa dioxygenase activities have been isolated from the fly agaric (Girod and Zryd, 1991; Terradas and Wyler, 1991a) and shown to catalyse the extradiol cleavage leading to betalamic acid and the minor pigment, muscaflavin. In addition, the expected intermediates, 2,3- and 4,5-*seco*-Dopa, were identified in dioxygenase enzyme assays (Terradas and Wyler, 1991a) as well as in *Amanita muscaria* and *Hygrocybe conica* extracts (Terradas and Wyler, 1991b).

The gene encoding the fly agaric Dopa dioxygenase was cloned (Hinz *et al.*, 1997) and expressed in *E. coli* (Mueller *et al.*, 1997a). The recombinant enzyme catalysed both the 4,5- and the 2,3-extradiol cleavage of Dopa. This was an unexpected result in the light of previous suggestions that the two ring cleavages were catalysed by two different enzymes (Girod and Zryd,

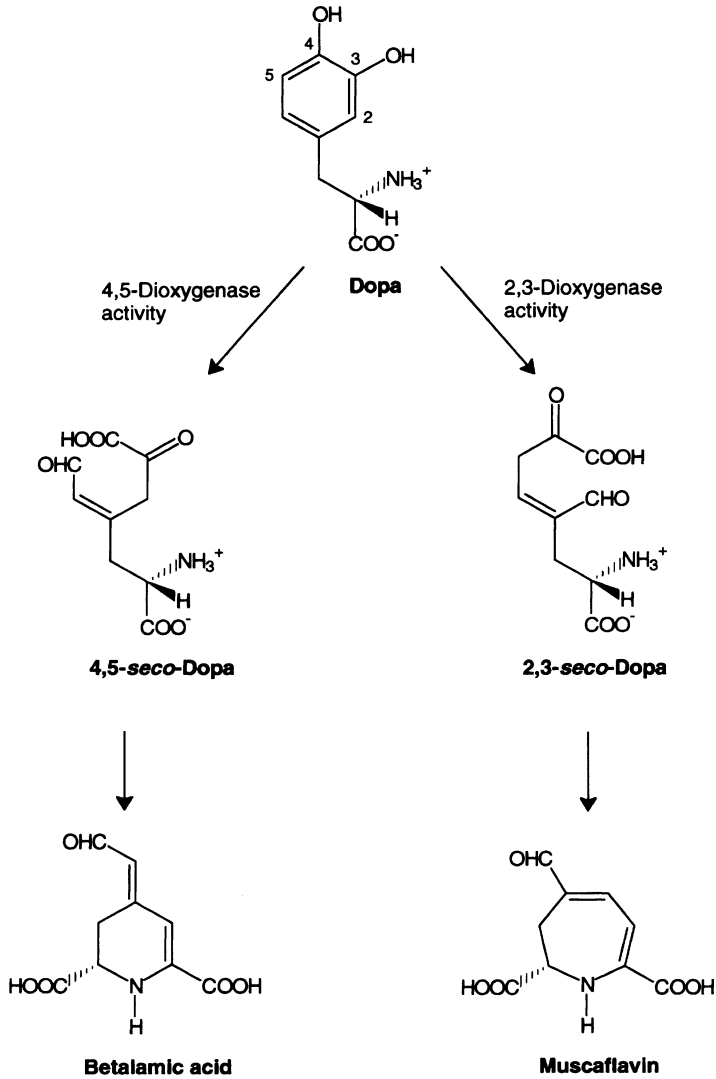


Figure 2.24 Enzymatic Dopa extradiol cleavages leading through spontaneous dihydropyridine ring closure to betalamic acid or dihydroazepine ring closure to muscaflavin. The Dopa dioxygenase from higher fungi catalyses both ring cleavages, the putative plant enzyme exclusively the 4,5-ring cleavage.

1991; Terradas and Wyler, 1991a). The cDNA clone encoding the fly agaric Dopa dioxygenase was introduced into white petals of *Portulaca grandiora*, using particle bombardment (Mueller *et al.*, 1997b). Expression of the clone complemented the betalain pathway in some cells of these petals, indicating that the Dopa extradiol cleavage is the pivotal reaction in betalain biosynthesis. The Dopa dioxygenase activity from betalain-producing higher plants has

not yet been demonstrated and attempts to detect the plant enzyme by using antibodies directed against the fly agaric dioxygenase have failed (Mueller *et al.*, 1997a). The authors concluded that the dioxygenases involved in betalain formation in fungi and in plants are different in structure and probably do not share a common evolutionary origin.

Based on the conclusions of Trezzini and Zryd (1990) following their crossbreeding experiments with the model system *Portulaca grandiora*, only two enzymes are necessary for the biosynthesis of the betacyanin aglycone, betanidin and the betaxanthins, i.e. tyrosinase and Dopa dioxygenase generating *cyclo*-Dopa and betalamic acid, respectively. According to this model, the subsequent formation of betanidin and betaxanthins (imine formation) should proceed spontaneously, which can easily be demonstrated under in vitro conditions (Terradas and Wyler, 1991a). This has been confirmed by recent results from a two-step in vitro assay (Schliemann *et al.*, 1998). By combining the Dopa dioxygenase from the fly agaric with the tyrosinase from *Portulaca grandiora*, the formation of betanidin from Dopa was demonstrated (Schliemann *et al.*, 1998).

Support for the existence of an analogous in vivo reaction, at least in the case of betaxanthin formation, came from amino acid feeding experiments with hairy roots and seedlings of *Beta vulgaris* (Hempel and Bbhm, 1997). Administration of various amino acids led to the appearance of the corresponding betaxanthins, irrespective of the *S*- or *R*-isomers applied. Following this study, extensive feeding experiments were carried out using *S*- and *R*-isomers of proteinogenic and nonproteinogenic amino acids with hairy roots of *Beta vulgaris* (yellow cultivar) (N. Kobayashi, W. Schliemann, D. Strack, unpublished); these confirmed the lack of amino acid specificity and stereoselectivity in betaxanthin formation. Furthermore, feeding of 2-aminoindan 2-phosphonic acid (AIP), a specific inhibitor of phenylalanine ammonialyase (PAL; EC 4.3.1.5) (Zont and Amrhein, 1992), led to an endogenous increase of the phenylalanine level and thereby to the formation of the betaxanthin derived from phenylalanine. In addition, by feeding of *cyclo*-Dopa to *Beta vulgaris* seedlings (yellow cultivar), it could be shown that the normally yellow coloured hypocotyls turned red due to the formation of betanidin. The yellow colour of these hypocotyls originates mainly from high concentrations of betalamic acid (N. Kobayashi, W. Schliemann, D. Strack, unpublished), which obviously reacts with *cyclo*-Dopa taken up by the seedlings. In summary, these results indicate that indeed the condensation of betalamic acid with amino acids (including *cyclo*-Dopa) or amines in plants is a spontaneous rather than an enzyme-catalysed reaction. However, this hypothesis still awaits proof. Further studies should prove the spontaneous betaxanthin formation in the plant vacuole, controlled at the site of transport of betalamic acid into the vacuole, as well as the betanidin formation in the plant cytoplasm, as postulated by Trezzini and Zryd (1990).

Considering the complexity of betacyanin structures (Heuer *et al.*, 1994), the 'final enzymes' involved in betacyanin biosynthesis, i.e. glucosyltransferases

and acyltransferases, might be as diverse as those in anthocyanin biosynthesis (Strack and Wray, 1994b). Glucosylation of betanidin can proceed at the *cyclo*-Dopa moiety (C-5 and C-6 hydroxyl groups). Feeding experiments using *cyclo*-Dopa and its 5-*O*-glucoside, as well as betanidin, have indicated two possible levels of sugar attachment in the formation of betacyanins, glucosylation of betanidin (Sciuto *et al.*, 1972) or glucosylation of *cyclo*-Dopa prior to condensation with betalamic acid (Sciuto *et al.*, 1974). The latter has been supported by the identification of free *cyclo*-Dopa 5-*O*-glucoside in betacyanin-accumulating red beet roots (Wyler *et al.*, 1984). However, the first description of a glucosyltransferase involved in betalain biosynthesis demonstrated, at least for cell cultures of *Dorotheanthus bellidiformis*, that betanidin is the acceptor for glucose attachment via uridine diphosphate (UDP)-glucose (Heuer and Strack, 1992; Heuer *et al.*, 1996). There are two different regiospecific glucosyltransferases, the UDP-glucose:betanidin 5-*O*- and 6-*O*-glucosyltransferases (5-GT and 6-GT), leading to betanin (betanidin 5-*O*-glucoside) and gomphrenin I (betanidin 6-*O*-glucoside), respectively. Both enzymes have been purified to near homogeneity and characterized (Vogt *et al.*, 1997).

Further glycosylations of betanin and gomphrenin I as well as acylations, mainly with hydroxycinnamates, lead to complex polyacylated oligoglyco sides of betanidin. Enzymes responsible for acylation of amaranthin (betanidin 5-*O*-glucuronosylglucoside) to form celosianin I (4-coumaroylamaranthin) and celosianin II (feruloylamaranthin) have been characterized from *Chenopodium rubrum* cell cultures (Bokern *et al.*, 1992). The formation of betacyanins acylated with ferulate has been demonstrated to proceed via 1-*O*-feruloylglucose in eight members from four different families within the Caryophyllales (Bokern *et al.*, 1992). In addition, the respective 1-*O*-acylglucosides regularly co-occur with the acylated betacyanins (Strack *et al.*, 1990). It has not yet been possible to demonstrate the acceptance of the alternative acyldonors, hydroxycinnamoyl-coenzyme A thioesters, in betacyanin acylation. The 1-*O*-hydroxycinnamoylglucose-dependent acylation is presumably the only mechanism of acylation in betacyanin-producing plants. In contrast, most studies on the acyltransferases involved in flavonoid biosynthesis, including anthocyanins, report the acceptance of the coenzyme A ester, e.g. in the acylation of anthocyanins in *Silene dioica* (Kamsteeg *et al.*, 1980), *Matthiola incana* (Teusch *et al.*, 1987), *Ajuga reptans* (Callebaut *et al.*, 1996) and *Gentiana triora* (Fujiwara *et al.*, 1997). However, the acceptance of a 1-*O*-acylglucoside has been demonstrated, i.e. the formation of cyanidin hydroxycinnamoyltriglycoside in *Daucus carota* (Gläβgen and Seitz, 1992).

Unexpectedly, purified betanidin glucosyltransferases from *D. bellidiformis*, besides betanidin regioselectively, also accepted highly active flavonoids (Vogt *et al.*, 1997). The 5-GT preferentially catalysed the transfer of glucose to the C-4′ hydroxyl function of flavonoids (flavonols, flavones, anthocyanidins) with B-ring ortho-dihydroxyl groups, with quercetin as the preferred substrate. The 6-GT instead catalysed the glycosylation of the C-3 hydroxyl function of flavonoids (flavonols, anthocyanidins), with cyanidin as the

preferred substrate. The speculation of Vogt and co-workers (1997) that these betanidin glucosyltransferases might be phylogenetically related to flavonoid glucosyltransferases concerns the basic question on the phylogenetic origin of betanidin biosynthesis. The question to be addressed is as follows: are 5-GT and 6-GT phylogenetically derived from quercetin 4′-0- and cyanidin 3-*O*-glucosyltransferases, respectively? The latter implies – in agreement with the hypothesis of Ehrendorfer (1976) – that the biosynthesis of the betacyanins appeared later than the flavonoid pathway in the evolution of higher plants. The discovery of enzymes able to accept substrates of the mutually exclusive anthocyanin and betalain pathways may shed new light on the evolution of both classes of pigments.

Detection of Dopa 4,5-dioxygenase in higher plants and clarification of the level of glucosylation, at betanidin and/or *cyclo*-Dopa, are the last two steps in betalain biosynthesis to be confirmed. Molecular studies are still needed to elucidate the evolutionary mechanisms of the mutual exclusion of the two pathways (Stafford, 1994), one leading to the ubiquitously occurring anthocyanins and the other to the rare betalains.

2.11 Conclusions

These examples of alkaloid biosynthesis serve to indicate how isolation of the enzymes of whole pathways has clarified our understanding of alkaloid biosynthesis and enabled investigations to take place at the molecular level. The pathways leading to *Nicotiana*, tropane, isoquinoline and monoterpene indol alkaloids have been analysed both at the enzymic and genetic levels. There is still a need to investigate systems of vesicular transport and alkaloid sequestration. In many instances, little is known about sites of alkaloid synthesis, location of sequestration and means of translocation, although the isolation and heterologous expression of an increasing number of genes are producing a new insight into this area. Investigations with plant cell cultures have suggested that, in many plants, alkaloid production is developmentally regulated and this may account for the lack of production of some alkaloids in cell culture. This must be a major area of study in the future if commercial exploitation is to take place. Several genes of biosynthetic pathways have been cloned during the past two decades; this provides a possibility to produce valuable alkaloids in recombinant microorganisms. The formation of reticuline and magnoflorine in recombinant *E. coli* and *S. cerevisiae* can be seen as a proof of the concept (Hawkins and Smolke, 2008; Keasling, 2008; Minami *et al*., 2008; Schäfer and Wink, 2009).

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Annual Plant Reviews (2010) **40**, 182–257
doi: 10.1002/9781444320503.ch4

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Chapter 4

BIOSYNTHESIS OF PHENYLPROPANOIDS AND RELATED COMPOUNDS

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Abstract: Phenolic compounds are ubiquitous in the plant kingdom. A main pathway for the formation of these compounds starts with the aromatic amino acids L-phenylalanine and – to a lesser extent – L-tyrosine. In the general phenylpropanoid pathway, these are transformed to coenzyme A-activated 4-coumaric acid by phenylalanine ammonia-lyase, cinnamic acid 4-hydroxylase and 4-coumarate CoA-ligase. 4-Coumaroyl-CoA gives rise to a large number of different natural products, e.g. flavonoids, lignans, coumarins, tannins, hydroxycinnamic acid esters and amides as well as lignin monomers. This review gives an insight into recent findings concerning the general phenylpropanoid pathway as well as the biosyntheses of hydroxycinnamoyl conjugates, phenolic aroma and fragrance compounds, lignans, coumarins and gallo-/ellagitannins.

Keywords: phenylpropanoid metabolism; hydroxycinnamic acid conjugates; lignans; coumarins; furanocoumarins; gallotannins; ellagitannins; metabolic channelling

4.1 Introduction

The biosyntheses of phenylpropanoids and the natural compounds derived thereof are among the most thoroughly investigated biosynthetic pathways leading to plant natural products. Our knowledge of enzymes, genes and their transcriptional control has increased dramatically since the last review in this series (Petersen *et al.*, 1999). Nowadays, research is focused mainly on the molecular and genetic levels, the elucidation of the regulatory principles of the biosyntheses and the functions of natural products for their producers. We are just beginning to understand the importance of natural compounds

 ISBN: 978-1-405-18397-0

in the interactions of plants with their environment. Upcoming knowledge concerns the metabolic channelling in biosynthetic pathways (Dixon *et al.*, 2001; Winkel, 2004; Jörgensen *et al.*, 2005) as well as the regulation of phenolic biosynthetic pathways on the level of transcription factors (e.g. Vom Endt *et al.*, 2002) and the structural elucidation of biosynthetic enzymes involved in natural product biosyntheses (see, e.g. Noel *et al.*, 2005). Furthermore, interest in the evolutionary origin of secondary metabolic enzymes in enzymes from primary metabolism is steadily rising (see the special volumes of *Phytochemistry*, 66(11), 2005, and 70 (15–16) 2009 'Evolution of Metabolic Diversity'). This review provides a broad overview of our knowledge of the biosynthesis and molecular biology of a number of compounds related to phenylpropanoid metabolism. It is not meant to be comprehensive and the reader is referred to other recent reviews to get answers for specific questions. Since the field of flavonoids, isoflavonoids and stilbenes has extended very much and has been reviewed recently (see, e.g. Dixon *et al.*, 2004; Schijlen *et al.*, 2004; Williams and Grayer, 2004; Martens and Mithöfer, 2005; Andersen and Markham, 2006; Ververidis *et al.*, 2007a,b), it is not treated in this chapter. For achievements gained before 1995, the reader is referred to the previous review published in this series under the same title in 1999 (Petersen *et al.*, 1999, and the literature cited therein).

4.2 General phenylpropanoid pathway and formation of hydroxycinnamate conjugates

Phenylpropanoids and their derivatives are compounds containing a C_6C_3-moiety derived from the amino acid L-phenylalanine (and less frequently L-tyrosine). Channelling of the amino acids from primary into secondary metabolism is achieved by the activity of phenylalanine/tyrosine ammonia-lyases (PAL, TAL). The core reactions of the general phenylpropanoid pathway are the deamination by PAL, the introduction of a 4-OH group into the aromatic ring by cinnamate 4-hydroxylase (CAH, C4H) and the activation of the acid as coenzyme A thioester (catalysed by 4-coumarate CoA-ligase, 4CL) or less frequently as glucose ester. The introduction of further substituents (see Fig. 4.1) into the aromatic ring has been a hot research topic for decades but seems to be solved now (see Section 4.2.1.4). Phenylpropanoids are used as the monomers for lignin and lignan formation and as starters for the addition of acetate units by polyketide synthases leading to, e.g., flavonoids and stilbenes. Hydroxycinnamic acids are found as 'decorating units' in many natural compounds, e.g. flavonoids and anthocyanins. Moreover, hydroxycinnamate derivatives comprise different esters and amides that are formed with all kinds of molecules as acceptors. Additionally, hydroxycinnamic acid moieties are found as phenolic constituents in cutins and suberins.

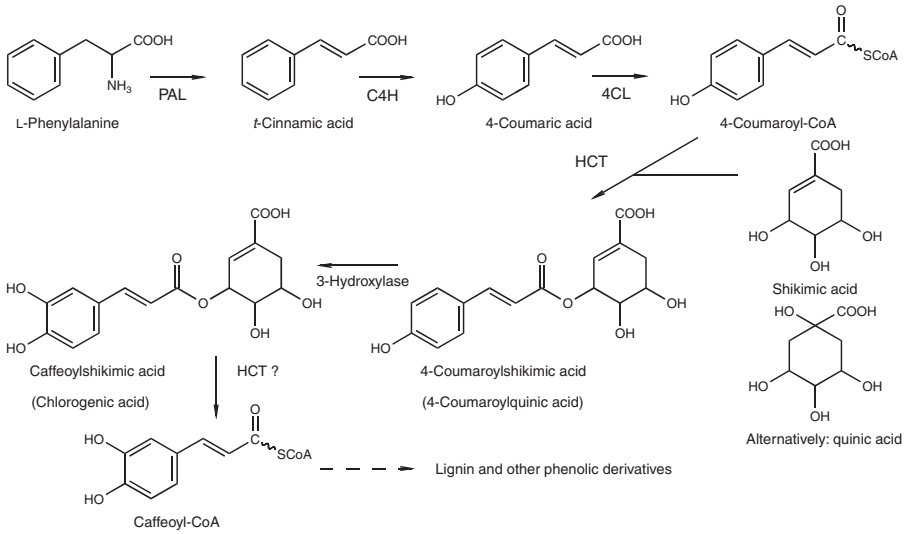


Figure 4.1 Current view of the phenylpropanoid metabolism. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA-ligase; HCT, hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase; 3-hydroxylase, 4-hydroxycinnamoylshikimate/quinate 3-hydroxylase.

4.2.1 The general phenylpropanoid pathway

4.2.1.1 Phenylalanine ammonia-lyase

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) channels phenylalanine into the general phenylpropanoid pathway by removing the nitrogen as ammonia while introducing the *trans*-double bond between C7 and C8 of the side chain (Fig. 4.1). Mainly in grasses, an enzyme having a higher affinity for tyrosine (tyrosine ammonia-lyase, TAL) is found. Since its first description by Koukol and Conn in 1961, PAL ranges among the most and best studied enzymes in plant secondary metabolism. In many plants, PAL isoforms are encoded by multi-gene families (see, e.g. Wanner *et al.*, 1995). Members of these families are differentially expressed and the encoded PAL isoforms play specific roles in plant metabolism. The four annotated PAL genes from *Arabidopsis thaliana* have been heterologously expressed and characterized showing slightly different properties while one isoform showed a very low activity (Cochrane *et al.*, 2004). Although PAL is generally considered as a soluble enzyme, varying sub-cellular localizations of different PAL isoforms (cytoplasmic – membrane-bound) and association with the ER-bound cinnamate 4-hydroxylase has been postulated, thus enabling an effective channelling of metabolites through the phenylpropanoid pathway (Rasmussen and Dixon, 1999; Achnine *et al.*, 2004; Sato *et al.*, 2004). Engineering of PAL and C4H into yeast, however, did not support this view (Ro and Douglas, 2004); here, however, it must be taken into account that this system is highly artificial. The

putative organization of enzymes involved in biosynthetic pathways leading to different phenolic compounds in so-called metabolons has been reviewed by Winkel (2004) and Jörgensen *et al.* (2005).

PAL is induced together with other genes/enzymes of phenolic metabolism by environmental factors, e.g. pathogen attack or UV light. The involvement of transcription factors in the coordinated induction of genes leading to specific compounds has been demonstrated in different plants. In maize, PAL and the following enzymes leading to anthocyanin formation and vacuolar storage are induced by a pair of transcription factors, the MYB transcription factor C1 and R, which belongs to the basic helix–loop–helix (bHLH) factors. A different MYB factor P also induces PAL, but also the enzymes involved in the biosynthesis of flavan-4-ols (Grotewold *et al.*, 1998; Mol *et al.*, 1998; review by Vom Endt *et al.*, 2002). Similar situations are found in other well-investigated flavonoid/anthocyanin-synthesizing plants. The regulation of PAL activity on the protein level by phosphorylation has been described by Bolwell *et al.* (1996).

PAL is active as a homotetrameric protein without any co-factor. The electrophilic group necessary for the removal of nitrogen as ammonia is provided by three amino acids (Ala–Ser–Gly) which autocatalytically form a 4-methylidene-imidazole-5-one (MIO) group (Langer *et al.*, 2001; Poppe, 2001) as seen in the first crystal structure of a plant PAL (Ritter and Schulz, 2004). This structure also demonstrated the high structural similarity of PAL to histidine ammonia-lyase (HAL) from the degradation pathway of histidine to glutamate, which was crystallized from *Pseudomonas putida* (Schwede *et al.*, 1999). Therefore, an evolutionary origin of PAL in HAL, which catalyses a similar deamination reaction, was postulated (Ritter and Schulz, 2004).

4.2.1.2 Cinnamic acid 4-hydroxylase

The hydroxylation step in the core phenylpropanoid pathway transforming cinnamic acid to 4-coumaric acid is catalysed by cinnamic acid 4-hydroxylase (C4H, CAH; EC 1.14.13.11; Fig. 4.1) which probably is the best investigated cytochrome P450 monooxygenase in plant metabolism. After extensive biochemical characterization (see review by Werck-Reichhart, 1995) from its time of detection by Nair and Vining (1965), the first cDNA was isolated in 1993 from *Helianthus tuberosus* and classified as CYP73A1 (Teutsch *et al.*, 1993). To date, more than 80 members of the CYP73A family have been listed on the cytochrome P450 homepage of Dr David Nelson (http://drnelson.utmem.edu/CytochromeP450.html) and all actively expressed members of the family exert C4H activity. Many assignments, however, are only based on sequence similarities and not on the determination of catalytic activities. C4Hs exist as multi-gene families in many species (Lu *et al.*, 2006). From sequence data, two classes I and II can be distinguished which are differently abundant in monocotyledonous and dicotyledonous plants (Ehlting *et al.*, 2006). Heterologous expression of the open reading frames

(ORFs) encoding C4H is in most cases achieved in *Saccharomyces cerevisiae* since this organism, as a eukaryote, has endoplasmic reticulum membranes to localize cytochrome P450s as well as NADPH:cytochrome P450 reductase (CPR). Yeast has an own CPR which is able to provide the electrons for the C4H reaction, but in many cases yeast cells engineered with *Arabidopsis* CPR or the species-derived CPR have been used. C4H directly isolated from plant cells as well as heterologously expressed proteins exert a high substrate specificity, essentially only accepting *t*-cinnamic acid as a substrate. Increased C4H transcript levels and activities are found in correlation with lignification, synthesis of phenolic defence compounds (induction by fungal elicitors and/or jasmonates) as well as wounding. Promoter analyses have shown regulatory boxes for binding of transcription factors also involved in the regulation of other genes of phenolic metabolism (see, e.g. Lu *et al.*, 2006). Down-regulation of C4H usually resulted in lower C4H activities and a reduced and/or altered lignin content (Sewalt *et al.*, 1997; Chen *et al.*, 2006). The latter was surprising since C4H activity is needed for the formation of all lignin monomers, and an explanation for this observation has not yet been found.

A new method to quantify C4H (as an example for a cytochrome P450) was established with the help of fluorescence-coupled immunodetection as a substitute and with a lower detection limit as CO difference spectra (Humphreys and Chapple, 2004).

An engineered water-soluble C4H (originally from *Helianthus tuberosus*) has been expressed and purified from yeast and used for ^{1}H-NMR studies in order to investigate the active site and the substrate positioning. The initial placement of the cinnamate parallel to the heme was not able to explain the exclusive 4-hydroxylation of the substrate and it was suggested that the substrate has to shift during the catalytic cycle (Schoch *et al.*, 2003).

4.2.1.3 Activation of hydroxycinnamic acids and their derivatives

(Hydroxy)cinnamic acids themselves are metabolically rather inert. In order to undergo further metabolism, generally activation of the carboxyl group is necessary. Activation of (hydroxy)cinnamic acids can be achieved by either ATP-dependent transfer to coenzyme A (CoA) or UDP-glucose (UDPG)-dependent transfer to glucose. Enzymes catalysing the former reaction are named hydroxycinnamic acid:CoA-ligases or 4-coumarate:CoA-ligases (4CL; Fig. 4.1), although differently substituted hydroxycinnamic acids may be accepted by the enzymes. Generally, 4-coumaric, caffeic and ferulic acids are considered good substrates, whereas cinnamic and sinapic acids are poorly or not at all converted. The coenzyme A thioesters will enter different further reactions such as reduction to aldehydes and alcohols (monolignols), addition of acetate units from malonyl-CoA by polyketide synthases leading to the formation of, e.g., flavonoids, isoflavonoids and stilbenes or acyl transfer to varying acceptor molecules.

During the reaction of 4CL, the (hydroxy)cinnamic acid is first activated by AMP (from ATP) and then transferred to coenzyme A. 4CLs share common

peptide domains and a common reaction mechanism with other members of the AMP-binding protein family such as firefly luciferases, nonribosomal peptide synthetases and acyl-CoA synthetases (Cukovic *et al.*, 2001; Ehlting *et al.*, 2001). By sequence comparison, two evolutionary ancient sub-groups (classes I and II) can be distinguished (Ehlting *et al.*, 1999). Usually 4CLs are encoded by several genes within one organism. However, from the 11 annotated putative 4CL genes from *Arabidopsis thaliana*, only four proteins did really catalyse a 4CL reaction accepting different cinnamic acids with different affinities (Costa *et al.*, 2005). Only one of the enzymes was capable of effectively activating sinapic acid as previously described by Hamberger and Hahlbrock (2004) and Lindermayr *et al.* (2002) for soybean 4CLs. In *Populus tremuloides*, two isoforms of 4CL showed substrate preferences towards 4-coumaric and caffeic acids, respectively (Harding *et al.*, 2002). Feeding experiments with different plant species performed by Yamauchi *et al.* (2003) suggested that different pathways towards syringyl (S) lignin units may occur in different plant species. Different *cis*-regulatory elements in the promoter regions of the four 4CL genes (At4CL1-At4CL4) of *Arabidopsis thaliana* were shown to mediate differential regulation through developmental and wounding signals (Soltani *et al.*, 2006).

Structural investigations on the 4CLs from several plant species helped to identify the substrate binding motif responsible for the discrimination between highly and less substituted cinnamic acids (Ehlting *et al.*, 2001; Stuible and Kombrink, 2001; Lindermayr *et al.*, 2003; Schneider *et al.*, 2003). The amino acid residues responsible for 4CL catalytic activity were identified by mutational analysis of At4CL2 from *Arabidodpsis thaliana* (Stuible *et al.*, 2000).

An unexpected reaction, the synthesis of (di)adenosine polyphosphate by 4CL in the presence of cinnamic acids, was described by Pietrowska-Borek *et al.* (2003).

Although 4CLs were believed to be specific for plants, a cinnamic acid CoA-ligase has recently been cloned from *Streptomyces coelicolor* A3(2). The heterologously expressed enzyme predominantly accepted 4-coumarate and cinnamate and with lower affinity caffeate, while ferulate was not accepted. Mutations of amino acid residues in the substrate-binding pocket were able to alter the substrate affinity (Kaneko *et al.*, 2003). This bacterial 4CL gene was used for the biotechnological production of flavonoids in *E. coli* (see, e.g. Miyahisa *et al.*, 2006, and the literature cited therein).

Similar CoA-ligases, but with distinct substrate specificities, seem to be active in other metabolic pathways. 3-Hydroxybenzoate:CoA-ligase from *Centaurium erythraea* involved in xanthone biosynthesis showed very similar characteristics to the hydroxycinnamate:CoA-ligase from the same species, but did not accept hydroxycinnamates (Barillas and Beerhues, 1997). Three CoA-ligases, namely cinnamate:CoA-ligase, benzoate:CoA-ligase and 4-coumarate:CoA-ligase, were separated in cell extracts from *Hypericum androsaemum* attributed to xanthone, benzoic acid and phenylpropanoid metabolism, respectively (Abd El-Mawla and Beerhues, 2002).

Transfer of glucose to hydroxycinnamic acids or alcohols can either occur at aromatic OH groups or at the aliphatic side chain (see, e.g. the review by Bowles *et al.*, 2006). The former is usually considered to lead to storage and transport compounds, e.g. in lignification, whereas the activation of a hydroxycinnamic acid as a glucose ester parallels the activation as a coenzyme A thioester, but is not as abundant (see below). UDP-glucose (UDPG)-dependent glucosyltransferases forming hydroxycinnamic acid glucose esters in a freely reversible reaction have already been known for a long time (see Mock and Strack, 1993). Recently, more than 100 secondary metabolite glucosyltransferase genes have been identified in the *Arabidopsis* genome on the basis of conserved sequence motifs in the binding region for the sugar donor (Paquette *et al.*, 2003). Some of them were shown to also glucosylate xenobiotics. A UDPG:cinnamate glucosyltransferase from *Fragaria ananassa* catalyzes the formation of (hydroxy)cinnamoyl glucose esters involved in the formation of aroma compounds (Lunkenbein *et al.*, 2006). In Brassicaceae a number of sinapate esters (e.g. sinapoylmalate, sinapoylcholine) play important roles in defence and as storage compounds. The transfer of the sinapate moiety to different acceptors is mediated from sinapoyl-glucose. UDP-glucose:sinapate glucosyltransferase has been extensively characterized, e.g., from the economically important plants *Raphanus sativus* and *Brassica napus* (Nurmann and Strack, 1981; Wang and Ellis, 1998). Genes encoding the enzymes involved in the formation of hydroxycinnamate glucose esters have recently been identified in, e.g., *Arabidopsis thaliana* and seeds of *Brassica napus* (Milkowski *et al.*, 2000a,b; Lim *et al.*, 2001; Mittasch *et al.*, 2007, and the cited literature). The enzyme sinapate 1-glucosyltransferase (EC 2.4.1.120) exclusively forms glucose esters and prefers sinapate as acceptor, but also takes other hydroxycinnamates. RNAi-mediated silencing of sinapoyl 1-glucosyltransferase in *Brassica napus* resulted in the reduction of a number of sinapate esters (Baumert *et al.*, 2005). The formation of these esters is catalysed by serine carboxypeptidase-like acyltransferases (see below). A hydroxycinnamate glucosyltransferase from tomato usually transferring the glucose to the aromatic OH group (EC 2.4.1.126) was reported to additionally form glucose esters with some other substrates (Fleuriet and Macheix, 1980). Similarly, a glucosyltransferase with a very broad substrate specificity (e.g. glucoside formation with stilbenes, flavonoids, coumarins and glucose esters of hydroxycinnamic and hydroxybenzoic esters) was found in grapes (Hall and De Luca, 2007). Hydroxycoumarins as well as hydroxycinnamic acids were glucosylated by salicylic acid-inducible enzymes from tobacco (Fraissinet-Tachet *et al.*, 1998).

A cross-talk between the two activation pathways of hydroxycinnamic acids by CoA or glucose was shown in an *Arabidopsis* mutant with hyperfluorescence: upon reduction of UDP-glucose:sinapate glucosyltransferase activity a part of the sinapic acid was activated as CoA thioester and directed into flavonoid biosynthesis (Sinlapadech *et al.*, 2007).

Hydroxycinnamic acid esters, e.g. chlorogenic acid, can act as 'activated' hydroxycinnamoyl donors as well, and thus represent an activated form of a hydroxycinnamic acid. This will be exemplified in more detail in Sections 4.2.1.4 and 4.2.2.

4.2.1.4 Establishing the caffeoyl substitution pattern: hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase and 4-coumaroylshikimate/quinate 3-hydroxylase

For decades the introduction of the 3- or meta-hydroxyl group into 4-coumaroyl derivatives has been an open question and several enzyme types have been proposed to be involved in this reaction (see Petersen *et al.*, 1999 and Ehlting *et al.*, 2006 for an overview). The importance for one or the other of these previously described 3-hydroxylation enzymes in specific biosynthetic pathways or in specific plants remains to be newly elucidated. The 3-hydroxylation of a 4-coumaroyl moiety at the ester stage had been described for the biosynthesis of chlorogenic acid or caffeoylshikimate (Heller and Kühnl, 1985; Kühnl *et al.*, 1987) as well as for rosmarinic acid biosynthesis (Petersen, 1997). The importance of 4-coumaroyl esters (4-coumaroylquinate and -shikimate) for the formation of caffeoyl moieties for many, if not all, phenolic pathways was detected in 2001 and 2002 independently by four groups (Schoch *et al.*, 2001; Anterola *et al.*, 2002; Franke *et al.*, 2002a; Nair *et al.*, 2002). The 4-coumaroyl esters of shikimate and quinate are hydroxylated by a cytochrome P450 monooxygenase of the CYP98 family; the enzyme thus should be named 4-coumaroylshikimate/quinate 3-hydroxylase (C3H).

The formation of the 4-coumaroyl ester as well as – putatively – the retransfer of the newly established caffeoyl moiety to coenzyme A is catalysed by a hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase (Fig. 4.1) of the BAHD superfamily of acyltransferases (Hoffmann *et al.*, 2003). Older reports had already shown that enzymes transferring hydroxycinnamoyl moieties to shikimate and/or quinate might show more or less pronounced substrate specificities (Rhodes *et al.*, 1979; Ulbrich and Zenk, 1979, 1980). The cDNA isolated by Hoffmann *et al.* (2003) from tobacco encoded a protein that preferred shikimate as a substrate, whereas the protein encoded by a similar but slightly different cDNA from tobacco and tomato preferentially accepted quinate with a 20-fold lower K_m value than for shikimate (Niggeweg *et al.*, 2004). The latter enzyme was proposed to be active in chlorogenic acid biosynthesis since over-expression of the cDNA led to an enhanced chlorogenic acid content of the plant material and increased antioxidant activity and pathogen resistance. Down-regulation resulted in 98% decreased chlorogenic acid levels, while the content of other phenolics and of lignin remained unchanged. On the other hand, down-regulation of the shikimate hydroxycinnamoyltransferase resulted in strongly altered lignin content and composition, and thus revealed that this enzyme might be the basic enzyme necessary for introduction of the 3-OH group into

phenylpropanoid moieties and therefore also lignin precursors (Hoffmann *et al.*, 2004, 2005). Similar hydroxycinnamoyltransferases are currently under investigation, e.g. from *Cynara cardunculus* with cynarin (dicaffeoylquinic acid) as one of the main secondary compounds (Comino *et al.*, 2006, 2007) as well as 'rosmarinic acid synthase' from *Coleus blumei* (Berger *et al.*, 2006).

The CYP98 family is responsible for the introduction of 3-hydroxyl groups into 4-coumaroyl moieties (Fig. 4.1). CYP98A3 from *Arabidopsis thaliana* heterologously expressed in yeast was shown to only slowly catalyse the 3-hydroxylation of 4-coumaric acid, while 4-coumaric acid esters, notably shikimate and quinate esters, were converted much more rapidly (Schoch *et al.*, 2001; Nair *et al.*, 2002). A coordinate regulation of the corresponding CYP98 in *Pinus taeda* with other genes involved in monolignol biosynthesis has been shown by Anterola *et al.* (2002). The ref8 mutation in *Arabidopsis thaliana* resulted in a reduced epidermal fluorescence due to reduced levels of sinapate esters. Franke *et al.* (2002a) showed that this was caused by the inability of the mutants to produce caffeic acid; thus the ref8 mutant was traced back to a cytochrome P450 involved in the 3-hydroxylation of 4-coumarate. Down-regulation or mutation of C3H resulted in a shift of hydroxycinnamic acid and hydroxycinnamic acid ester accumulation towards the 4-hydroxy substitution pattern and in reduced lignin contents and altered lignin composition with higher levels of units with 4-hydroxylated aromatic rings (Anterola and Lewis, 2002; Franke *et al.*, 2002b; Reddy *et al.*, 2005).

The number of CYP98 genes varies in plants between one and several, while dicotyledonous plants seem to have evolved a higher degree of diversification (Ehlting *et al.*, 2006).

Recently two CYP98 cDNAs (CYP98A35, CYP98A36) have been isolated from *Coffea canephora* with different substrate preferences and different intron–exon arrangements (Mahesh *et al.*, 2007). Two introns were found in CYP98A35, but only one in CYP98A36. CYP98A35 was able to metabolize 4-coumaroylshikimate and -quinate with the same rate, while the other enzyme only accepted the shikimate ester.

4.2.1.5 Further hydroxylation and methylation reactions at the aromatic ring

According to the above-mentioned hypothesis, the caffeic acid moiety is retransferred to coenzyme A for further modification reactions. Methylation of the caffeoyl moiety in position 3 is achieved by *S*-adenosyl-L-methionine (SAM)-dependent *O*-methyltransferases (OMTs) either acting on the level of the free acid or the coenzyme A thioester. Hydroxylation in position 5 is catalysed by a cytochrome P450 of the CYP84 family which will be described in more detail. Establishment of the sinapoyl substitution pattern by adding another methyl group will be depicted below.

A cytochrome P450 with ferulic acid 5-hydroxylase (F5H) activity was first detected by Grand (1984) in poplar. The gene for this cytochrome P450 defining the new CYP84 family was described and isolated with the help of an

Arabidopsis mutant (*fah1*) defective in the accumulation of sinapic acid esters (Chapple *et al.*, 1992; Meyer *et al.*, 1996). Corresponding genes/cDNAs have successively been isolated from several other plants. In some species more than one F5H gene is present (Nair *et al.*, 2000; Kim *et al.*, 2006a). Biochemical characterization of this hydroxylase showed that coniferaldehyde and coniferyl alcohol are much better substrates than ferulic acid (Humphreys *et al.*, 1999; Osakabe *et al.*, 1999). Thus, the enzyme should be re-named coniferyl aldehyde/alcohol 5-hydroxylase (CA5H) but F5H still is used in the scientific literature. Expression of F5H is generally found in correlation to lignification (Ehlting *et al.*, 2006). Over-expression and down-regulation of the gene resulted in altered lignin composition mainly with respect to the content of syringyl units in lignin. This demonstrated the importance of this reaction in monolignol formation for lignin biosynthesis (see, e.g. Meyer *et al.*, 1998; Franke *et al.*, 2000; Sibout *et al.*, 2002; Higuchi, 2003; Huntley *et al.*, 2003). Sinapic acid ester accumulation in Brassicaceae species is affected as well by the expression of F5H (Ruegger *et al.*, 1999; Nair *et al.*, 2000).

Methylation of the 3- and 5-hydroxyl group in order to achieve the coniferyl or syringyl substitution pattern is catalysed by different *S*-adenosyl-L-methionine (SAM)-dependent OMTs active on the levels of the co-enzyme A thioester (caffeoyl-CoA *O*-methyltransferase, CCoAOMT) or the acid, aldehyde or alcohol (caffeate *O*-methyltransferase, COMT); the latter enzyme was first thought to be active on the hydroxycinnamic acid level, but was later shown to prefer aldehydes and/or alcohols. This corresponds to the finding that not ferulate but coniferaldehyde and/or coniferyl alcohol are the substrates for 5-hydroxylation. COMT can efficiently methylate 5-hydroxyconiferaldehyde to sinapaldehyde (Humphreys *et al.*, 1999; Osakabe *et al.*, 1999). The aldehydes will then be reoxidized to the acids by sinapaldehyde/coniferaldehyde dehydrogenase(s) (Nair *et al.*, 2004) or reduced to the alcohols/monolignols for lignin/lignan biosynthesis. Recent investigations with *Arabidopsis* mutants gave the hint that COMT and CCoAOMT are both involved in 3- and 5-*O*-methylation (Do *et al.*, 2007). An OMT methylating free acid as well as CoA-esters with similar efficiency was reported from a gymnosperm (Li *et al.*, 1997). Nowadays, it seems to be clear that both types of enzymes, COMTs and CCoAOMTs, are involved in the formation of monolignols. While CCoAOMT is preferentially involved in the guajacyl pathway, COMT more strongly affects the formation of syringyl units and may be the evolutionarily younger enzyme (Meng and Campbell, 1998).

A general review on the group of small molecule OMTs was recently published by Noel *et al.* (2003).

COMT (EC 2.1.1.68) was detected as an enzyme methylating caffeic acid to ferulic acid with the help of SAM and later it was shown that 5-hydroxyferulic acid is also methylated to sinapic acid. Mostly, other substrates will be accepted as well (Roje, 2006). Nowadays, however, there are strong indications that the hydroxycinnamic aldehydes and/or alcohols will be preferentially methylated by COMTs (Parvathi *et al.*, 2001). Kinetic investigations of the

aspen enzyme revealed 5-hydroxyconiferyl aldehyde as the best substrate (Li *et al.*, 2000). COMTs belong to the class of small molecule OMTs (SMOMT), usually enzymes with a molecular mass of approximately 40 kDa acting as homodimers. COMT crystals with bound substrates were structurally investigated providing the structural basis for mutational alterations of the substrate preferences (Eckardt, 2002; Zubieta *et al.*, 2002). Two COMT classes (I and II) were defined in tobacco (Maury *et al.*, 1999). In many plant species, down-regulation of COMT was used to reduce lignin levels, e.g. in order to improve digestibility (see, e.g. Chen *et al.*, 2004). Especially S lignin units were virtually absent after strong down-regulation of COMT in alfalfa (Guo *et al.*, 2001) or poplar (Jouanin *et al.*, 2000). A COMT knockout mutant of *Arabidopsis* was affected in lignin as well as in sinapate ester levels (Goujon *et al.*, 2003a).

CCoAOMT (EC 2.1.1.104) transfers the methyl group of SAM to the aromatic 3-OH group of caffeoyl-CoA and the 5-OH group of 5-hydroxyferuloyl-CoA, thus forming feruloyl-CoA and sinapoyl-CoA, respectively. Generally, however, caffeoyl-CoA is strongly preferred as a substrate, and nowadays a physiological role in methylation of 5-hydroxyferuloyl-CoA seems unlikely (Ye *et al.*, 2001). The enzyme was first detected in elicited carrot and parsley suspension cultures in 1988/89 (Matern *et al.*, 1988; Kühnl *et al.*, 1989; Pakusch *et al.*, 1989) and cloned after purification from parsley by Schmitt *et al.* (1991). Afterwards, CCoAOMTs were cloned from many other plants (see Ye *et al.*, 2001). In tobacco, three different classes were defined (Maury *et al.*, 1999). Except for the SAM and metal binding sites, the sequence similarities to COMTs on amino acid levels are very low, but interestingly similarities to mammalian catechol OMTs were detected (Ye *et al.*, 2001). The crystal structure of CCoAOMT from *Medicago sativa* has been solved, showing that the enzyme is active as a homodimer of a 28 kDa protein. Sequence comparison as well as structural features showed that the enzyme belongs to a structurally and mechanistically distinct family of plant SMOMTs (Ferrer *et al.*, 2005). As for mammalian catechol OMTs, the reaction mechanism is dependent on divalent cations (Ibrahim *et al.*, 1998; Ferrer *et al.*, 2005). In certain plants, CCoAOMT expression is observed mainly in correlation with lignification, although a role in plant defence was suggested as well. As an example, a coordinate induction of CCoAOMT and hydroxycinnamoyl-CoA:hydroxyanthranilate hydroxycinnamoyltransferase activities finally leading to avenanthramide phytoalexins was observed in oat after treatment with victorin or *Puccinia coronata* (Yang *et al.*, 2004). Similarly, CCoAOMT and stilbene synthase activities were induced by elicitor or salicylic acid treatments in grapevine (Busam *et al.*, 1997). In some cases, CCoAOMT down-regulation resulted in lower total lignin contents and altered lignin composition (see, e.g. Meyermans *et al.*, 2000; Zhong *et al.*, 2000; Pincon *et al.*, 2001; Lu *et al.*, 2004). In *Medicago sativa*, however, neither the formation of S lignin units nor the levels of cell wall-bound ferulate were affected by CCoAOMT down-regulation (Chen *et al.*, 2006).

Two OMTs methylating specifically the outer hydroxyl groups of a 3,4,5-trihydroxylated aromatic ring (like, e.g., in gallic acid) have been described

from *Vanilla planifolia*. These enzymes showed similarities to caffeate OMTs, and an evolutionary origin in caffeate OMTs was suggested (Li *et al.*, 2006). COMTs isolated from alfalfa displayed higher affinities towards benzaldehyde derivates compared to caffeic or 5-hydroxyferulic acids, thus showing multi-functionality in phenylpropanoid metabolism (Kota *et al.*, 2004). Acceptance of such different substrates as caffeoyl-CoA, caffeoyl glucose and flavonols was shown for the Mg^{2+}-dependent *O*-methyltransferase cloned and heterologously expressed from *Mesembryanthemum crystallinum* and a novel subclass of the CCoAOMT family was proposed (Ibdah *et al.*, 2003).

4.2.1.6 Metabolic channelling in phenylpropanoid metabolism

Most enzymes of phenylpropanoid metabolism have been characterized as 'soluble' enzymes with the exception of the mainly ER-located cytochrome P450-dependent enzyme systems. Solubility of a protein after cell disruption must, however, not indicate that the proteins move freely in the cytoplasm or respective organelle, and binding of a substrate is more or less random. Already, Stafford (1974) proposed that the enzymes of phenylpropanoid metabolism may be organized in complexes, and in the 1980s it was suggested that soluble enzymes may be associated to membrane-bound ones in order to channel metabolites through the biosynthetic pathway (Hrazdina and Jensen, 1992, and the literature cited therein). This might be the explanation for sometimes surprisingly low substrate specificities of enzymes (Winkel, 2004): if they are organized in 'metabolons', they are served with specific substrates by neighbouring enzymes and must not express own high substrate specifity. On the other hand, the low effectivity of some precursor feeding experiments can be explained by such a channelling as well (Dixon *et al.*, 2001). In phenylpropanoid biosynthesis, C4H and F5H may be the anchoring enzymes since these cytochrome P450s are attached by their N-terminal domain into ER-membranes. For PAL from tobacco, different isoform families seem to be localized to different compartments, PAL-1 to the ER and the cytosol and PAL-2 to the cytosol (Rasmussen and Dixon 1999; Achnine *et al.*, 2004). Similarly, different isoforms of 4CL displaying different expression patterns and substrate specificities may be involved in such a 'metabolon' formation (Ehlting *et al.*, 1999). Metabolic channelling involving coniferaldehyde 5-hydroxylase and caffeic acid 3-*O*-methyltransferase in the biosynthesis of syringyl monolignols via coniferaldehyde was suggested by Guo *et al.* (2002) in *Medicago sativa*. Aspects of metabolic channelling in natural product biosynthetic pathways have been reviewed recently by Dixon *et al.* (2001), Winkel (2004) and Jörgensen *et al.* (2005).

4.2.2 Formation of hydroxycinnamate conjugates

Important hydroxycinnamic acid conjugates from plants are hydroxycinnamic acid esters and hydroxycinnamic acid amides (see Petersen *et al.*, 1999).

Formation of hydroxycinnamic acid esters is catalysed by three different enzyme classes and from three differently activated forms of hydroxycinnamic acids. Acyltransferases belonging to the superfamily of BAHD acyltransferases have been detected in fungi and plants (St Pierre and De Luca, 2000; D'Auria, 2006). They use acids activated as coenzyme A thioesters (e.g. acetyl-CoA, hydroxycinnamoyl-CoA, benzoyl-CoA, anthraniloyl-CoA) as acyl donors. Acyltransferases of this type are widely active in plant secondary metabolism, for instance in the biosynthesis of hydroxycinnamoylshikimate and -quinate (Hoffmann *et al.*, 2003; Niggeweg *et al.*, 2004), rosmarinic acid (Berger *et al.*, 2006), fragrance compounds (Gang, 2005), acylated anthocyanins (Nakayama *et al.*, 2003), alkaloids (e.g. Bayer *et al.*, 2004) or the diterpenoid alkaloid paclitaxel (e.g. Walker *et al.*, 2002). A recent compilation is found in D'Auria (2006). First crystal structures of enzymes of this class were solved for vinorine synthase (an acetyltransferase; Ma *et al.*, 2005) and for an anthocyanin malonyltransferase (Unno *et al.*, 2007) showing the involvement of the conserved sequence motif HxxxD(G) in catalysis, but a more peripheral situation of the second conserved motif DFGWG.

The second group of acyltransferases uses 1-*O*-β-acetal esters of glucose as acyl donors. Enzymes of this class are involved in the formation of, e.g., sinapoylmalate or sinapoylcholine in Brassicaceae (Lehfeldt *et al.*, 2000; Shirley and Chapple, 2003; Milkowski *et al.*, 2004), acylated betacyanins in *Chenopodium rubrum* (Bokern *et al.*, 1992) or diacylated glucose in tomato (Li and Steffens, 2000). The substrate for these acyltransferases, sinapoylglucose, is formed by UDP-glucose:sinapate glucosyltransferase which was cloned from *Brassica napus* (Milkowski *et al.*, 2000a) and which also accepts other cinnamic acids. Different (hydroxy)cinnamic acid glucosyltransferases cloned from *Arabidopsis thaliana* revealed more distinct substrate specificities (Milkowski *et al.*, 2000b). Molecular investigations have shown that the acyltransferases have evolved from serine carboxypeptidases, and they have therefore been named serine carboxypeptidase-like acyltransferases (see reviews by Steffens, 2000, and Milkowski and Strack, 2004). Molecular analyses as well as immunological investigations have shown that these enzymes are targeted to the vacuole (Hause *et al.*, 2002). Modelling of the protein structures of 1-*O*-sinapoyl-β-glucose:l-malate sinapoyltransferase (SMT) and 1-*O*-sinapoyl-β-glucose:choline sinapoyltransferase (SCT) led to the identification of the catalytic triad (Ser–His–Asp) and of amino acid residues crucial for substrate positioning (Stehle *et al.*, 2006/2007). A survey of the genome of *Arabidopsis thaliana* revealed 51 genes encoding serine carboxypeptidase-like enzymes. A clade of 19 quite similar genes being specific for plants comprised several sinapoyltransferases utilizing sinapoylglucose involved in secondary metabolism (Fraser *et al.*, 2007).

In several cases, it has been shown that the same product is formed by different types of acyltransferases in different plant species. Chlorogenic acid is formed by an acyltransferase of the BAHD superfamily

from hydroxycinnamoyl-CoA in most species, but from hydroxycinnamoylglucose in *Ipomoea batatas* (Villegas and Kojima, 1986). Caffeoylglucarate is similary synthesized from caffeoyl-CoA in rye (Strack *et al.*, 1987a), but from 1-*O*-caffeoylglucose in *Cestrum elegans* (Strack *et al.*, 1988). In addition, transacylation of hydroxycinnamic acid esters is possible (see below) since caffeoylglucarate and -galactarate are synthesized with chlorogenate as caffeoyl donor in *Lycopersicon esculentum* (Strack *et al.*, 1987b; Strack and Gross, 1990).

The above-mentioned hypothesis (see Section 4.2.1.4) that 4-coumaroylshikimate is hydroxylated to caffeoylshikimate and then the caffeoyl moiety is re-transferred to coenzyme A by the same acyltransferase is an example for caffeoylshikimate as a caffeoyl donor. As already indicated before, this is also true for the formation of caffeoylglucarate and -galactarate in tomato (Strack *et al.*, 1987b; Strack and Gross, 1990). Also dicaffeoylquinic acids can be formed by caffeoyl transfer from chlorogenic acid to another chlorogenic acid molecule in sweet potato (Kojima and Kondo, 1985; Villegas *et al.*, 1987).

Hydroxycinnamic acid amides are, to our knowledge, only formed from CoA-activated hydroxycinnamic acids (Strack and Mock, 1993). The first hydroxycinnamoyltransferase from plants transferring hydroxycinnamoyl or benzoyl residues from the corresponding CoA-esters to anthranilate was cloned from *Dianthus caryophyllus* (Yang *et al.*, 1997, 1998). This enzyme is involved in the formation of dianthramides, the carnation phytoalexins. It represents the 'H' in the name of the enzyme class of BAHD acyltransferases (D'Auria, 2006). Hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyltransferase (HHT, EC 2.3.1.) from *Avena sativa*, which catalyses the last step in the formation of avenanthramides, is a member of the same enzyme class (Matsukawa *et al.*, 2000). cDNAs encoding different isoforms of these enzymes were isolated from oat (Yang *et al.*, 2004). Hydroxycinnamoyl agmatine hydroxycinnamoyltransferases represent a novel class of hydroxycinnamoyltransferases (agmatine coumaroyl transferase, ACT, EC 2.3.1.64; Burhenne *et al.*, 2003; Kristensen *et al.*, 2004). Corresponding cDNAs were isolated from barley and wheat. Spermine/spermidine *N*-hydroxycinnamoyltransferase activities are involved in the biosynthesis of aphelandrine, a polyamine alkaloid from *Aphelandra tetragona* consisting of a spermine and two hydroxycinnamic acid units (Hedberg *et al.*, 1996). The formation of *N*-hydroxycinnamoylamines is induced by infection and/or wounding in Solanaceae or maize (e.g. Pearce *et al.*, 1998). This reaction is catalysed by hydroxycinnamoyl-CoA:tyramine *N*-hydroxycinnamoyltransferases (THT; EC 2.3.1.110; Negrel and Martin, 1984; Negrel and Javelle, 1997) that are encoded by a gene family which does not show similarity to the BAHD gene family, but to mammalian diamine acetyltransferases. These enzymes accept various donor and acceptor molecules (Farmer *et al.*, 1999; Schmidt *et al.*, 1999; Ishihara *et al.*, 2000). Main products are 4-coumaroyltyramine and feruloyltyramine, but also cinnamoyl-, caffeoyl-, sinapoyl-CoA and tryptamine were accepted. In *Capsicum annuum* a separate

serotonin *N*-hydroxycinnamoyltransferase (SHT) was detected synthesizing 4-coumaroylserotonin and feruloylserotonin (Kang *et al.*, 2006).

4.2.3 Phenylpropanoid-derived aroma and fragrance compounds

The smell and taste of plants rely on aroma and fragrance compounds, many of which (besides the terpenoids) are derived from phenylpropanoid metabolism. In food and cosmetic industry, such fragrance and aroma compounds play an important economical role. Simple phenolic fragrance compounds are, e.g., eugenol, isoeugenol or (methyl)chavicol (Fig. 4.2), the biosynthesis of which has been clarified recently; more complex compounds are phenolic esters. Evolutionary aspects of the biosynthesis of flavours and scents have been reviewed by Gang (2005).

Eugenol and isoeugenol are synthesized via coniferyl alcohol which is acylated with acetate (from acetyl-CoA; Dexter *et al.*, 2007) and then reductively cleaved by eugenol or isoeugenol synthase to form eugenol or isoeugenol, respectively (Koeduka *et al.*, 2006). Similarly, 4-coumaryl alcohol is esterified and the ester cleaved in dependence on NAD(P)H to achieve chavicol which can then be methylated to methylchavicol (Vassao *et al.*, 2006). In *Clarkia breweri*, four phenylpropanoids ((iso)eugenol, methyl(iso)eugenol) are members of the strong and sweet smell emitted by the flowers. A SAM-dependent methyltransferase methylates the aromatic 4-hydroxyl groups of (iso)eugenol and thus forms methyl(iso)eugenol (Wang *et al.*, 1997; Wang and Pichersky, 1998). This methyltransferase was proposed to have evolved by gene duplication from COMT (Barkman, 2003). A similar (iso)eugenol-methylating enzyme was cloned from roses (Wu *et al.*, 2003). An independent evolution was suggested for chavicol *O*-methyltransferase and eugenol *O*-methyltransferase from *Ocimum basilicum* catalysing the formation of methylchavicol and methyleugenol, respectively. The nucleotide sequences showed high similarity to isoflavone OMTs and low similarity to the methyltransferases from *Clarkia*. Mutational change of only one

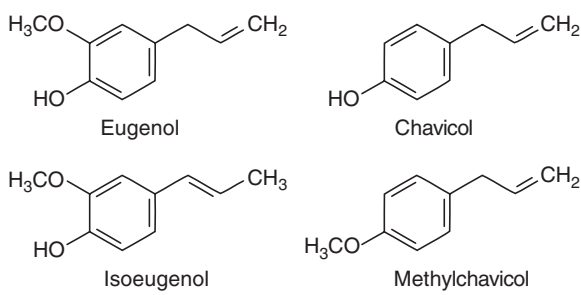


Figure 4.2 Structures of some phenylpropanoid-derived fragrance compounds.

amino acid (as previously predicted to be involved in substrate binding by molecular modelling) in eugenol and chavicol OMTs from basil were sufficient to interchange the substrate specificities (Gang *et al.*, 2002).

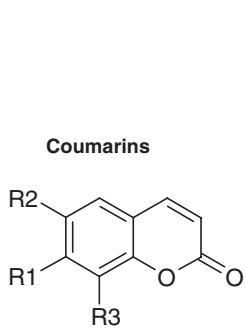
Other fragrance and aroma compounds derived from phenylalanine are phenylethyl alcohol, phenylacetaldehyde and phenylethyl acetate or benzenoid compounds like methyl benzoate, benzyl alcohol, benzylaldehyde and benzyl benzoate (Boatright *et al.*, 2004; Dudareva *et al.*, 2004; Kaminaga *et al.*, 2006; Tieman *et al.*, 2006). A number of acyltransferases (belonging to the above-mentioned BAHD superfamily of acyltransferases) have been identified that are active in the formation of different esters, mostly displaying rather broad substrate specificities and thus leading to diverse products, e.g. acetyl-CoA:benzyl alcohol acetyltransferase or benzoyl-CoA:benzyl alcohol benzoyltransferase from *Clarkia breweri* (Dudareva *et al.*, 1998; D'Auria *et al.*, 2002) or melon acyltransferases producing typical volatiles (El-Sharkawy *et al.*, 2005). Transgenic *Petunia* flowers carrying a rose acyltransferase gene produced benzyl and phenylethyl acetates (Guterman *et al.*, 2006).

Methylcinnamate and methyl-4-coumarate as widely distributed floral scent and communication compounds are synthesized by a carboxyl methyltransferase belonging to the SABATH family. The enzyme from *Ocimum basilicum*, where three isoforms were detected, preferably accepts cinnamate and 4-coumarate as substrates (Kapteyn *et al.*, 2007).

4.3 Coumarins

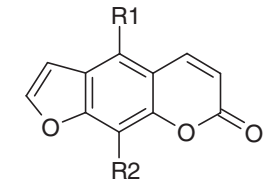
4.3.1 Classification and recent advances

Naturally occurring coumarins are classified by their benzopyran-2-on nucleus (Fig. 4.3) and have been isolated from numerous plants, particularly among the Apiaceae, Rutaceae and Ficaceae as well as from some genera of the Fabaceae. A comprehensive collection of structures was published recently (Murray, 2002) as an update of previous publications (Murray *et al.*, 1982; Estévez-Braun and González, 1997; Murray, 1997; Malikov and Saidkhodzhaev, 1998), but new derivatives are still being added to the list (e.g. Kuo *et al.*, 2004). Plant coumarins originate from the shikimate and general phenylpropanoid pathways yielding cinnamic acid as the immediate product (Murray *et al.*, 1982) which is derivatized in various ways, and the pattern of coumarins was proposed occasionally as a parameter of taxonomic identification (Zschocke *et al.*, 1998; Herde, 2005). Furthermore, the glucosidation-type of coumarins might be a characteristic for a plant family (Nasipuri and Ramstad, 2006). This chapter is confined to plant-produced coumarins and focuses on the most recent findings concerning their biosynthesis and evolution. The few coumarins reported from microorganisms arise through the polyketide pathway (Inoue *et al.*, 1989) and will not be considered any further.

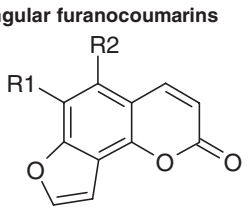


Isopimpinellin

R1 = R2 = R3 = H	Coumarin
R1 = OH, R2 = R3 = H	Umbelliferone
R1 = OCH_3, R2 = R3 = H	Herniarin
R1 = R2 = OH, R3 = H	Esculetin
R1 = OH, R2 = OCH_3, R3 = H	Scopoletin
R1 = OGlc, R2 = OCH_3,R3 = H	Scopolin
R1 = OCH_3, R2 = OH, R3 = H	Isoscopoletin
R1 = R2 = OCH_3, R3 = H	Scoparone
R1 = R3 = OH, R2 = H	Daphnetin
R1 = OH, R3 = OCH_3, R2 = H	Hydrangetin
R1 = R3 = OH, R2 = OCH_3	Fraxetin
R1 = OH, R2 = R3 = OCH_3	Isofraxidin



R1 = R2 = H	Psoralen
R1 = OCH_3, R2 = H	Bergapten
R1 = OCH_2-CH=CMe_2; R2 = H	Imperatorin
R1 = H, R2 = OCH_3	Xanthotoxin
R1 = R2 = OCH_3	



R1 = R2 = H	Angelicin
R1 = OCH_3, R2 = H	Sphondin
R1 = R2 = OCH_3	Pimpinellin

Figure 4.3 Structures of coumarins, linear furanocoumarins and angular furanocoumarins.

A multitude of bioactivities have been ascribed to coumarins, most of which were reviewed before (see Keating and O'Kennedy, 1997; Matern *et al.*, 1999; Petersen *et al.*, 1999; Bourgaud *et al.*, 2006). Nevertheless, new aspects are still being disclosed, such as the effect of daphnetin (Fig. 4.3) on the differentiation of human renal carcinoma cells (Finn *et al.*, 2004) or as an iron chelator against *Pneumocystis carinii* (Ye *et al.*, 2004), which also applies to other hydroxycoumarins (Fylaktakidou *et al.*, 2004; Lacy and O'Kennedy, 2004) and prenyloxy-coumarins (Curini *et al.*, 2006). Metal complexation was generally proposed to enhance the antibacterial and antifungal activities of coumarins (Rehman *et al.*, 2005). Furthermore, the larvicidal activity of pimpinellin (Fig. 4.3) and its photodimerization product against *Aedes aegypti* were recognized (De Oliveira *et al.*, 2005), while the antifungal activity of angular furanocoumarins was studied in a systematic approach (Sardari *et al.*, 1999). The differential response of linear and angular furanocoumarins (Fig. 4.3) in psoralen plus UV-A (PUVA)-induced apoptosis (Viola *et al.*, 2004) was reported. While linear furanocoumarins (psoralens) show impressive antiproliferative activity, modulate chloride secretion (Devor *et al.*, 1997), and inhibit the metalloproteinase-2 secretion from brain tumor cells (Ngameni *et al.*,

2006), angelicin (Fig. 4.3) was a powerful inducer of erythroid differentiation (Lampronti *et al.*, 2003). The bioactivities of coumarins, however, also raised toxicological concerns. Coumarin which is present in a number of plants used as spice and medicinal herb, i.e. cinnamon, or for cosmetic purposes is a potentially harmful compound causing liver damage and other failures. This has recently caused a formal initiative (opinion) by the EU Scientific Committee On Consumer Products at the European Commission (eighth plenary meeting of 20 June 2006; SCCP/0935/05). The problem seemed even more pronounced in coumarin-producing vegetables, i.e. celery root or petiole and wild parsnip accumulating linear furanocoumarins. Nevertheless, negligible plasma levels were measured after extensive celery consumption (Gral *et al.*, 1993), and sensitive methodologies have also been developed for esculetin and daphnetin quantitation in plasma and urine (Egan *et al.*, 2003).

A particularly fascinating field of research concerns the ecotoxicological relevance and the cost of furanocoumarin biosynthesis in terms of reproductive fitness (Carroll and Berenbaum, 2006). Insect herbivores colonizing furanocoumarin-producing plants developed resistance by faster and more efficient metabolism of these compounds (Berenbaum and Zangerl, 1998; Nitao *et al.*, 2003). This phenomenon was studied in the black swallowtail caterpillar (*Papilio polyxenes*) feeding primarily on host plants of the Apiaceae and Rutaceae families and in the parsnip webworm, *Depressaria pastinacella*. Both species metabolize psoralens, i.e. xanthotoxin (Fig. 4.3), through a pathway of oxidative furan-ring fission involving cytochrome P450 monooxygenases, CYPs (Nitao *et al.*, 2003). In *Papilio polyxenes*, the isozyme CYP6B1 specifically breaks down psoralens, whereas the analogous catabolism of the angular furanocoumarin angelicin (Fig. 4.3) was shown to occur less readily (Ivie *et al.*, 1986) and likely involves CYP6B3 attacking both linear and angular furanocoumarins (Hung *et al.*, 1995). Conserved amino acids in the substrate recognition sites (SRS) nos 1, 4 and 6 in CYP6B1 were considered critical for coumarin catabolism (Chen *et al.*, 2002). In *Depressaria pastinacella*, however, the catabolism of angular furanocoumarins does not affect the furan moiety but proceeds primarily by dealkylation of *O*-alkylated furanocoumarins, e.g. sphondin and imperatorin (Fig. 4.3) (Nitao *et al.*, 2003; Mao *et al.*, 2006, 2007), and allelic variation of CYP6AB3 was suggested to affect the rate of imperatorin dealkylation (Mao *et al.*, 2007). A molecular model of CYP6AB3v1 revealed the three-dimensional similarity of SRS1, 4 and 6 to the corresponding catalytic sites in CYP6B of *Papilio polyxenes* (Mao *et al.*, 2007). Photo-activation was proposed to be important for the overall toxicity of furanocoumarins. However, angular furanocoumarins are generally considered less phototoxic (Lampronti *et al.*, 2003). While numerous plant species in the Apiaceae or Rutaceae produce exclusively psoralens, the accumulation of angular furanocoumarins always occurs concomitant with psoralens, and thus the angular biosynthetic pathway was proposed to have evolved later than the linear pathway. It is conceivable that genes of the angular pathway developed from the corresponding genes for the production of psoralens

and angular furanocoumarins may contribute to the defence against herbivores by inhibition of furanocoumarin detoxifying enzymes (Berenbaum and Zangerl, 1998).

The accumulation of identical furanocoumarins in multiple unrelated families suggests that the biosynthetic capacity has evolved independently several times (Berenbaum and Zangerl, 1996) and that relevant enzymes are related to ubiquitous enzymes of the general plant phenolic metabolism. So far, however, the mechanistic details of coumarin biosynthesis are incompletely understood. Major steps forward have been accomplished only very recently and are summarized below, while for the basic information the reader is referred to the literature (Murray *et al.*, 1982; Hakamatsuka *et al.*, 1991; Keating and O'Kennedy, 1997; Matern *et al.*, 1999; Petersen *et al.*, 1999; Bourgaud *et al.*, 2006). The classification of coumarins is problematic and follows mostly a biogenetic scheme which distinguishes the monomeric from oligomeric compounds and considers the number and position of oxygen atoms attached to the coumarin nucleus as well as the length and the form of carbon side chains (Murray *et al.*, 1982; Estévez-Braun and González, 1997; Murray, 2002). For simplicity, in this chapter coumarin derivatives which include hydroxylated, alkoxylated or alkylated and glucosidic benzopyran-2-ons are distinguished from furanocoumarins. Numbering of the carbon skeleton refers to Fig. 4.4.

4.3.2 Coumarin derivatives

4.3.2.1 Coumarin and umbelliferone

The biosynthesis of coumarin and umbelliferone starts out from L-phenylalanine which is converted to *trans*-cinnamic and *trans*-4-coumaric acid (Fig. 4.4) through the shikimate and general phenylpropanoid pathways (Knaggs, 2003; Ro and Douglas, 2004). While the shikimate pathway was assigned to the plastids (Schmid and Amrhein, 1995; Herrmann and Weaver, 1999), the enzymes of the general phenylpropanoid pathway, PAL, cinnamate 4-hydroxylase (C4H) and 4-coumarate:CoA-ligase (4CL), are considered to be active in the cytosol as soluble entities (PAL, 4CL) or bound to the endoplasmic reticulum (C4H). C4H genes from coumarin-producing *Petroselinum crispum* (Koopmann *et al.*, 1999), *Ammi majus* (Hübner *et al.*, 2003) or *Ruta graveolens* (Gravot *et al.*, 2004) have been cloned and expressed in yeast cells, and the translated polypeptides share high sequence similarity; i.e. sequences from *Petroselinum* and *Ammi* differ by only seven residues (five conservative exchanges). C4H is a cytochrome P450 monooxygenase (CYP), and psoralens are known to affect various CYPs by mechanism-based inhibition (Gravot *et al.*, 2004); however, enhanced psoralen tolerance was documented for C4Hs from coumarin-producing plants. In addition, two 4CL genes were cloned very recently from *Ruta graveolens* and shown to encode fully functional soluble enzymes, but one of these sequences was flanked N-terminally by a typical plastid transit peptide (Endler *et al.*, 2008). The latter enzyme was predominantly expressed in the stem and flower tissues, less in the leaf

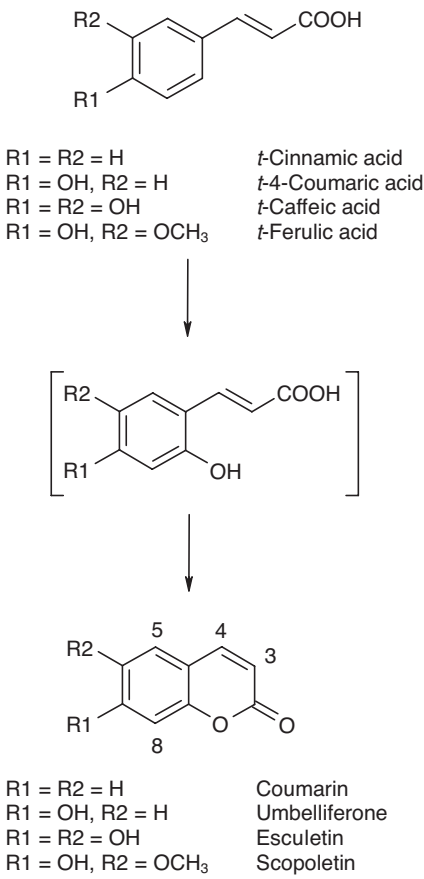


Figure 4.4 Proposed formation of coumarins from cinnamic acids.

and minimally in the root of *Ruta graveolens* plants. Although the sub-cellular location of this 4CL still requires verification, the compartmentalization correlates to a recent report assigning a 4CL-like enzyme to peroxisomes in *Arabidopsis* (Koo *et al.*, 2006). Unfortunately, a rate-limiting step for coumarin biosynthesis has not been identified and the relevance of 4CL in this context is still a matter of discussion. The cyclization of the cinnamoyl or 4-coumaroyl moiety to benzopyran-2-on is the pivotal reaction in the coumarin pathway, but the mode of cyclization and the immediate substrate in this process have remained elusive.

The cyclization proposal for cinnamic acid and 4-coumaric acid, respectively, to yield coumarin or umbelliferone is firmly established in the literature (Murray *et al.*, 1982; Bourgaud *et al.*, 2006), although very few plants have the potential to hydroxylate coumarin to umbelliferone (Fig. 4.4) (Murray *et al.*, 1982). The cyclization depends most likely on the intermediate

2′-hydroxylation (*ortho*-hydroxylation), which is supported by the formation of *trans*-2′-hydroxycinnamate-2′-*O*-glucoside in *Melilotus alba* mesophyll cells. This glucoside is transported through the tonoplast and then trapped in the vacuole by light-independent isomerization to coumarinyl glucoside (*cis-o*-hydroxycinnamic acid glucoside) (Rataboul *et al.*, 1985). Three laboratories independently reported some 30 years ago the 2′-hydroxylation of cinnamic acids in vitro and ascribed the reaction to chloroplasts from *Melilotus*, *Hydrangea* and *Petunia* (Murray *et al.*, 1982). Unfortunately, these early findings could not be confirmed by subsequent enzymatic studies. The discrepancies, which have been outlined elsewhere (Conn, 1984; Matern *et al.*, 1999; Bourgaud *et al.*, 2006), conceivably resulted from limitations in the separation techniques. Nevertheless, feeding of (*ortho*-^{3}H, *ring*-1-^{14}C)cinnamic acid as a precursor to *Melilotus* or *Gaultheria* had revealed an National Institutes of Health (NIH) shift during *o*-coumaric acid formation suggesting the involvement of a CYP (Ellis and Amrhein, 1971). It is thus possible that an unusually labile monooxygenase is responsible for the *ortho*-hydroxylation of cinnamic or 4-coumaric acid, but experimental proof is still lacking. Other than the '*ortho*-hydroxylation' issue, the hydrolysis of coumarinyl glucoside was investigated further, because upon tissue disruption of *Melilotus alba* a cell wall associated β-glucosidase activity releases coumarinic acid (*cis-o*-coumaric acid), which spontaneously lactonizes to coumarin. cDNAs encoding two coumarin pathway β-glucosidases (GLU1 and GLU2) were cloned by RT-PCR from *Melilotus alba* (Karam, 2001). These enzymes are both 506 amino acids in length, share 89.5% amino acid identity and possess multiple N-glycosylation sites as well as an N-terminal signal sequence. Furthermore, based on their NEP and ITENG motifs, they belong to glycoside hydrolase (GH) family 1. Several mutant *Melilotus alba* lines are available (Vogel *et al.*, 2005), and two allele pairs control the levels of coumarin glucosides (*Cu/cu*) or coumarinyl glucoside β-glucosidases (*B/b*). Together with relative RT-PCR analysis, Northern and Western blottings of the *BB* and *bb* genotypes of *Melilotus* suggested that the *B* gene is a regulatory gene controlling the expression of *GLU1* and *GLU2* genes (Karam, 2001; Tabor, 2001). Supporting this hypothesis, the promoter region of *GLU2*, like *GLU1*, is highly conserved between both genotypes (Laust, 2003). In contrast, high sequence identity between *GLU1* and *GLU2* promotor regions of the *BB* genotype was confined to the 3′-portion of the sequence. The significant differences in upstream sequences may be responsible for the observed 12-fold higher *GLU1* expression (Tabor, 2001). During these studies, three additional closely related β-glucosidase genes (*GLU3*–*GLU5*) were amplified; it remains unknown whether they are actively expressed and involved in coumarin biosynthesis (Laust, 2003). It is noteworthy in this context that the accumulation of *ortho*-hydroxylated cinnamic acids has rarely been reported from plants; i.e. *ortho*-coumaric acid was secreted from jasmonate-treated roots of wild-type *Arabidopsis* (Walker *et al.*, 2003), while untreated roots accumulate scopoletin and scopolin (Fig. 4.3) (Kai *et al.*, 2006).

4.3.2.2 Alkylated umbelliferone

Umbelliferone is the pivotal metabolite in the pathways to substituted coumarins and furano- or pyranocoumarins. Its 7-*O*-methylation to herniarin (Fig. 4.3) or *O*-prenylation and *C*-alkylation reactions are often observed in coumarin-producing higher plants where the prenylation at C-6 or C-8 mark the entry to the routes branching linear from angular furano- and pyranocoumarins (Fig. 4.5). In *Ruta graveolens* and *Ammi majus*, the prenylation reactions are catalysed by particulate enzymes (Hamerski *et al.*, 1990), and

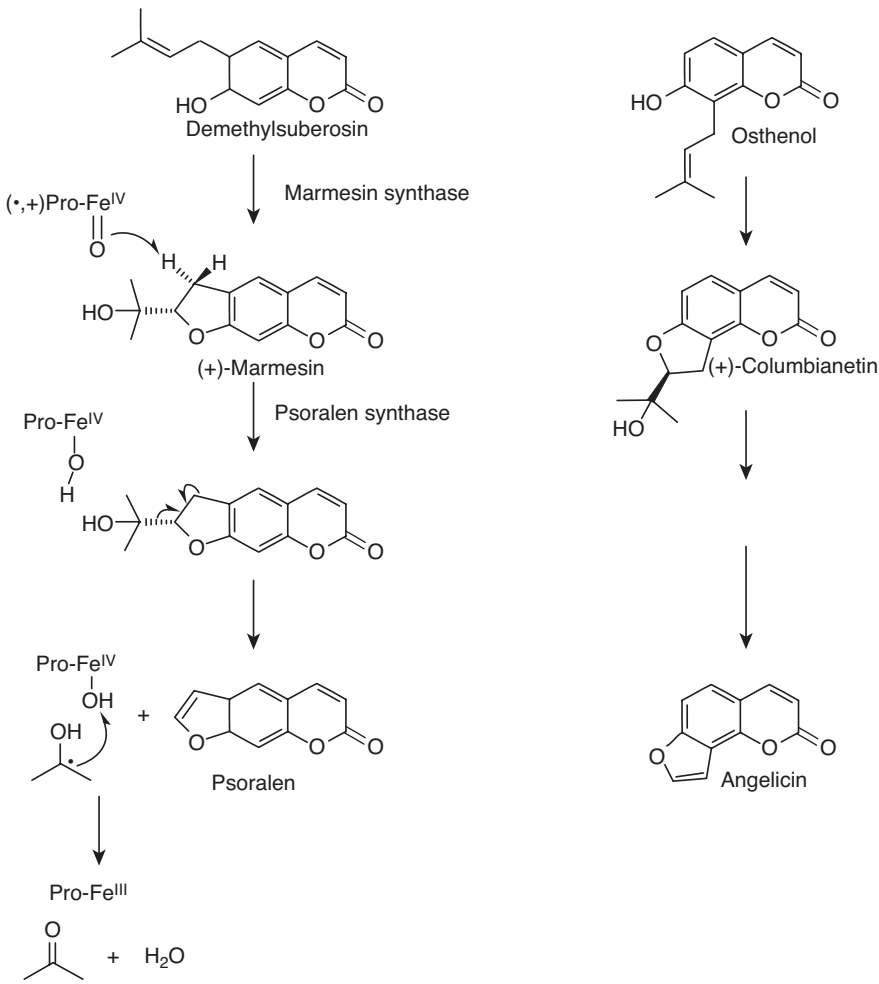


Figure 4.5 Cytochrome P450-dependent reactions forming psoralen from 6-prenylumbelliferone (demethylsuberosin). The analogous pathway yielding angelicin from 8-prenylumbelliferone (osthenol) is shown for comparison, but has not been confirmed in vitro.

some plants, i.e. *Ammi majus*, are capable of catalysing all three prenylation reactions (Hamerski *et al.*, 1990; Elgamal *et al.*, 1993). The first plant prenyltransferase to be characterized in vitro was purified from *Ruta graveolens* (Dhillon and Brown, 1976) accumulating psoralens only. The enzyme was identified as a Mn^{2+}-dependent dimethylallyl diphosphate:umbelliferone 6-*C*-dimethylallyltransferase and assigned to the chloroplast membranes (Dhillon and Brown, 1976). Together with the allocation of the shikimate pathway, this emphasizes the importance of plastids for the accumulation of coumarins.

4.3.2.3 Polyoxygenated coumarins

Umbelliferone may be hydroxylated or methoxylated at the 6- and/or 8-positions yielding the most common polyoxygenated coumarins esculetin, daphnetin, scopoletin and scoparone (Fig. 4.3), and glucosidic conjugates have been reported. Successive hydroxylation and methylation may lead to esculetin or daphnetin and scopoletin/isoscopoletin/scoparone or hydrangetin (Fig. 4.3), while 6- and 8-dihydroxylations and methylation are necessary for the formation of fraxetin and isofraxidin (Fig. 4.3). Coumarins hydroxylated at the 6-, 7- and 8-positions were also reported from *Pelargonium sidoides* (Kayser and Kolodziej, 1995), including 6,7-dihydroxy-coumarin-8-sulfate (Gödecke *et al.*, 2005). While scopoletin and related compounds function as phytoalexins (Serghini *et al.*, 2001; Shimizu *et al.*, 2005; Prats *et al.*, 2006), some of the coumarins may possess unusual bioactivities. For example, hydrangetin was proposed to act as a protein kinase inhibitor (Yang *et al.*, 1999) that modulated the response of rye during high photosystem II excitation and cold acclimation (Ndong *et al.*, 2003).

Two aspects of the biosynthesis received considerable attention recently. Alternative pathways for the biosynthesis of scopoletin in tobacco had been proposed involving the methoxylation of umbelliferone or the cyclization of ferulic acid (Fig. 4.4). Scopoletin may then be glucosylated to scopolin by non-specific glucosyltransferases for storage in the vacuole (Taguchi *et al.*, 2001). *Arabidopsis* mutants were used to determine the contents of scopoletin and scopolin (Fig. 4.3) in the roots. In comparison to wild-type *Arabidopsis*, the level of scopoletin and scopolin was greatly diminished in a mutant that carried a T-DNA insertion within the CYP98A3 gene encoding *p*-coumaroylshikimate/quinate 3′-hydroxylase (C3′H) (Kai *et al.*, 2006). This suggested that the biosynthesis of scopoletin and scopolin depends on the 3′-hydroxylation of *p*-coumarate to caffeate prior to cyclization or *O*-methylation to yield esculetin (Fig. 4.3) and ferulic acid, respectively. The *O*-methylation of esculetin has not been studied in this plant. Nevertheless, broad specificity OMTs may be present, such as the enzymes recombinantly expressed from other sources and shown to methylate esculetin to scopoletin, isoscopoletin and scoparone (Kim *et al.*, 2006b). The coumarins accumulate in the form of their glucosides because *Arabidopsis* encodes numerous glucosyltransferases recognizing hydroxycoumarins (Lim *et al.*, 2003a).

4.3.3 Furanocoumarins

4.3.3.1 The furanocoumarin skeleton

A flow chart of furanocoumarin formation from umbelliferone and dimethylallyl diphosphate was drawn many years ago from precursor feeding studies (Murray *et al.*, 1982). Accordingly, the C-6- or C-8-prenylation of umbelliferone was proposed to yield demethylsuberosin and osthenol (Fig. 4.5) at the branch point to linear and angular furanocoumarins. The assignment of the C-6 prenyltransferase to chloroplast membranes (Dhillon and Brown, 1976) received further support recently by precursor feeding studies in celery which demonstrated the formation of the prenyl moiety in psoralens through the deoxy-D-xylulose pathway (Stanjek *et al.*, 1999a) associated with plastids. It is conceivable that the same applies to angular furanocoumarins, although equivalent incorporation studies have not been undertaken.

The identification of furanocoumarins as phytoalexins and their rapid de novo induction in dark-grown cell cultures of various Apiaceae upon treatment with fungal elicitor (Matern *et al.*, 1999) greatly stimulated more detailed in vitro investigations. Microsomal fractions from induced *Petroselinum crispum* or *Ammi majus* cells were shown to convert demethylsuberosin via (+)-marmesin and psoralen to bergaptol (Fig. 4.5) in the presence of molecular oxygen and NADPH. Kinetic studies with various chemicals known to inhibit CYPs moreover suggested the consecutive action of multiple CYPs in this pathway, which was confirmed through the blue-light reversible inhibition of individual reaction steps by CO (Hamerski and Matern, 1988a,b). Formally, these CYPs catalyse very different reaction steps involving the cyclization of the prenyl side chain, the cleavage of a carbon–carbon bond and the 5-hydroxylation of the coumarin nucleus to bergaptol, which poses interesting mechanistic questions. Moreover, an analogous reaction sequence converts osthenol to angelicin and sphondin (Fig. 4.5), and the superimposable dihydrofuran configuration of (+)-marmesin and (+)-columbianetin (Fig. 4.5) as the immediate precursors of psoralen or angelicin suggests a high level of similarity for psoralen and angelicin synthases. The mechanistic considerations seem to support the assumption that angelicin synthase has evolved from psoralen synthase (Berenbaum and Zangerl, 1998). This might also apply to 'columbianetin synthase' and 'marmesin synthase' forming the dihydrofuran moieties by oxidative cyclization of osthenol or demethylsuberosin (Hamerski and Matern, 1988a; Matern *et al.*, 1999). Whereas angelicin and columbianetin synthase activities have not been recorded in vitro, marmesin synthase assays were accomplished with microsomes from elicited *Ammi majus* or *Petroselinum crispum* cells (Matern *et al.*, 1999). Although P450 monooxygenases likely epoxidize olefins by insertion of an 'oxen' (Bolwell *et al.*, 1994), marmesin synthase catalysed the instant cyclization without release of an intermediate, presumably due to delocalization of the double bond electrons by the 7-hydroxy group. Such an effect appears feasible, at least, from model studies (Halkier, 1996). The biosynthesis of linear dihydropyrano- or

pyronocoumarins which occasionally accumulate concomitantly with psoralens can be explained by a related mechanism (Beier *et al.*, 1994; Matern *et al.*, 1999).

The formation of psoralen from (+)-marmesin was initially considered to proceed via a C-4′ carbocation of (+)-marmesin and subsequent 1,3-elimination of acetone and psoralen (Murray *et al.*, 1982). The release of acetone, however, was unprecedented in plant secondary metabolism, and a two-step oxidation mechanism removing consecutively one and two of the side chain carbons was therefore also considered (Stanjek *et al.*, 1999b); the latter is analogous to the carbon–carbon bond cleavage in steroid metabolism (Halkier, 1996; Ortiz de Montanello and De Voss, 2002). Incubations employing microsomes from elicited cells of *Ammi majus* and other Apiaceae (Matern, 1991) with deuterated marmesin substrates provided an answer (Stanjek *et al.*, 1999b) because the release of acetone from (+)-marmesin was confirmed concomitant with the formation of psoralen. Psoralen synthase was classified as a P450 monooxygenase which abstracts hydrogen from its substrate via an iron(IV)oxo porphyrin cation radical yielding an iron tethered hydroxyl radical (Halkier, 1996; Ortiz de Montanello and De Voss, 2002), and two modes of operation had been envisaged for the psoralen synthase reaction. Homolytic abstraction of one of the 3′-hydrogens from (+)-marmesin and instantaneous disproportionation of the marmesin carbon radical should release psoralen and an isopropyloxy side chain radical which recombines with the enzyme hydroxyl radical to produce acetone and water (Hakamatsuka *et al.*, 1991). Alternatively, the enzyme radical might be used for a 'rebound' hydroxylation reaction (Ortiz de Montanello and De Voss, 2002) to yield 3′-hydroxymarmesin prior to the elimination of psoralen and acetone. The latter assumption would require the proper 3′-configuration for base-catalysed *anti*-elimination (Zou *et al.*, 2005). However, no intermediate was observed in psoralen synthase incubations. Moreover, *syn*-eliminations were exclusively observed in the formation of psoralen from (+)-marmesin (Stanjek *et al.*, 1999b) or angelicin from columbianetin (Stanjek and Boland, 1998). Therefore, the plausible mechanism of the psoralen synthase reaction is presented with a carbon radical intermediate (Stanjek *et al.*, 1999b) (Fig. 4.5), although the nonconcerted reaction mechanism (hydrogen abstraction preceding carbon–carbon bond cleavage and hydroxylation) has not yet been established.

The psoralen synthase gene from *Ammi majus* was recently cloned and expressed in yeast cells (Larbat *et al.*, 2007). The cDNA was isolated as one of several CYPs differentially expressed in dark-grown *Ammi* cells upon treatment with fungal elicitor, and the ephemeral transcript abundance showed a sharp maximum at 4 h. The gene was classified as *CYP71AJ1* and represents the first cloned monooxygenase sequence committed to coumarin biosynthesis. Several obstacles had to be overcome for functional identification; e.g. the expression of the genuine sequence was negligible in yeast cells and the activity of the recombinant enzyme was rather labile as had been observed before with

microsomal fractions from elicited *Ammi majus* cells (Hamerski and Matern, 1988a). Therefore, the N-terminal membrane anchor sequence was replaced by that of CYP71A1 (C4H from *Helianthus tuberosus*) and assays were run with fresh microsomes only. Most notably, the recombinant enzyme was specific for (+)-marmesin and did not release any intermediate. (+)-Columbianetin (Fig. 4.5), the substrate of angelicin synthase, however, competitively inhibited psoralen synthase activity. Preliminary homology modelling suggested that the distance of the 3′-hydrogens of (+)-columbianetin to the active site iron(IV)oxo porphyrin cation radical exceeds 6 Å which is too far for the radical interaction (Larbat *et al.*, 2007). Nevertheless, it is to be expected that homology cloning might also give access to monooxygenases of the angular furanocoumarin pathway.

4.3.3.2 Oxygenated psoralens

The hydroxylation of psoralen at the 5- and/or 8-positions is most likely necessary for the formation of bergaptol (5-hydroxypsoralen), xanthotoxol (8-hydroxypsoralen) and 5,8-dihydroxypsoralen, because 5-hydroxymarmesin was neither produced in incubations with microsomes from elicited *Ammi majus* cells (Hamerski and Matern, 1988b) nor readily accepted by the recombinant psoralen synthase (Larbat *et al.*, 2007), and the resulting phenols are methylated to bergapten, xanthotoxin and isopimpinellin (Fig. 4.6). 5,8-Dihydroxypsoralen is a labile hydroquinone assumed as an intermediate in isopimpinellin biosynthesis, but the sequence of hydroxylations and methylations has not been unravelled (Murray *et al.*, 1982). Conceivably, angular methoxyfuranocoumarins, i.e. sphondin (Fig. 4.6), arise by analogous consecutive hydroxylation and methylation of angelicin, but experimental proof is lacking. Only one of these hydroxylases has so far been characterized in vitro. Psoralen 5-monooxygenase was described as a particulate P450 monooxygenase from elicited *Ammi majus* cells and clearly distinguished from marmesin and psoralen synthases (Hamerski and Matern, 1988b). The enzyme exclusively converts bergaptol to psoralen, thus suggesting additional hydroxylases for the formation of 8-hydroxypsoralen (xanthotoxol) and 5,8-dihydroxypsoralen or its monomethylether.

Bergapten, xanthotoxin and isopimpinellin accumulate in various species of the Apiaceae (Beier *et al.*, 1994; Manderfeld *et al.*, 1997; Ekiert and Gomolka, 2000; Abu-Mustafa *et al.*, 2006) or Rutaceae (Poutaraud *et al.*, 2000; Milesi *et al.*, 2001) and at least two distinct OMTs methylate bergaptol or xanthotoxol to bergapten (BMT) and xanthotoxin (XMT) (Fig. 4.6). Both OMTs were purified from *Ruta graveolens* (Murray *et al.*, 1982) and *Petroselinum crispum* (Hauffe *et al.*, 1986). Precursor feeding studies with *Ruta graveolens* plants, however, revealed that bergapten or xanthotoxin may be converted further to isopimpinellin with a slight bias towards xanthotoxin, posing the question for another set of OMTs as well as bergapten 8- and xanthotoxin 5-hydroxylases. The *Petroselinum* XMT showed marginal OMT activity only

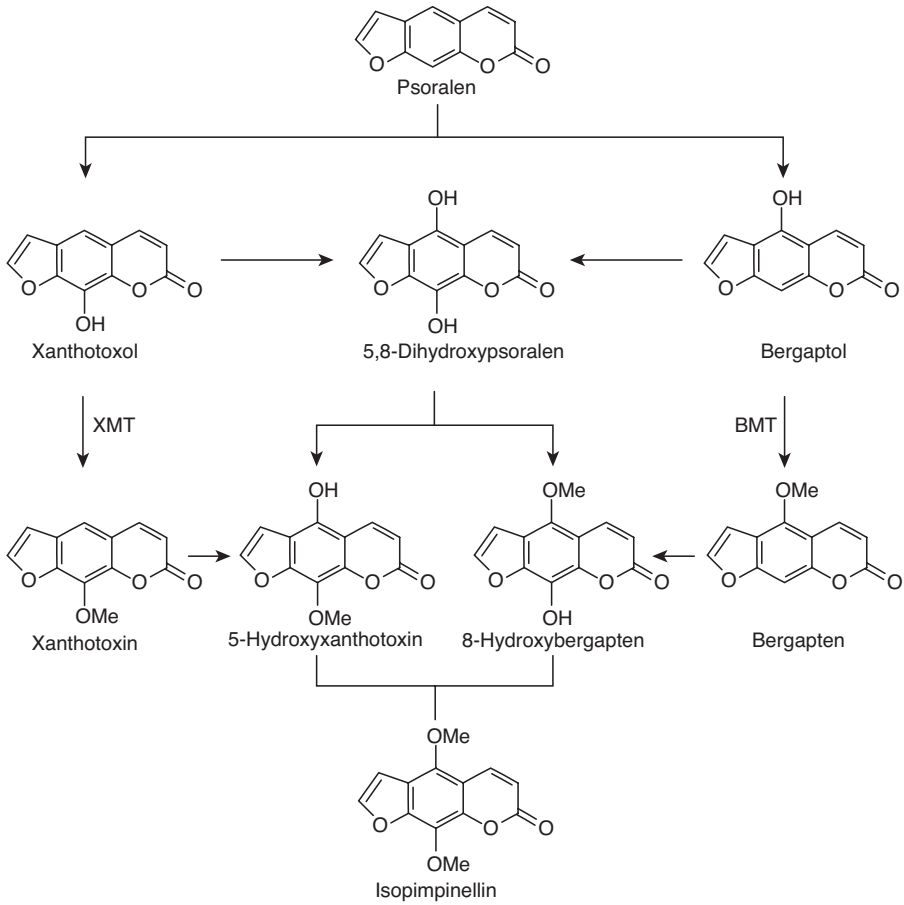


Figure 4.6 Schematic routes leading from psoralen to isopimpinellin.

with 5-hydroxyxanthotoxin, 8-hydroxybergapten, bergaptol and daphnetin as a substrate, whereas the corresponding BMT catalysed considerable 5- and 8-*O*-methylations of 5,8-dihydroxypsoralen with a pronounced preference for 5-hydroxyxanthotoxin over bergaptol. These ambiguous results do not illuminate the pathway to isopimpinellin, particularly because the methylation of coumarins by broad substrate OMTs has been reported (i.e. Kim *et al.*, 2006b), and suggest the expression of additional coumarin-specific OMTs in this plant. Dark-grown cultures of various Apiaceae, e.g. *Petroselinum crispum* and *Ammi majus*, do not produce psoralens, but their accumulation is rapidly induced upon the addition of fungal elicitor (Tietjen *et al.*, 1983; Hamerski and Matern, 1988a), which set the stage for biochemical and molecular studies. Accordingly, the BMT gene was cloned from elicited *Petroselinum crispum* cells and used to monitor the induction process (i.e. Lozoya *et al.*, 1991), but

the sequence has not been released to the public. Maximal BMT transcript abundance was observed in these studies beyond 25 h of elicitation, which is rather late compared to other enzymes of the coumarin pathway (Lozoya *et al.*, 1991; Larbat *et al.*, 2007). More recently, the cDNA of BMT from *Ammi majus* cells was cloned and accessed to databases (Hehmann *et al.*, 2004). Gene expression was induced with fungal elicitor revealing a transient maximum of BMT transcript abundance at 7 h of elicitation. Narrow substrate specificity for bergaptol was recorded with the recombinant enzyme, although the BMT polypeptide showed 64% identity (78.4% similarity) with a caffeic acid OMT (COMT) isolated also from *Ammi majus* (Hehmann *et al.*, 2004).

The narrow substrate specificity of *Ammi majus* BMT prompted a theoretical study (Han *et al.*, 2006) based on crystal structures and homology models of chalcone OMT, isoflavone OMT and COMT. Peculiar features were delineated for BMT to explain the specificity with emphasis on the restricted access to the active site cavity. The BMT cavity entrance is gated by Ser260 and Gly317 rather than Asp268 and Gly324 as in COMT, which was supposed to affect the conformational change required for substrate access to the buried BMT active site. Furthermore, Ser260 was situated proximal to the catalytic His259 bracketed through the hydrogen bonding by Glu287 and Glu320 and assumed to promote the transfer of a proton from bergaptol hydroxyl. Docking studies of BMT with 5-hydroxyferulate, caffeate or bergaptol proposed that only the hydroxyl of bergaptol approached the SAM sulfur close enough for reaction. Four of the bergaptol binding residues in BMT assigned by docked solutions remarkably differed from those in COMT with hydrophobic Val315 replacing the equivalent Asn324 in COMT which conceivably interacts with the phenolic hydroxyl and positions the phenol-ring. If these four amino acids in BMT are replaced by the corresponding residues of COMT, the total energy calculated for the interaction of bergaptol with the mutant BMT is higher than that for 5-hydroxyferulate or caffeate. Thus, the four amino acid differences in the binding site were assumed to define the substrate specificity of BMT. However, experimental evidence for this claim was not provided, and the level in COMT and BMT homology of only 65% compromises the conclusions. The salient points of this concept remain to be supported by molecular characterization of further coumarin-specific OMTs, including BMTs from other plants.

4.4 Lignans

Lignans in the strict sense are phenylpropanoid dimers linked by a C–C bond between carbons 8 and 8′ in the side chain (Haworth, 1942). Dimers linked by other carbon atoms have been named neolignans; here 3,3′-, 8,3′- or 8-*O*-4′-linkages are most frequently found (Davin and Lewis, 1992). Higher oligomers also occur: sesquilignans and dilignans (Dewick, 1989). Davin and Lewis (2003), however, have suggested that the term lignans

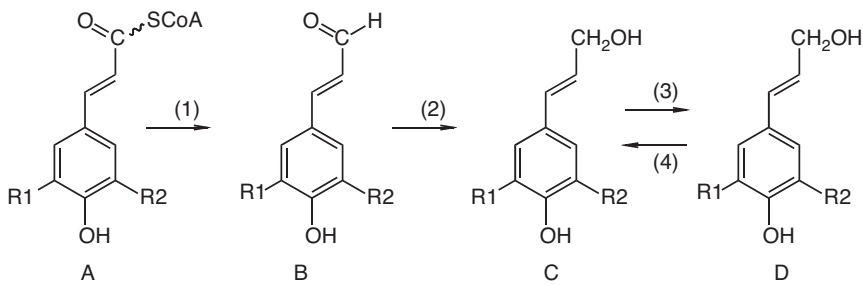
should be used for all coupling products of hydroxycinnamoyl-derived compounds, irrespective of the molecular size (dimers, trimers and higher oligomers), the interunit linkages (e.g. 8–8′, 8–1′, 8–5′, 8-*O*-4′, 5–5′, 3-*O*-4′, 7–1′, 8–7′, 1–5′, 2-*O*-3′ and others) as well as the nature of the coupled units (e.g. (hydroxy)cinnamyl alcohol, allylphenol). Lignans can be divided into several sub-groups, depending on other linkages and substitution patterns introduced into the original hydroxycinnamyl alcohol dimer. Lignans are widely distributed in the plant kingdom. More than 55 plant families contain lignans (Dewick, 1989), mainly gymnosperms and dicotyledonous angiosperms. Although there are no reports about lignans in algae, liverworts and hornworts already contain lignan-like compounds (Takeda *et al.*, 1990; Cullmann *et al.*, 1993, 1995; Tazaki *et al.*, 1995). Norlignans are phenolic compounds mainly occurring in monocotyledonous plants and conifers with a diphenylpentane skeleton (C6–C5–C6). Since the same phenylpropanoid precursors are used as for lignan formation, the biosynthetic pathway must comprise a loss of a carbon (Suzuki and Umezawa, 2007).

The same monomeric precursors (monolignols, e.g. coniferyl or 4-coumaryl alcohols) are used in lignin and lignan formation. Due to the high economical importance of lignin, the investigations on monolignol biosynthesis have been focused on lignification. It is rather unclear whether specific isoenzymes, regulatory properties or compartmentalizations exist for the biosynthesis of monolignols that will be directed into lignans. Recent feeding studies with labelled precursors to seeds of *Carthamus tinctorius* suggested the involvement of ferulic acid in lignan formation, which is in contrast to the current view of lignin precursor formation (Sakakibara *et al.*, 2007). The main three monomers found in lignin are 4-coumaryl, coniferyl and sinapyl alcohol giving the H (hydroxycinnamyl), G (guajacyl) and S (syringyl) units in lignin, respectively. These monolignols arise from cinnamic acid by hydroxylation and methylation reactions as well as the activation and successive reduction of the carboxyl group of the side chain to an alcohol. Although a metabolic grid was suggested in which these modification reactions could occur on virtually all levels, this is questioned nowadays and a more ordered sequence is more likely (see Dixon *et al.*, 2001 for a review). This would also be supported by the current view of lignification (not discussed here) where differently substituted monolignols are incorporated at specific sites and at specific time points. Lignin formation as well as its manipulation for a better commercial use of wood has been of great interest in this and the last decades. The understanding of lignin formation in plant cell walls has increased tremendously and the former view of a solely random coupling of lignin monomers by radical coupling seems to be incorrect. Dirigent proteins and sites have been found and template models postulated. Numerous publications deal with the genetic manipulation of lignin contents and composition. Since a detailed review of lignin biosynthesis and its manipulation would go beyond the special limit of this chapter, the reader is referred to recent reviews: e.g. Lewis *et al.* (1999), Dixon *et al.* (2001), Anterola and Lewis (2002), Humphreys and

Chapple (2002), Boerjan *et al.* (2003), Rogers and Campbell (2004), Davin and Lewis (2005b) and Chiang (2006).

4.4.1 Biosynthesis of monolignols

Monolignols are the hydroxycinnamyl alcohol monomers entering lignan and lignin biosynthesis, e.g. 4-coumaroyl, coniferyl and sinapyl alcohol. They are directly derived from the general phenylpropanoid pathway (see Section 4.2.1) by reduction of the coenzyme A-thioesters of 4-coumaric, ferulic and sinapic acid to the corresponding alcohols via the respective aldehydes. The enzymes involved are cinnamoyl-CoA:NADPH oxidoreductase (CCR; EC 1.2.1.44) and cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) (Fig. 4.7). Fluxes and regulatory features in the phenylpropanoid and monolignol pathways have been monitored mainly with respect to lignification and suberinization (Anterola *et al.*, 1999; Bernards *et al.*, 2000). Further steps involved in monolignol formation are the glucosylation of monolignols for storage and/or transport and the cleavage of the respective glucosides by β-glucosidases. Most publications concerning monolignol(-glycoside)



	A	B	C	D
R1 = H R2 = H	4-Coumaroyl-CoA	4-Coumar-aldehyde	4-Coumaryl alcohol	4-Coumaryl alcohol 4-*O*-β-glucoside
R1 = H R2 = OCH_3	Feruloyl-CoA	Coniferaldehyde	Coniferyl alcohol	Coniferyl alcohol 4-*O*-β-glucoside (Coniferin)
R1 = OCH_3 R2 = OCH_3	Sinapoyl-CoA	Sinapaldehyde	Sinapyl alcohol	Sinapyl alcohol 4-*O*-β-glucoside (Syringin)

Figure 4.7 Monolignol biosynthesis. Enzymes involved are cinnamoyl-CoA:NADPH oxidoreductase (CCR; (1)), cinnamyl alcohol dehydrogenase (CAD; (2)), UDP-glucose:cinnamyl alcohol 4-*O*-glucosyltransferase (3), cinnamyl alcohol 4-*O*-glucoside glucosidase (4).

biosynthesis and usage concern lignin formation. Studies especially aiming at the biosynthesis of monolignol(-glycosides) with respect to lignan formation are scarce.

Reviews that include aspects of monolignol biosynthesis have been published by, e.g., Lewis and Yamamoto (1990), Davin and Lewis (1992), Whetten and Sederoff (1995), Boudet (1998), Van Rensburg *et al.* (2000), Dixon *et al.* (2001), Anterola and Lewis (2002), Boerjan *et al.* (2003) and Davin and Lewis (2003), Dixon and Reddy (2003), Goujon *et al.* (2003b), Chiang (2006).

4.4.1.1 Cinnamoyl-CoA:NADPH oxidoreductase

Cinnamoyl-CoA:NADPH oxidoreductase (cinnamoyl-CoA reductase, CCR) converts CoA-activated cinnamic acids to the corresponding aldehydes, preferentially using NADPH as reductant (Fig. 4.7). The reaction is readily reversible. First reports about this enzyme date from the 1970s (see Petersen *et al.*, 1999, for further information). Sequence analysis of CCR from *Eucalyptus gunnii* revealed the membership of CCR in the mammalian 3b-hydroxysteroid dehydrogenase/plant dihydroflavonol reductase superfamily (Lacombe *et al.*, 1997). Two genes, CCR1 and CCR2, were cloned from *Arabidopsis thaliana* and expression studies suggested that CCR2 is involved in lignification, whereas CCR2 takes part in pathogen defence reactions (Lauvergeat *et al.*, 2001). Nine more CCR-like sequences have been found in the *Arabidopsis* genome without biochemical proof of CCR activity. Elicitation with several pathogens resulted in increased CCR activity and lignin/lignan accumulation in *Linum usitatissimum* (Hano *et al.*, 2006a). As expected, down-regulation of CCR in *Nicotiana tabacum* resulted in a reduction in lignin content by 50% (Piquemal *et al.*, 1998). In tomato, RNAi inhibition of CCR also resulted in reduced lignin levels and concurrently the amounts of different soluble phenolics were increased (Van Der Rest *et al.*, 2006). Similar strong reduction in lignin (syringyl units more severely reduced than guajacyl units) as well as an increased incorporation of ferulic acid was observed in poplar upon CCR down-regulation (Leple *et al.*, 2007). Investigations of a xylem-specific CCR from *Populus tremuloides* showed a high preference for feruloyl-CoA as a substrate and suggested that this substrate was formed from caffeoyl-CoA by CCoAOMT (Li *et al.*, 2005). Such a high substrate preference was, however, not seen in CCRs from all plant species.

4.4.1.2 Cinnamyl alcohol dehydrogenase

The activity of cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) was already described in the 1970s and was mainly investigated with respect to lignin biosynthesis (see Petersen *et al.*, 1999, for further information). It catalyses the reduction of cinnamaldehydes to cinnamyl alcohols with the help of NADPH; the reaction is readily reversible (Fig. 4.7). From a functional point of view, CAD activity is involved in developmental lignification and in the formation of defence compounds. Several reviews have treated the involvement of this enzyme in lignin monomer formation (Boudet *et al.*, 1998,

2004; Anterola and Lewis, 2002). As already found in protein purification studies, CADs can exist as monomeric or dimeric enzymes. Cloning of CAD1 and CAD2 from *Eucalyptus* revealed that they belong to two different families with only marginal amino acid sequence homologies (Boudet *et al.*, 2004). CAD1 is active as monomer and has similarities with CCR and dihydroflavonol reductases belonging to the family of short-chain reductases. It seems to be highly conserved in tracheophytes. CAD1 has a rather broad substrate specificity accepting 4-coumaryl, coniferyl and sinapyl alcohols as substrates. CAD2, on the other hand, is a member of the zinc-dependent medium chain dehydrogenase/reductase (MDR) superfamily and is active as dimer. It preferably accepts 4-coumaryl and coniferyl alcohols as substrates. Antisense studies showed a preferential involvement in lignification. This opened up the discussion about how syringyl units in lignin are synthesized. Among the CAD isoforms from *Arabidopsis thaliana* a specific sinapyl alcohol dehydrogenase was not detected (Kim *et al.*, 2004), although such an enzyme was postulated in aspen by Li *et al.* (2001). In *Arabidopsis*, CAD-C and CAD-D were reported to be active in coniferyl as well as sinapyl aldehyde reduction (Sibout *et al.*, 2005). Differential substrate specificities might (according to Boudet *et al.*, 2004) also be due to different combinations of monomeric isoforms to homo- and/or heterodimers. Feeding studies revealed that angiosperms obviously have two different paths towards sinapyl alcohol, one via coniferyl alcohol with hydroxylation and methylation on the alcohol stage and the other via sinapoyl-CoA with two final reduction steps (Chen *et al.*, 1999; Yamauchi *et al.*, 2003).

Originally, 17 genes from *Arabidopsis thaliana* were annotated as CADs, eight of which were wrongly annotated and only six of the heterologously expressed proteins revealed considerable CAD activity (Kim *et al.*, 2004). The substrate acceptance varied somewhat between isoforms, but generally showed redundance in the catalytic activities explaining the often low impact of down-regulation of single genes on lignin contents and/or composition. The expression pattern of the so-called CAD genes was investigated and showed distinct tissue/organ specificity. Mainly two isoforms (AtCAD4/5; CAD-C/D) and perhaps to a lesser extent two others (AtCAD7/8) were assigned to monolignol/lignan formation (Sibout *et al.*, 2003; Kim *et al.*, 2007). Down-regulation of AtCAD4 and 5 resulted in a strongly reduced and altered lignin deposition (Jourdes *et al.*, 2007).

From *Helicobacter pylori,* a dismutating CAD was cloned that has a similar substrate specificity as the enzyme from plants, but can catalyse a dismutation reaction, e.g., of benzaldehyde to benzyl alcohol and benzoic acid (Mee *et al.*, 2005).

Crystal structures are known from a putative yeast CAD (Valencia *et al.*, 2004), an aspen sinapyl alcohol dehydrogenase (SAD; Bomati and Noel, 2005) and a zinc-dependent CAD (AtCAD5) from *Arabidopsis thaliana.* The latter was solved in the apo-form and together with NADP and the structure of AtCAD4 accordingly modelled (Youn *et al.*, 2006a). The structural features

supported the membership in the MDR superfamily. Although the overall topology of the known structures was similar, the *Arabidopsis* CADs had a smaller substrate-binding pocket than SAD from aspen.

4.4.1.3 Glycosyltransferases and glycosidases

Monolignols as well as lignans are often glycosylated for storage, frequently in the plant's vacuoles (see, e.g. Leinhos and Savidge, 1993 and Henges, 1999). The release is coupled with cleavage of the glycosides by glycosidases. The enzyme glycosylating coniferyl alcohol (UDP-glucose:coniferyl alcohol glucosyltransferase, EC 2.4.1.111), but also other monolignols (4-coumaryl, sinapyl alcohol), has already been known for some time. This glucosyltransferase attaches the glucose moiety to the aromatic hydroxyl group in the para-position of the side chain of the monolignols (Fig. 4.7) (Ibrahim and Grisebach, 1976; Ibrahim, 1977; Schmid and Grisebach, 1982; Schmid *et al.*, 1982). The glucosylation of the side chain hydroxyl function (e.g. the formation of isoconiferin) occurs much more rarely. Three closely related genes encoding glucosyltransferases (UGT72E1–E3) were identified in *Arabidopsis*. They accept hydroxycinnamic acids and aldehydes besides monolignols (Lanot *et al.*, 2006). From the same species a specific 3-*O*-glucosyltransferase of caffeic acid, UGT71C1, was cloned and characterized (Lim *et al.*, 2003b). Many glucosyltransferases also accepted unnatural substrates in in vitro assays (Messner *et al.*, 2003). Glucosyltransferases accepting sinapate have already been described in Section 4.2.1.3. In *Sphagnum fallax* a specific *cis*-4-coumaric acid glucosyltransferase is active which does not accept the more common *trans*-4-coumaric acid (Rasmussen and Rudolph, 1997). In *Ginkgo biloba*, a 'living fossil', coniferaldehyde glucoside seems to play a role in lignification in addition to coniferin (Tsuji *et al.*, 2005).

Coniferin (the β-D-glucoside of coniferyl alcohol) is accumulated prior to lignin formation as well as in lignan-synthesizing cell cultures (e.g. Berlin *et al.*, 1986; Van Uden *et al.*, 1991; Smollny *et al.*, 1998). Coniferin and lignan contents were found to be inversely correlated in cell cultures. However, there is no direct proof for a transformation of stored coniferin to lignans. Feeding of coniferin resulted in an enhanced podophyllotoxin accumulation in cell cultures of *Podophyllum hexandrum* (Van Uden *et al.*, 1990).

Cell wall associated β-glucosidases (e.g. coniferin β-glucosidase; EC 3.2.1.126; Fig. 4.7) have been described in relation to lignification (e.g. Marcinowski and Grisebach, 1978; Marcinowski *et al.*, 1979; Burmeister and Hösel, 1981; Hösel *et al.*, 1982) and purified from, e.g., *Pinus* species (Leinhos *et al.*, 1994; Dharmawardhana *et al.*, 1995; Dharmawardhana and Ellis, 1998). The cDNA encoding a coniferin β-glucosidase in *Pinus contorta* contained a signal peptide targeting the protein to the endoplasmic reticulum and thus suggesting that the protein is secreted into the extracellular space (Dharmawardhana *et al.*, 1999). A cluster of glycosidase genes belonging to the GH family 1 has been identified in *Arabidopsis thaliana*. The substrate specificities of the heterologously expressed proteins differed between the members (Escamilla-Trevino

et al., 2006). Similar specific glycosidases involved in lignan biosynthesis have not yet been described, although β-glucosidase activities towards coniferin and lignan glycosides have been observed in lignan-accumulating cell cultures of *Linum* (Smollny *et al.*, 1998). In *Linum*, at least part of the coniferin was localized in the vacuole (Henges and Alfermann, unpublished results). Therefore, vacuolar or cytoplasmic β-glucosidases might be involved in the hydrolysis.

The lignans podophyllotoxin, 6-methoxypodophyllotoxin, and α- and β-peltatin are present as glucosides (e.g. Berlin *et al.*, 1988; Broomhead and Dewick, 1990; Heyenga *et al.*, 1990; Wichers *et al.*, 1991; Van Uden *et al.*, 1993; Smollny *et al.*, 1998) and the feeding of lignan aglyca to plants or plant cells resulted in the formation of glucosides (Van Uden *et al.*, 1995, 1997). Glucosylation takes place at the free 6- and 7-hydroxyl groups. Recently, separate UDP-glucose-dependent glucosyltransferases from *Linum nodiflorum* have been described catalysing the 7-*O*-glucosidation of podophyllotoxin, 6-methoxypodophyllotoxin and 5′-demethoxy-6-methoxypodophyllotoxin on the one side and the 6-*O*-glucosidation of β-peltatin on the other (Berim *et al.*, 2008). Arctigenin, matairesinol, phillygenin and epipinoresinol have been isolated as glucosides (arctiin, matairesinoside, phillyrin and epipinoresinol glucoside, respectively) from *Forsythia* (Rahman *et al.*, 1986, 1990; Ozawa *et al.*, 1993). In these cases, the *para*-located hydroxyl group in one of the aromatic rings was glucosylated. In *Sesamum indicum*, hexoses are linked to pinoresinol, sesaminol and sesamolinol (Ogasawara *et al.*, 1997; Moazzami *et al.*, 2006). Here, however, the glycosyltransferases have not yet been described.

The isolation of glycosylated lignans together with aglyca or the complete loss of glycosylated products is mostly due to β-glucosidases, and it must be assumed that all, or at least part, of the lignans are present in the glycosidic form in the plant itself. A highly specific podophyllotoxin-glucoside glucosidase has been isolated from *Podophyllum peltatum* (Dayan *et al.*, 2003).

4.4.1.4 Other modification reactions

Monolignol units usually maintain their *trans*-double bond in the side chain. During heartwood formation in gymnosperms, however, this double bond is reduced by phenylpropenal double bond reductases (PPDBR). The enzyme from *Pinus taeda* accepted dehydrodiconiferyl and coniferyl aldehydes but not the respective alcohols and NADPH as substrates (Kasahara *et al.*, 2006). The structure of a very similar alkenal double bond reductase from *Arabidopsis* was studied (Youn *et al.*, 2006b).

A novel side chain methylating SAM-dependent *O*-methyltransferase accepting preferentially coniferyl alcohol was recently detected in suspension cultures of *Linum* species. The corresponding product, coniferyl alcohol 9-*O*-methyl ether, is present in the suspension-cultured cells. The heterologously expressed protein displayed strict substrate specificities since alterations in the substitution pattern of the aromatic ring, and the side chain length strongly affected the methylation activities (Berim *et al.*, 2007).

4.4.2 Lignan and norlignan biosynthesis

Most plant lignans exist as (+)- or (−)-enantiomers and the relative quantities of these may vary from plant species to plant species; e.g. predominantly (+)-pinoresinol was reported from *Forsythia suspensa* (Davin and Lewis, 2003) and (–)-pinoresinol from *Zanthoxylum ailanthoides* (Katayama *et al.*, 1997), whereas mixtures with varying precentages of the respective enantiomers are found in, e.g., *Arctium lappa* (Suzuki *et al.*, 2002b) or (but only for some lignans) *Wikstroemia sikokiana* (Okunishi *et al.*, 2000; see also the review by Umezawa *et al.*, 1998). The stereospecificity of lignan formation can also change in different organs as shown in *Arctium lappa* (Suzuki *et al.*, 1999). Recent investigations have shown that several stereospecific enzymes may be involved in lignan biosynthesis (see below), thus establishing the specific enantiomeric mixtures. Lignan accumulation and biosynthesis have been treated in several recent reviews, e.g. Umezawa *et al.* (1997), Lewis and Davin (1999), Davin and Lewis (2003), Fuss (2003), Umezawa (2003), Suzuki and Umezawa (2007).

4.4.2.1 Formation of phenylpropanoid dimers

Coupling of phenylpropanoid units can be achieved by peroxidases and/or laccases forming radicals that can dimerize by a radical coupling typically leading to racemic mixtures of the coupling products. This, however, mostly cannot explain the enantiomeric mixtures of the direct coupling products and/or further derived compounds found in plants. These may arise either by stereospecific coupling or by preferential metabolisation of specific enantiomers by successive stereospecific enzymes. After the first description of the stereospecific coupling of two coniferyl alcohol units by (+)-pinoresinol synthase in *Forsythia suspensa* to (+)-pinoresinol by the group of Norman Lewis (Davin *et al.*, 1997; Fig. 4.8), the same group has published more evidence for this mechanism of stereospecific synthesis mediated by an oxidizing enzyme and a 'dirigent protein' in the formation of, e.g., aryltetralin lignans in *Podophyllum peltatum* and *Linum flavum* (Xia *et al.*, 2000), lignans derived from allylphenol units in *Larrea tridentata* (Moinuddin *et al.*, 2003) or blechnic acids in *Blechnum spicant* (Wang *et al.*, 2001) which has been recently reviewed by Davin and Lewis (2000, 2003, 2005a, and the literature cited therein) and Lewis and Davin (1998, 2000). Corresponding genes have been cloned by Gang *et al.* (1999a) showing no relevant sequence similarities to known proteins. A compilation of the occurrence of genes encoding dirigent proteins or similar ones, which can meanwhile be grouped into five sub-families, can be read in the recent review of Suzuki and Umezawa (2007). The reaction catalysed by the 'pinoresinol synthase system' and related proteins/enzymes can be briefly summarized as follows: an oxidizing enzyme (laccase) forms radicals from phenylpropanoid units which are trapped by a 'dirigent protein' in order to orientate the radicals in such a way that stereospecific coupling is achieved. The 'dirigent protein' is reported to be active as dimer of 23–25 kDa units in

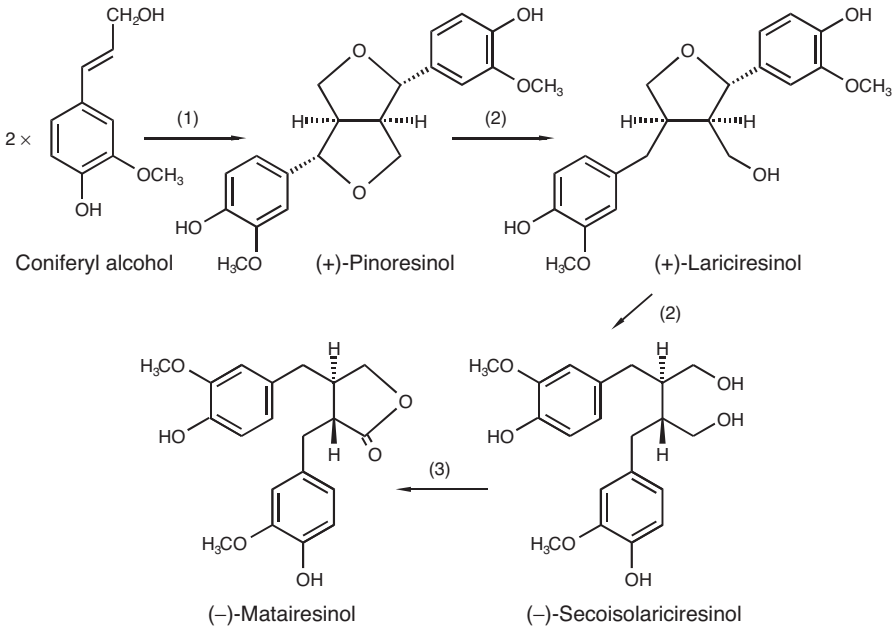


Figure 4.8 Biosynthetic pathway from coniferyl alcohol to (−)-matairesinol. Enzymes and proteins involved are (1) pinoresinol synthase (laccase + dirigent protein), (2) pinoresinol/lariciresinol synthase, (3) secoisolariciresinol dehydrogenase.

which each monomer binds one coniferyl alcohol radical (Halls and Lewis, 2002; Halls *et al.*, 2004). Feeding studies suggested an asymmetric stereoselective coupling of different units, e.g. coniferyl and sinapyl alcohols, for the biosynthesis of guaiacylglycerol-8-*O*-4′-sinapyl alcohol ether in *Eucommia ulmoides* (Katayama *et al.*, 2005). Several dirigent proteins or homologs seem to be involved in plant defence in spruce and pea (Wang *et al.*, 1999; Ralph *et al.*, 2006). A comparative mechanism ('dirigent sites') is postulated to be active in lignin formation (Burlat *et al.*, 2001; Davin and Lewis, 2000, 2005b, and the literature cited therein). Dirigent proteins seem to occur as gene families, e.g., in *Thuja plicata* with homologies between 72% and 99% to each other on amino acid level (Kim *et al.*, 2002a,b); the members of this gene family are differentially expressed in a spatial and temporal manner. The coding sequences encode proteins of 180 to 183 amino acid residues including a hydrophobic signal peptide. The localization of dirigent proteins/sites with respect to lignification has been determined in *Forsythia intermedia* by Kwon *et al.* (1999).

4.4.2.2 Pinoresinol/lariciresinol reductase

Pinoresinol/lariciresinol reductase (PLR) catalyses the stereospecific NADPH-dependent reduction of first pinoresinol to lariciresinol and afterwards lariciresinol to secoisolariciresinol (Fig. 4.8). The first PLR forming

(−)-secoisolariciresinol was characterized, purified and cloned from *Forsythia intermedia* (Chu *et al.*, 1992; Dinkova-Kostova *et al.*, 1996). This PLR reduces (+)-pinoresinol to (+)-lariciresinol and furtheron to (−)-secoisolariciresinol, thus conserving the conformation. Together with phenylcoumaran benzylic ether reductases (PCBERs) and isoflavonoid reductases, PLRs form the PIP family of reductases (Dinkova-Kostova *et al.*, 1996; Gang *et al.*, 1999b). The crystal structures of PLR as well as the related PCBER have been solved by Min *et al.* (2003) and thus helped in the understanding of the distinct enantio- and regiospecificity of these dimeric enzymes.

PLRs with different enantioselectivities have been detected in and cloned from different *Linum* species (*L. album*, *L. usitatissimum*, *L. perenne*) by the group of Fuss (von Heimendahl *et al.*, 2005; Hemmati *et al.*, 2007a). Enzymes of opposite enantioselectivity occur within one species, e.g. in *Thuja plicata* (Fujita *et al.*, 1999), in *Linum usitatissimum* (Hemmati *et al.*, personal communication) and in *Daphne* species or *Arctium lappa* (Okunishi *et al.*, 2001; Suzuki *et al.*, 2002b). Moreover, the reductase can change its enantioselectivity as demonstrated for (+)-pinoresinol/(−)-lariciresinol reductase from *L. perenne* involved in the biosynthesis of justicidin B. Down-regulation of the corresponding gene by RNAi reduced the accumulation of justicidin B in hairy root cultures of *L. perenne* down to 24% (Hemmati *et al.*, 2007a).

PLR was found to be expressed in the seed coats of maturing seeds of *Linum usitatissimum* where secoisolariciresinol diglucoside (as cell wall-bound 'polymer') is the main lignan stored (Teoh *et al.*, 2003; Hano *et al.*, 2006b). The cell wall-localized lignan complex was further identified by Ford *et al.* (2001) as secoisolariciresinol diglucoside-hydroxymethyl glutaryl-ester linked oligomers.

4.4.2.3 Secoisolariciresinol dehydrogenase

(−)-Secoisolariciresinol is oxidized to (−)-matairesinol by the NAD(P)-dependent secosiolariciresinol dehydrogenase (SDH; Fig. 4.8) identified, characterized and cloned from *Forsythia intermedia* and *Podophyllum peltatum* (Xia *et al.*, 2001). The enzyme showed similarities to NAD(H)-dependent short-chain dehydrogenases/reductases. The *Podophyllum* enzyme has been crystallized and its structure is solved (Youn *et al.*, 2005). It is active as a homotetramer. A highly conserved catalytic triad (Ser153, Tyr167 and Lys171) was identified in which Tyr167 functions as a general base. During catalysis NAD binds first, followed by (−)-secoisolariciresinol. The hydride abstracted from the substrate takes the *pro-S* position in NADH (Moinuddin *et al.*, 2006). The catalytic step catalysed by SDH is the last one to finally establish the configuration of the dibenzylbutyrolactone lignans which usually are accumulated optically pure in plants (Suzuki and Umezawa, 2007).

SDH in crude protein preparations from *Daphne* species (Thymelaeaceae) showed the preferential NADP-dependent formation of (−)-matairesinol, although the (+)-enantiomer is accumulated in the plant (Okunishi *et al.*, 2004).

4.4.2.4 Methylation reactions

Methylation of matairesinol with cell-free extracts of *Forsythia intermedia* leads to the formation of arctigenin and isoarctigenin (Ozawa *et al.*, 1993). SAM serves as methyl donor. With racemic matairesinol as a substrate, racemic arctigenin and isoarctigenin were formed, with a slight preference for the (−)-enantiomers. Only one methyl group was transferred to matairesinol. It was proposed that the formation of arctigenin proceeds from matairesinol via matairesinoside (matairesinol-glucoside) and arctiin to arctigenin. A cDNA encoding an *O*-methyltransferase catalysing this regiospecific methylation of matairesinol to arctigenin was recently cloned from *Carthamus tinctorius* seeds (Suzuki and Umezawa, 2007).

The non-phenolic furofuran lignan, (+)-eudesmin, from *Magnolia kobus* var. *borealis* is formed from pinoresinol by two SAM-dependent methylation steps (Miyauchi and Ozawa, 1998). Cell-free extracts catalysed the successive non-stereospecific methylation both of (+)- and (−)-pinoresinol. Thus, the stereospecificity has to be established in an earlier step.

4.4.2.5 Steps from matairesinol to deoxypodophyllotoxin and 4′-demethyldeoxypodophyllotoxin

Little information is available concerning biosynthetic steps from matairesinol to deoxypodophyllotoxin and 4′-demethyldeoxypodophyllotoxin. It is supposed that the transformation proceeds via yatein and the reactions include ring closure, the formation of the methylenedioxy bridge, and hydroxylation and methylation reactions in the pendant ring. Matairesinol is regarded as the key intermediate leading to two separate groups of lignans: those with a 3′,4′,5′-trimethoxy and those with a 4′-hydroxy-3′,5′-dimethoxy substitution pattern in the pendant ring (Kamil and Dewick, 1986a). Feeding of yatein or deoxypodophyllotoxin to *Podophyllum hexandrum* plants led to the formation of podophyllotoxin, whereas feeding of 4′-demethyldeoxypodophyllotoxin gave rise to 4′-demethylpodophyllotoxin. No interconversion occurred, indicating that the two groups arise separately from a common precursor (Jackson and Dewick, 1984; Kamil and Dewick, 1986b). Matairesinol was incorporated into both groups of lignans (Broomhead *et al.*, 1991) and may, therefore, represent the branch-point compound. The two groups of lignans then arise from yatein and 4′-demethylyatein, respectively.

Tracer experiments with *Anthriscus sylvestris* were conducted by Sakakibara *et al.* (2003) in order to elucidate the biosynthetic steps to yatein (Fig. 4.9) and bursehernin. These studies revealed that the 3′, 4′, 5′-trimethoxy-substitution pattern of the pendant ring is introduced first before introduction of the methylenedioxy bridge of yatein. Starting from matairesinol, first the 5′-OH group is introduced followed by its methylation, then the 4′-OH group is methylated and the resulting 4′, 5′-dimethylthujaplicatin is the precursor for yatein. Aromatic hydroxylases as well as OMTs have to be active in this part of the biosynthesis. The nature of the enzyme catalysing a possible ring closure

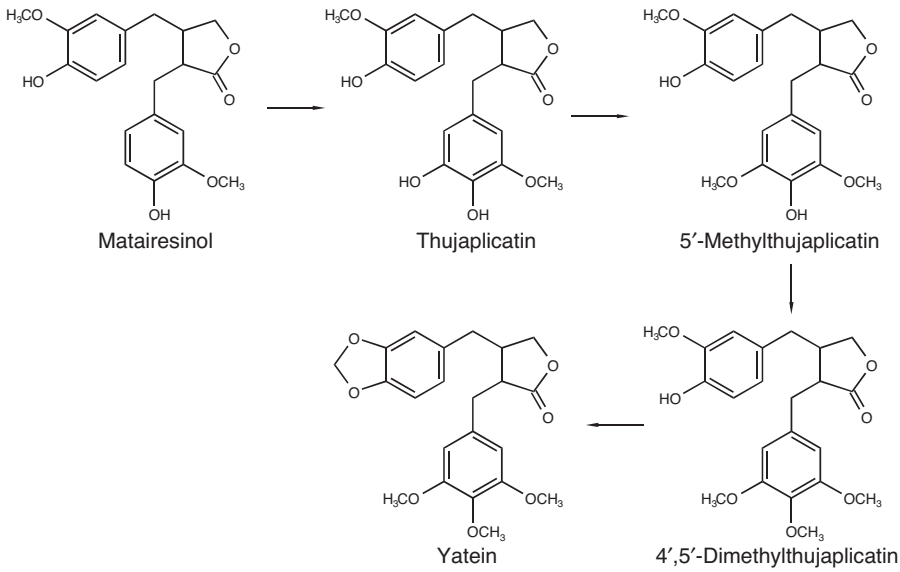


Figure 4.9 Biosynthetic steps from matairesinol to yatein as shown by feeding experiments with *Anthriscus sylvestris* according to Sakakibara *et al.* (2003).

from yatein to deoxypodophyllotoxin still remains unknown; a certain doubt may arise since usually deoxypodophyllotoxin is accumulated in *Anthricus sylvestris* (see, e.g. Koulman *et al.*, 2003), but this further step obviously was not investigated by Sakakibara *et al.* (2003). In the same series of experiments, bursehernin was established by methylenedioxy bridge formation yielding pluviatolide and subsequent 4′-*O*-methylation.

4.4.2.6 Formation of methylenedioxy bridges

A number of common lignans contain methylenedioxy bridges linked to aromatic rings, e.g. yatein, podophyllotoxin and its derivatives and some lignans from *Sesamum indicum* (Ogasawara *et al.*, 1997; Kato *et al.*, 1998). Feeding experiments with *Sesamum indicum* suggested the formation of (+)-sesamin from (+)-pinoresinol via (+)-piperitol (Kato *et al.*, 1998). Enzymological studies using microsomes from sesame seeds only showed the transformation of pinoresinol to piperitol, and therefore it was suggested that the two methylenedioxy bridges are introduced by two separate enzymes (Jiao *et al.*, 1998). A heterologously expressed cytochrome P450 (CYP81Q1) cloned from *Sesamum indicum*, however, was able to introduce both methylenedioxy bridges (Fig. 4.10; Ono *et al.*, 2006). The recombinant protein of a homologous CYP81Q3 from *Sesamum alatum* was not functional, which coincides with the absence of sesamin in this species. This CYP81Q (piperitol/sesamin synthase) is the first described so far to catalyse the sequential formation of two methylenedioxy bridges.

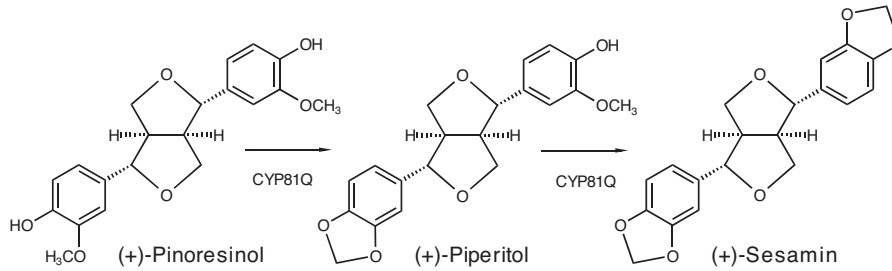


Figure 4.10 Biosynthesis of sesamin by the sequential introduction of two methylenedioxy bridges into pinoresinol by the cytochrome P450 CYP81Q (Ono *et al.*, 2006).

As indicated above, the methylenedioxy bridge of yatein is formed with 4′, 5′-dimethylthujaplicatin as a substrate. The methylenedioxy bridge of bursehernin is, however, directly introduced at the level of matairesinol prior to the methylation of the 4′-OH group (Sakakibara *et al.*, 2003).

4.4.2.7 Biosynthesis of podophyllotoxin, β-peltatin and 6-methoxypodophyllotoxin

Deoxypodophyllotoxin is regarded as a precursor for podophyllotoxin, β-peltatin and 6-methoxypodophyllotoxin (Fig. 4.11; Kuhlmann *et al.*, 2002), although Xia *et al.* (2000) demonstrated the 7-hydroxylation of matairesinol and the incorporation of 7-hydroxymatairesinol into 6-methoxypodophyllotoxin by feeding experiments with *Linum flavum* plants. Earlier feeding experiments with *Podophyllum hexandrum* plants and cell cultures (Jackson and Dewick, 1984; Kamil and Dewick, 1986b) as well as *Linum flavum* cell cultures (Van Uden *et al.*, 1995, 1997) supported the view of deoxypodophyllotoxin as the central precursor. Other possible pathways, however, cannot be ruled out. According to our view, a deoxypodophyllotoxin 7-hydroxylase yields podophyllotoxin. Unfortunately, this enzyme activity has not yet been unequivocally detected and characterized or the corresponding gene cloned. Hydroxylation in position 6 yielding β-peltatin is achieved by a cytochrome P450 monooxygenase, deoxypodophyllotoxin 6-hydroxylase, first described from suspension-cultured cells of *Linum flavum* and lateron *L. nodiflorum* (Molog *et al.*, 2001; Kuhlmann *et al.*, 2002). The corresponding enzyme from *L. album* was considered to play a regulatory role in distributing the precursor deoxypodophyllotoxin into either podophyllotoxin or 6-methoxypodophyllotoxin formation (Federolf *et al.*, 2007). β-Peltatin is methylated at the 6-OH group by a SAM-dependent methyltransferase. This enzyme was characterized from cell suspension cells of *Linum nodiflorum* by Kranz and Petersen (2003). The following 7-hydroxylation of β-peltatin to 6-methoxypodophyllotoxin has not yet been observed in vitro. Podophyllotoxin, β-peltatin and 6-methoxypodophyllotoxin are glucosylated in a

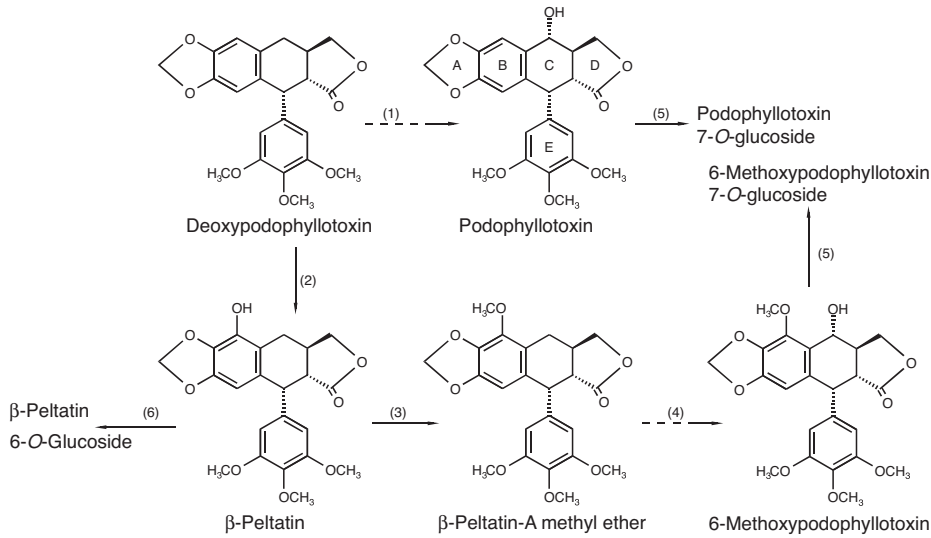


Figure 4.11 Proposed biosynthetic steps involved in the biosynthesis of podophyllotoxin(-glucoside) and 6-methoxypodophyllotoxin(-glucoside). Steps catalyzed by yet unidentified enzymes are shown with dashed arrows. Enzymes involved are as follows: (1) deoxypodophyllotoxin 7-hydroxylase, (2) deoxypodophyllotoxin 6-hydroxylase, (3) β-peltatin 6-*O*-methyltransferase, (4) β-peltatin-A methyl ether 7-hydroxylase, (5) (6-methoxy)podophyllotoxin 7-*O*-glucosyltransferase, (6) β-peltatin 6-*O*-glucosyltransferase.

UDP-glucose-dependent reaction by different glucosyltransferases (Berim *et al.*, 2008) and stored in the vacuole (Henges, 1999).

4.4.2.8 Biosyntheses of other lignans

The creosote bush *Larrea tridentata* is known to accumulate allylphenol-derived lignans like nordihydroguajaretic acid (NDGA) and derivatives. Moinuddin *et al.* (2003) suggested the couping of *p*-anol (*p*-propenylphenol) as first step towards *Larrea tridentata* lignans. Unexpectedly, a recently detected PLR-like enzyme from *Larrea tridentata* catalysed the conversion of 4-coumaryl and coniferyl esters to chavicol and eugenol, respectively, but not to anol (the expected NDGA precursor) and isoeugenol (Vassao *et al.*, 2007). During NDGA biosynthesis, (+)-larreatricin is enantio-specifically hydroxylated to (+)-3′-hydroxylarreatricin by a polyphenol oxidase (Cho *et al.*, 2003). This is one of the rare examples where a polyphenol oxidase was found to be substrate- and enantio-specific. A similar coupling of isoeugenol to finally afford verrucosin in *Virola surinamensis* was proposed by Lopes *et al.* (2004).

The hydroxylation of justicidin B in position 7 to diphyllin is catalysed by a cytochrome P450 monooxygenase as well (Hemmati *et al.*, 2007b).

A biosynthetic scheme for lyoniresinol, a syringyl lignan from *Lyonia ovalifolia* var. *elliptica*, has been proposed after feeding experiments with radioactively labelled precursors. Two moieties of sinapyl alcohol were

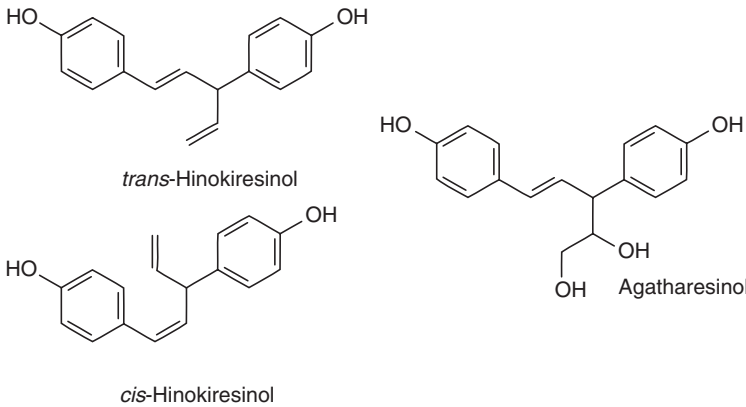


Figure 4.12 Structures of some norlignans.

found to be dimerized in an unspecific way followed by reduction (Rahman *et al.*, 2007).

4.4.2.9 Biosynthesis of norlignans

Norlignans lack one carbon atom in comparison to lignans. Three different linkage types are found: 8–8′, 7–8′ and 9–8′. They typically occur in monocotyledonous plants and in the heartwood of conifers, putatively as defence against rotting, but they have also been found in certain dicots (Suzuki and Umezawa, 2007).

The biosynthesis of norlignans such as *cis*- and *trans*-hinokiresinol and agatharesinol (Fig. 4.12) is under investigation in, e.g., *Cryptomeria japonica* and *Asparagus officinalis*. In hinokiresinol biosynthesis a phenylpropanoid dimer (4-coumaryl 4-coumarate) is formed from two different monomers, 4-coumaryl alcohol and 4-coumaroyl-CoA (Suzuki *et al.*, 2001, 2002a, 2004; Suzuki, 2002). This is essentially different from the 'normal' dimerization in lignan biosynthesis. Norlignans are synthesized under cleavage of the ester bond, decarboxylation and formation of a new bond. Agatharesinol in *Cryptomeria japonica* also originates from phenylpropanoid units, but hinokiresinol was excluded as a precursor (Imai and Nomura, 2005; Imai *et al.*, 2006a,b).

4.5 Gallotannins and ellagitannins

Plant tannins comprise structurally diverse plant secondary products that can be characterized by their ability to bind and consequently precipitate proteins (Haslam and Cai, 1994). This characteristic property of these substances led to their early and widespread use by humans, e.g. in the tanning process of animal hides to leather. Tannins have also been widely used in traditional folk medicine (Haslam, 1996). The pharmacological properties of the substances

are receiving considerable attention and are currently under investigation worldwide (see references in Gross *et al.*, 1999; Feldman, 2005).

Structurally, tannins can be subdivided into two major groups: condensed tannins (or proanthocyanidins), which are of flavonoid origin, and hydrolysable tannins, which are defined as esters of gallic acid (see Fig. 4.13) with a polyol moiety, mainly β-D-glucose. The last group can be further subdivided into gallotannins and ellagitannins (Khanbabaee and van Ree, 2001). Their basic building block is β-glucogallin (1-*O*-galloyl-β-D-

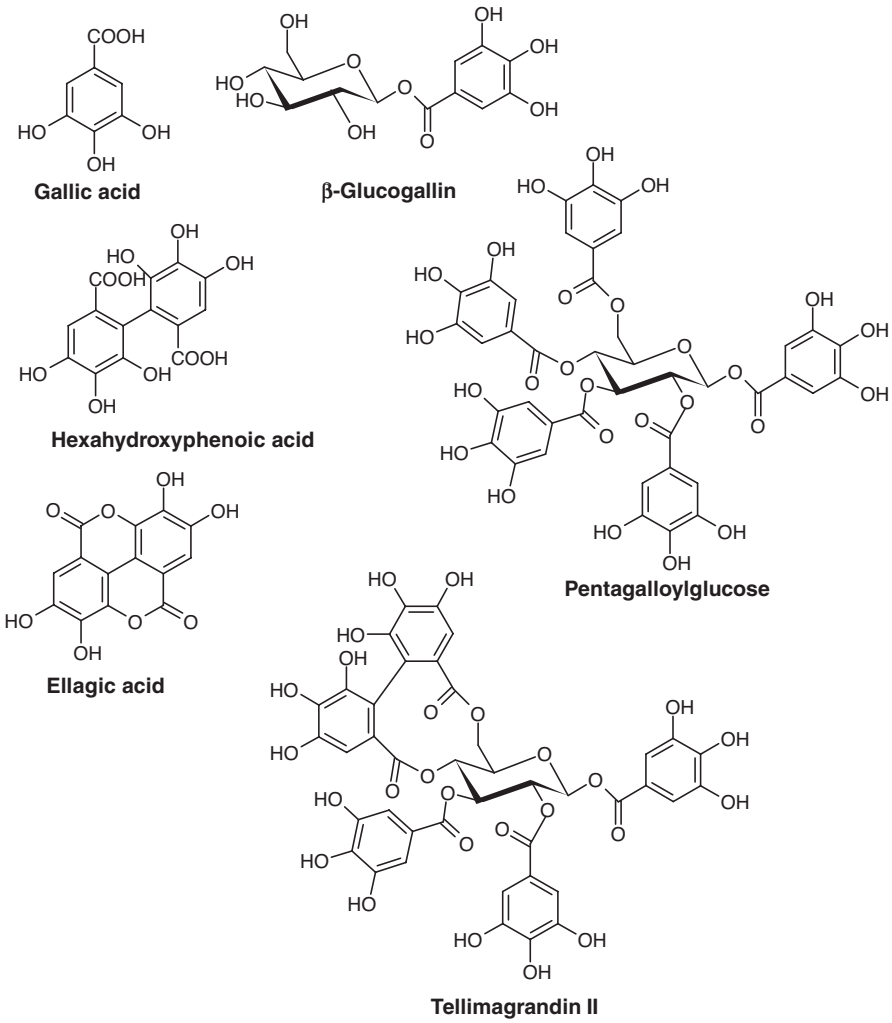


Figure 4.13 Structures of gallo- and ellagitannins and their precursors.

glucopyranose, Fig. 4.13), which is further esterified with additional gallic acid moieties, yielding 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (Fig. 4.13), the central intermediate of the pathway. Typical for the complex gallotannins is the addition of further gallic acid moieties and the formation of depsidic meta-bonds between suitably positioned galloyl residues in the molecule. Ellagitannins, however, are derived from pentagalloylglucose by oxidative linkage of the galloyl moieties, leading to the formation of 3,4,5,3′,4′,5′-hexahydroxydiphenoyl (HHDP) moieties (Fig. 4.13). They are named after their characteristic (but artifactual) hydrolysis product, ellagic acid (Fig. 4.13), the dilactone of HHDP.

Whereas gallotannins seem to be mainly restricted to woody dicotyledonous plants, ellagitannins are more widely spread in the plant kingdom. Hydrolysable tannins have also been employed as markers in chemotaxonomical studies (e.g. Haddock *et al.*, 1982).

4.5.1 Biosynthesis of gallic acid and pentagalloylglucose

The biosynthetic pathway to hydrolysable tannins can be separated into distinctive steps. Whereas the common building blocks of most hydrolysable tannins, β-glucogallin (Fig. 4.13) and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (Fig. 4.13), are produced via a ubiquitous route, the subsequent pathways leading to gallotannins and ellagitannins, respectively, are clearly distinguishable and will therefore be treated separately.

There has been an ongoing debate for a number of years on the biosynthetic origin of gallic acid, the phenolic building block of hydrolysable tannins. Only recently, retrobiosynthetic approaches using [^{13}C]-glucose feeding experiments and measurements of δ^{18}O values of gallic acid in *Rhus typhina* provided strong indications for 5-dehydroshikimate as one of the immediate precursors of gallic acids, thus ruling out the 'competing' theory of phenylpropanoids as intermediates (Werner *et al.*, 1997, 2004). This view was further corroborated by the demonstration of enzymatic conversion of 5-dehydroshikimate to gallate by extracts from leaves of *Betula pubescens* (Ossipov *et al.*, 2003).

Glucose is subsequently coupled to a gallate moiety in a glucosyltransferase- catalysed reaction using UDP-glucose as sugar donor, to yield β-glucogallin (Gross, 1983). In the following steps, further gallate moieties are attached to the β-glucogallin core. The order of galloylation is strictly fixed, yielding 1,6-digalloyl-glucose, 1,2,6-trigalloylglucose, 1,2,3,6-tetragalloylglucose and finally 1,2,3,4,6-pentagalloylglucose, with each of the steps being catalysed by a distinct acyltransferase (see references in Niemetz and Gross, 2005). The proteins, isolated from oak (*Quercus robur*, *Q. rubra*) and sumac (*Rhus typhina*), exhibit strikingly similar properties (e.g. slightly acidic pH optima and a uniform tendency to high molecular weights). All of the enzymes employ β-glucogallin (or higher substituted galloyl glucoses) as acyl donor, thus placing the 1-*O*-substituted galloylglucose into the category

of 'activated' energy-rich compounds, similar to acyl-CoA thioesters or sinapoylglucose esters (Mock and Strack, 1993). Interestingly, the sequence of galloylation reactions is comparable to chemical substitution reaction series on polyol moieties, arguing for steric or neighbour-activating effects rather than solely enzyme specificities as determinants of the sequence of reactions.

To shed some light onto the physiology of gallotannins *in planta*, localization studies were initiated to gain insights into the sub-cellular distribution of galloylglucoses and the enzymes responsible for their biosynthesis. Antisera against pentagalloylglucose and the 4-*O*-galloyltransferase from *Quercus robur* were raised and used in immunolocalization studies. Whereas signals for the metabolite were mainly detected in cell wall regions of leaves from *Q. robur*, labels for the enzyme (4-*O*-galloyltransferase) exhibited a slightly wider distribution in the cells, albeit with a clear focus in the apoplastic regions (Grundhöfer *et al.*, 2001). These findings, taken together with the acidic pH optima of the acyltransferases, analyses of gallotannin patterns in apoplastic washates from oak leaves and the pronounced cytotoxicity of hydrolysable tannins, argue strongly for the production and storage of galloylglucose esters in the walls of mesophyll cells.

4.5.2 Biosynthesis of gallotannins

The biosynthetic pathway to gallotannins proceeds by further sequential galloylation of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose, yielding the characteristic meta-depsidic bonds between neighbouring gallate moieties (Hofmann and Gross, 1990). It has become clear in recent years that although the chemical nature of the phenolic OH groups now used for the linkage of two gallate molecules is distinct from the aliphatic character of the polyol OH groups in the core of the molecule, the basic mode of reaction seems to be very similar, with β-glucogallin again serving as the acyldonor for the reactions.

Taking into account the large variety of gallotannins found in plants, the question arose about the specificity of the enzymes involved in the formation of this structural variety. Using enzyme extracts from *Rhus typhina*, several galloyltransferases could be purified and characterized (Niemetz and Gross, 1998, 1999, 2001; Fröhlich *et al.*, 2002). None of them seems to exhibit an exclusive substrate specificity towards a single acceptor molecule; however, calculations of catalytic efficiencies revealed a distinct spectrum of preferred substrates and main products for each of the enzymes. This led to the formulation of a grid-like scheme for the biosynthesis of higher substituted galloylglucoses (Fig. 4.14). The question remains unanswered to date whether those galloylglucoses not being the main product of a specific enzyme are formed as by-products by the action of several unspecific galloyltransferases. It seems also possible that the respective specific enzymes are not identified and characterized yet.

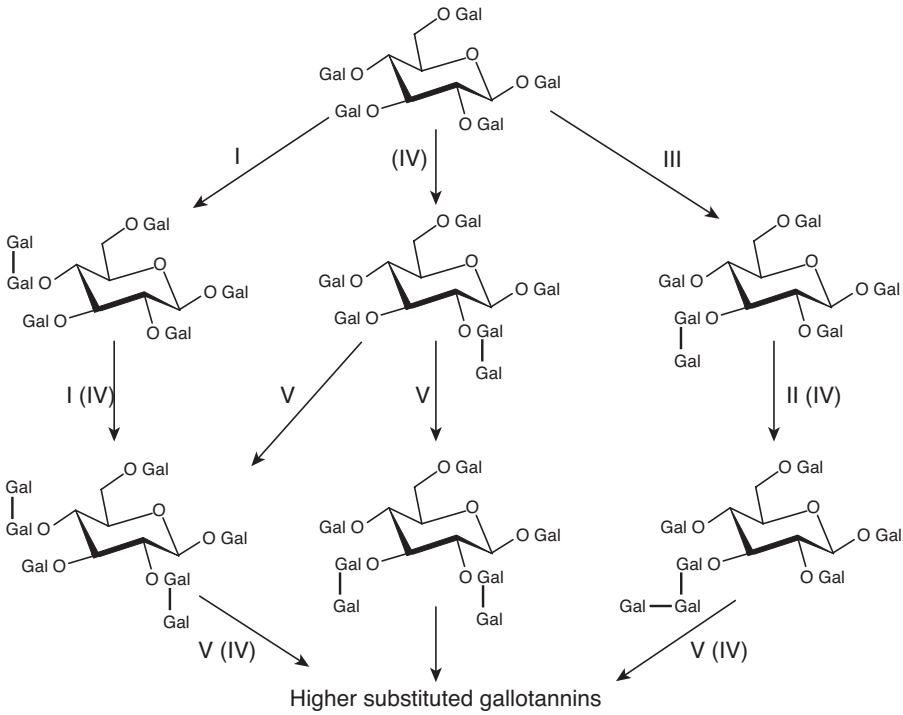


Figure 4.14 Proposed biosynthetic pathways of higher substituted gallotannins. Roman numerals represent galloyltransferases with different regioselectivities. Gal: gallic acid residue (bold print for newly attached residues; bonds in bold print: meta depsidic connections between gallate residues). (Redrawn from Niemetz and Gross, 2005.)

4.5.3 Biosynthesis of ellagitannins

Ellagitannins exhibit a large structural diversity due to the numerous possibilities of oxidative C–C and C–O couplings between the galloyl side chains of the molecules, leading to the characteristic hexahydroxydiphenoic (HHDP) moieties. This, together with the chemotherapeutical potential of some of the substances (e.g. Okuda *et al*., 1989; Miyamato *et al*., 1993), rendered ellagitannins a challenging task for chemical synthesis (Feldman, 2005) with remarkable progress being made in recent years.

In contrast to this, research on the biosynthesis of ellagitannins was hampered by methodological and analytical difficulties. The breakthrough in this area was the production of radiolabelled [U-^{14}C]-pentagalloylglucose (Rausch and Gross, 1996). Using this substrate, first hints towards the successful in vitro production of ellagitannins were obtained with enzyme preparations from *Tellima grandiflora* (Saxifragaceae). Several radiolabelled products were formed (Niemetz *et al.*, 2001), with the main product being identified as tellimagrandin II (Fig. 4.15). The enzyme responsible for this

oxidation reaction could subsequently be classified by inihibitor assays as a phenol oxidase of the laccase type (Niemetz and Gross, 2003a), thus ruling out the involvement of peroxidases or cytochrome P450 monooxygenases. Determination of the stereochemistry of tellimagrandin II revealed that the in vitro

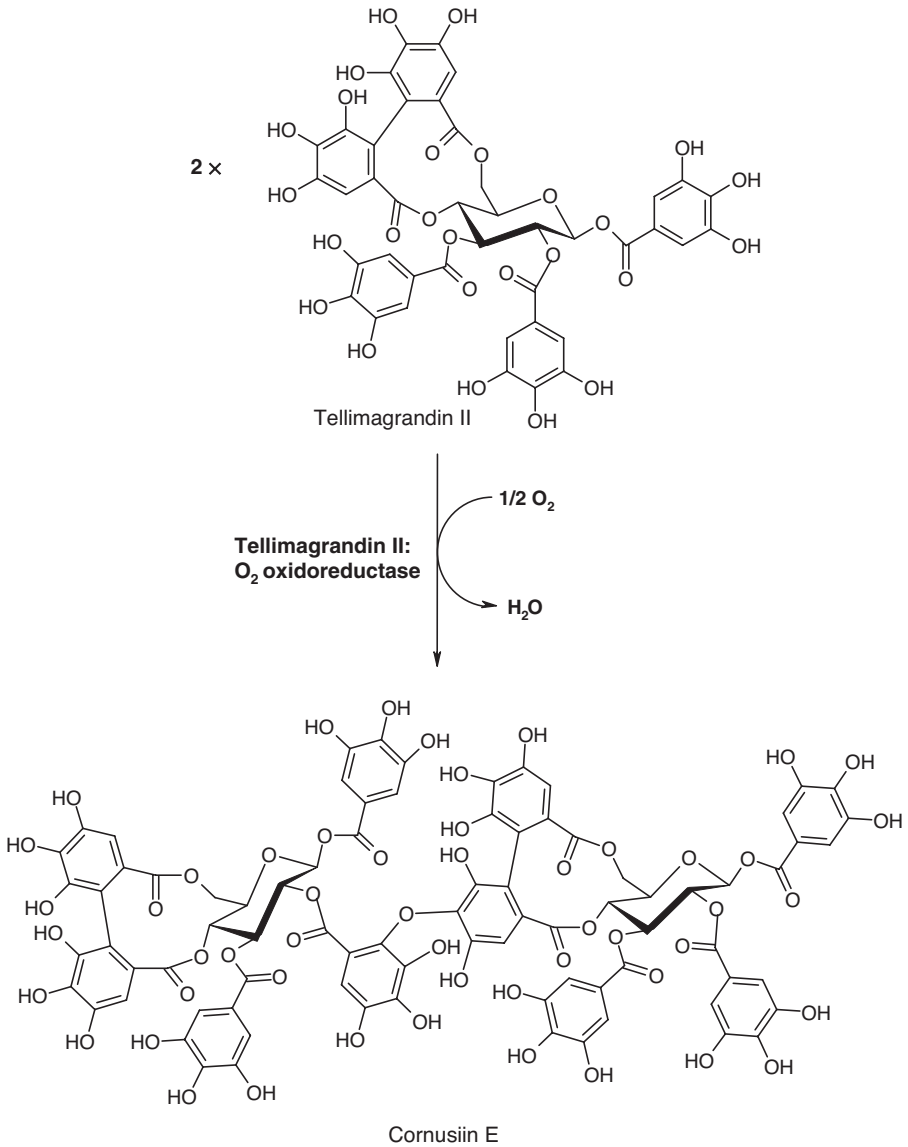


Figure 4.15 Oxidative coupling of two molecules tellimagrandin II to cornusiin E by the laccase-type tellimagrandin II:O_2 oxidoreductase from *Tellima grandiflora*.

product exhibited the same (*S*)-configuration of two neighbouring coupled galloyl moieties in the HHDP structure as found in tellimagrandin II isolated from natural sources. It is still under debate whether the stereoselectivity of the reaction is a consequence of the basic chemical or energetic properties of the molecules or whether a phenolic coupling mechanism employing a kind of 'dirigent factor' is involved (Davin and Lewis, 2005a). However, the strict stereoselectivity of tellimagrandin II formation by the purified laccase from *T. grandiflora* might point to a scenario without assistance from such a factor.

Oxidative coupling of ellagitannin monomers leads proposedly to the formation of more complex oligomeric structures. First evidence for this hypothesis was obtained by the characterization and purification of a tellimagrandin II:O_2 oxidoreductase from *T. grandiflora*. The enzyme could be enriched 344-fold from leaf extracts and catalyses the oxidative coupling of two molecules tellimagrandin II to yield cornusiin E (Niemetz and Gross, 2003b; Fig. 4.15). Inhibitor studies classified the enzyme again as a laccase, though its properties make it clearly distinguishable from the laccase converting pentagalloylglucose to tellimagrandin II.

4.6 Conclusion

The review has summarized recent findings concerning enzymes, genes, regulation and evolution of the general phenylpropanoid pathway and the biosyntheses of monolignols, lignans, hydroxycinnamate esters and amides, phenolic fragrance compounds, coumarins, furanocoumarins as well as gallotannins and ellagitannins. Important groups of phenolic compounds such as lignin, flavonoids/isoflavonoids and condensed tannins have not been touched or treated in detail since they would deserve own chapters.

Today, the research into the biosyntheses of the above-mentioned compounds is focussed on the molecular level. After having identified the biosynthetic enzymes by bioactivity assays, nowadays, cDNAs/genes are isolated and characterised. Important families of genes/enzymes have been identified, e.g. several methyltransferase families, acyltransferase families and families of cytochrome P450s especially involved in phenolic metabolism. Current and future research will aim at the identification of transcription factors and their binding sites and regulation in order to be able to switch on or off whole biosynthetic sequences. By gene transfer, biosynthetic pathways can be established in microorganisms in whole or partially for a heterologous production of plant natural products.

There are still many open questions even in the rather well understood phenolic metabolism which have to be taken into consideration in future. Thus, young investigators can still be encouraged to enter into this fascinating field of research, plant phenolic metabolism.

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Annual Plant Reviews (2010) **40**, 258–303
doi: 10.1002/9781444320503.ch5
www.interscience.wiley.com

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.

 ISBN: 978-1-405-18397-0

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

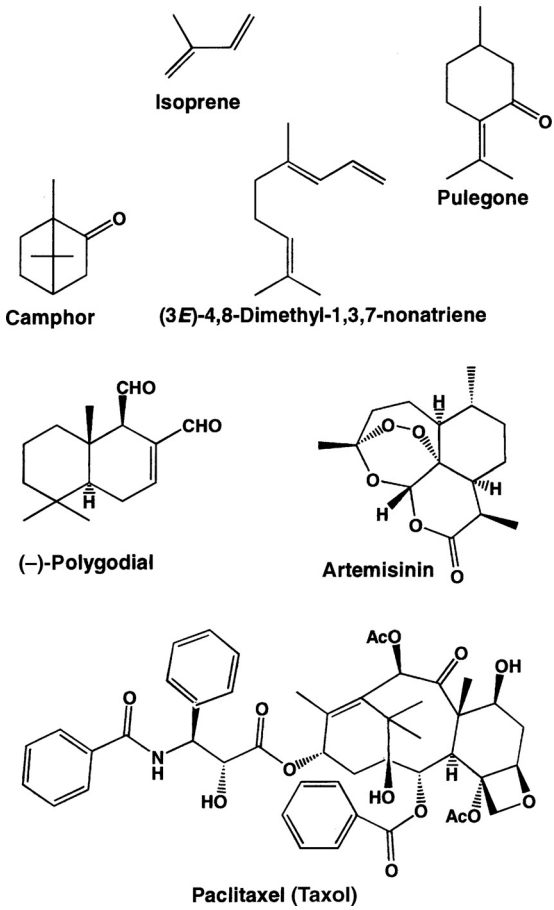

Figure 5.1 Examples of terpenoids that are of commercial importance or whose functional role in plants has recently been investigated. Isoprene may stabilize membranes at high temperatures. Camphor, artemisinin and paclitaxel (taxol) are valuable pharmaceuticals. The other three compounds appear to be 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,

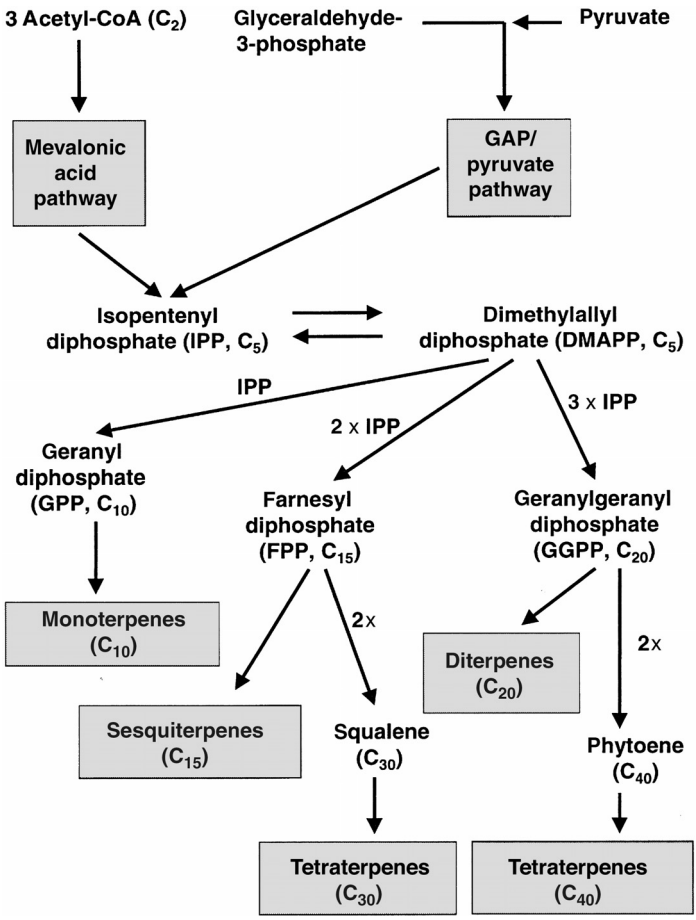

Figure 5.2 Overview of terpenoid biosynthesis in plants, showing the basic stages of this process and major groups of end products. CoA, coenzyme A; GAP, glyceraldehyde-3-phosphate.

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.

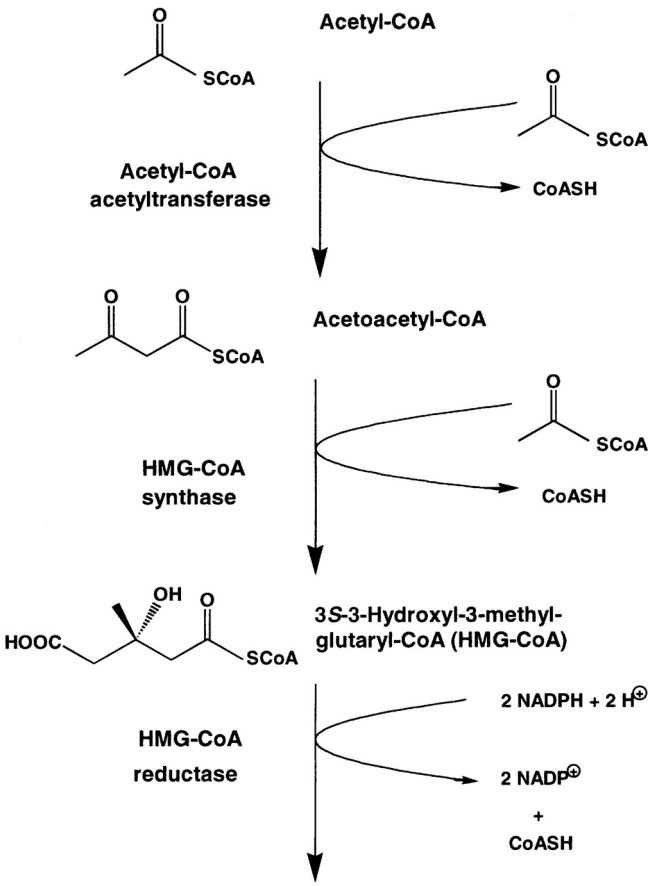

Figure 5.3 Outline of the mevalonate pathway for the formation of C_5 isoprenoid units. Most research has focused on HMG-CoA reductase (HMGR), the rate-determining step in terpenoid biosynthesis in mammals. P indicates a phosphate moiety. HMG-CoA, 3-hydroxy-3 methylglutaryl coenzyme A; NADPH, nicotinamide 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

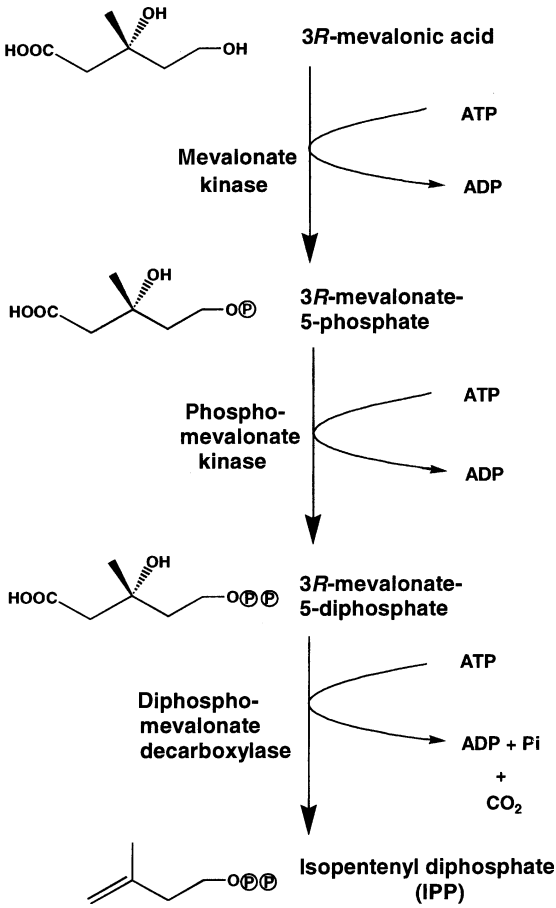

Figure 5.3 (*Continued*)

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 *Gossypium hirsutum*	Joost *et al.* (1995)
	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

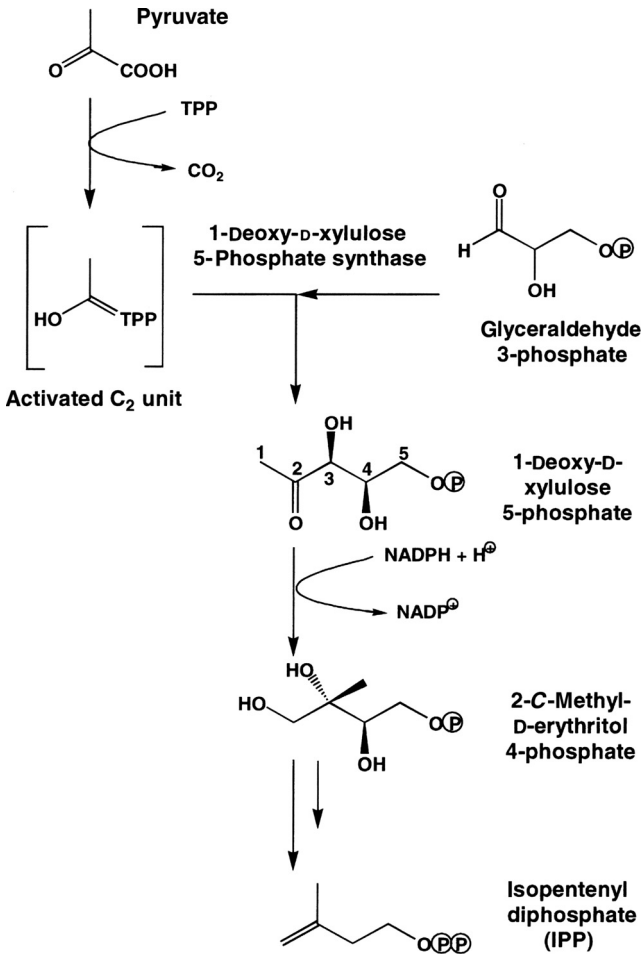

Figure 5.4 Outline of the newly discovered glyceraldehyde phosphate/pyruvate pathway for the formation of C_5 isoprenoid units. None of the intermediates after 2-C-methyl-D-erythritol 4-phosphate is known. P indicates a phosphate moiety. TPP, thiamine pyrophosphate; NADP, nicotinamide adenine dinucleotide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

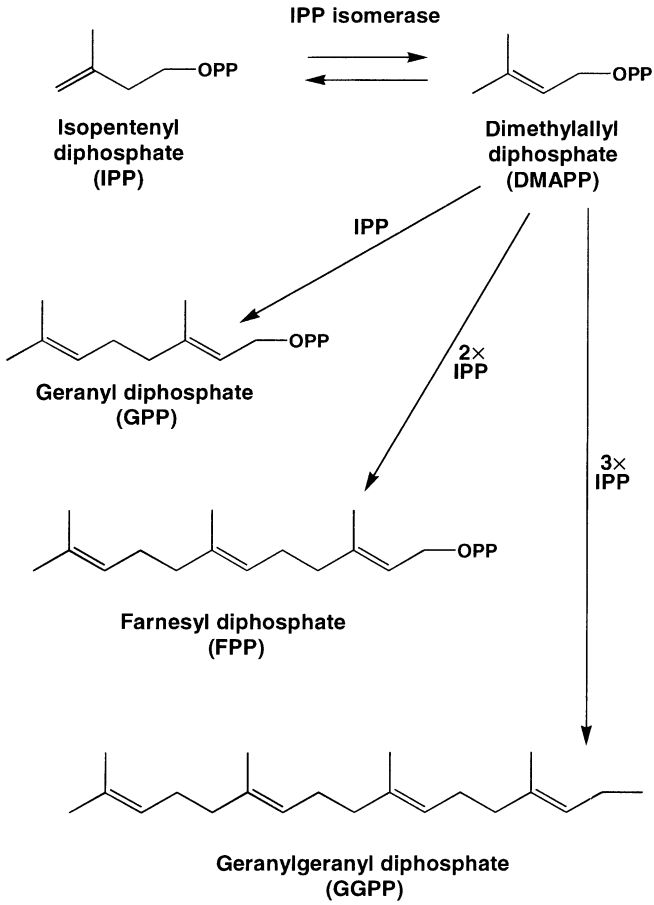

Figure 5.5 The formation of C_{10}, C_{15} and C_{20} prenyl diphosphates from the fusion of C_5 isoprenoid units. PP indicates a diphosphate moiety.

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).

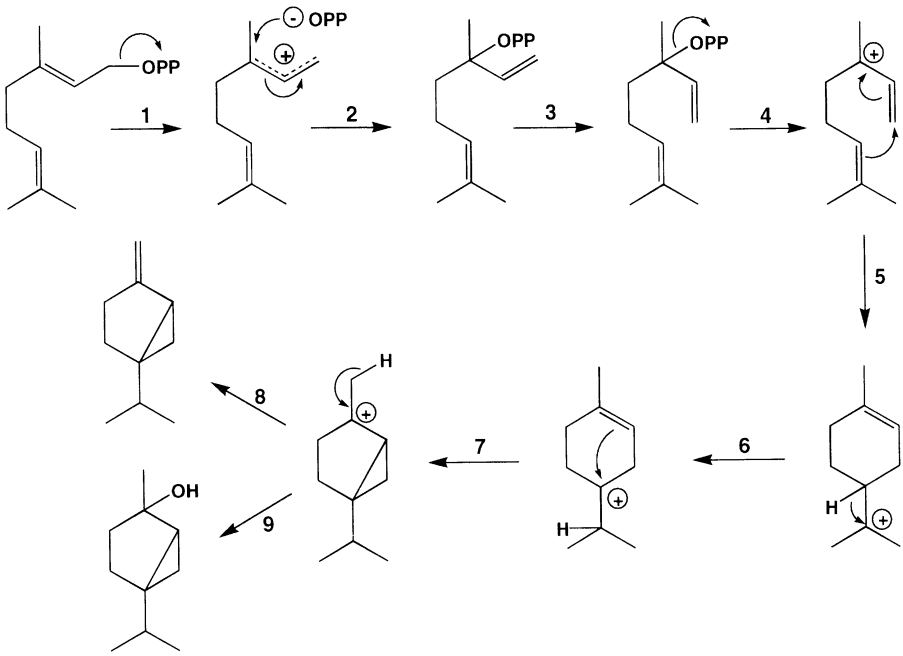

Figure 5.6 Proposed mechanism for the cyclization of geranyl diphosphate to sabinene and sabinene hydrate under catalysis by monoterpene synthases: the reaction begins with the hydrolysis of the diphosphate moiety to generate a resonance-stabilized carbocation (1); the carbocation then isomerizes to 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

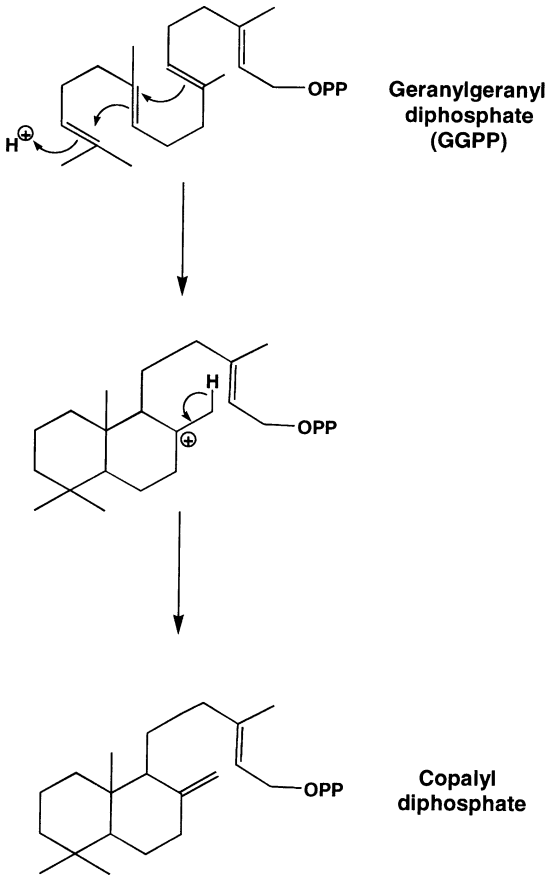

Figure 5.7 Proposed mechanism for the cyclization of geranylgeranyl diphosphate (GGPP) to the diterpene copalyl diphosphate, an example of terpene synthase-catalysed cyclization initiated by double-bond protonation, rather than by hydrolysis of the diphosphate ester. PP indicates a diphosphate moiety.

(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).

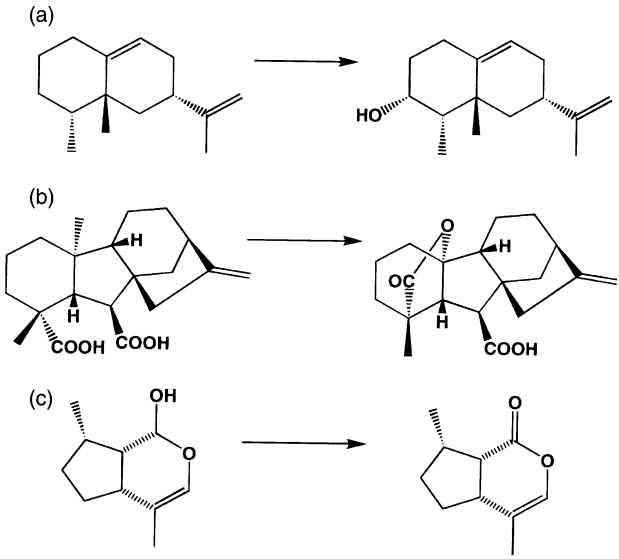

Figure 5.8 Examples of oxidative secondary transformations in terpenoid biosynthesis. (a) Hydroxylation of epi-aristolochene at the 3-position by a cytochrome P450-dependent terpene hydroxylase in *Capsicum annuum* (Hoshino *et al.*, 1995). (b) Conversion of GA_{12} to GA_9 by a 2-oxoglutarate-dependent 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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Annual Plant Reviews (2010) **40**, 304–363 www.interscience.wiley.com
doi: 10.1002/9781444320503.ch6

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

 ISBN: 978-1-405-18397-0

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).

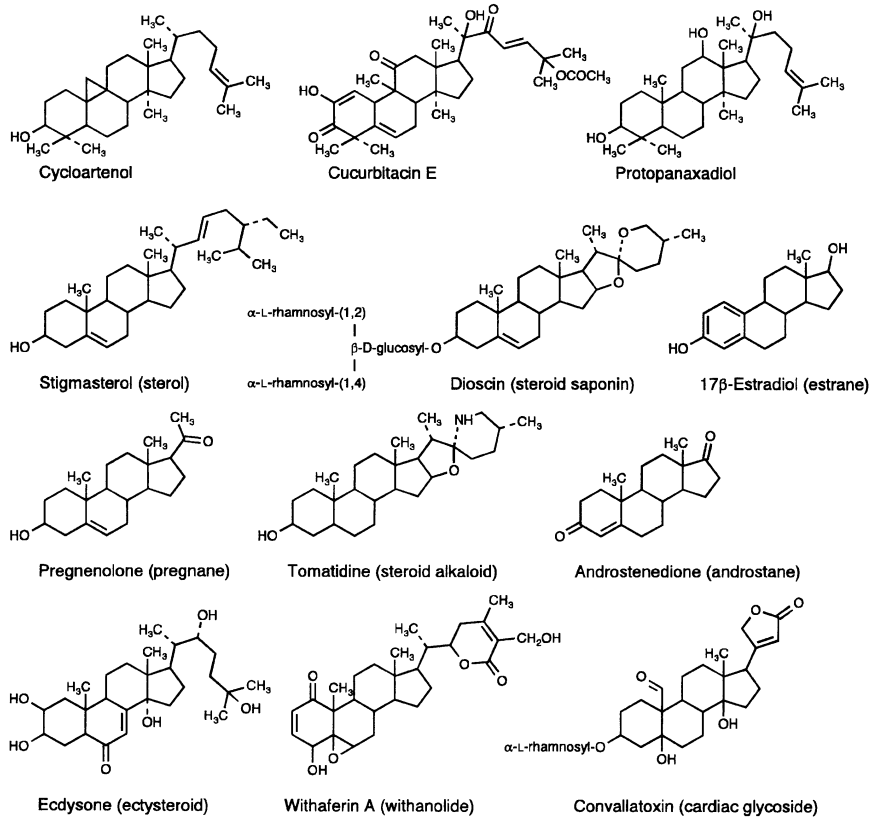


Figure 6.1 Chemical structures of plant metabolites synthesized from squalene-2,3-epoxide.

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

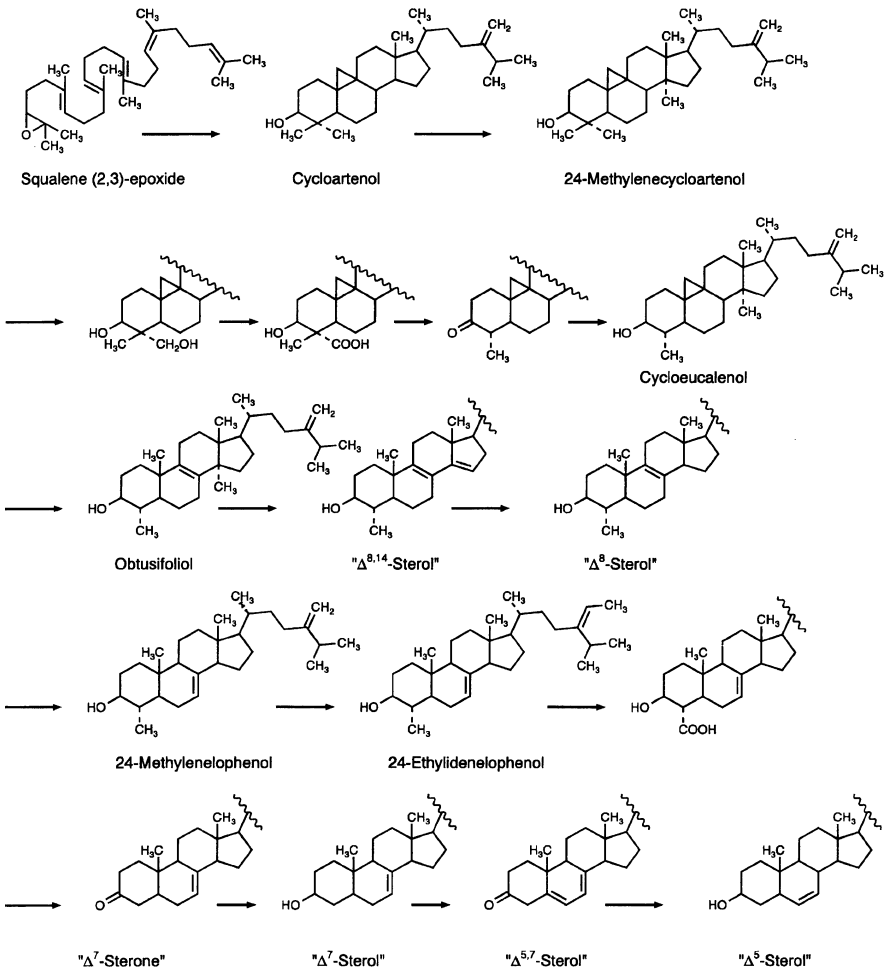


Figure 6.2 Proposed pathway for sterol biosynthesis in higher plants.

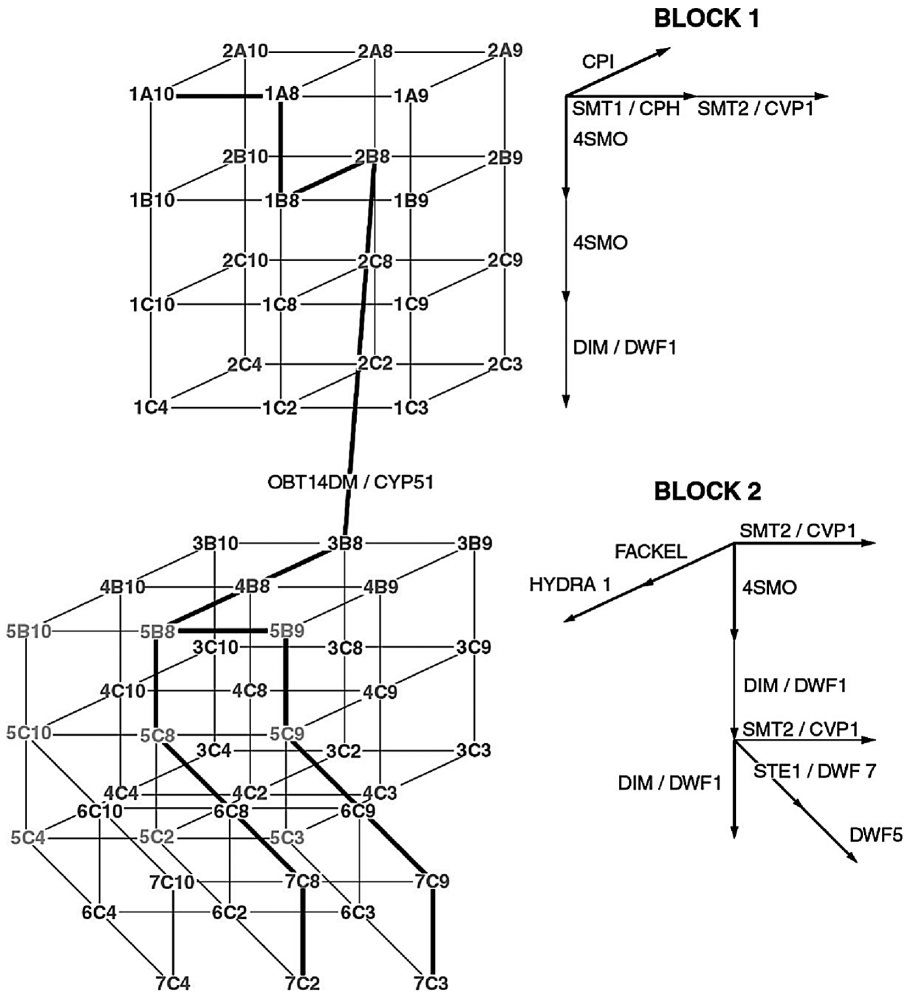


Figure 6.3 Biosynthesis of (24ξ)-24-methyl cholesterol (7C2) and (24R)-24-ethyl cholesterol (7C3) in vascular plants. A matrix of alternative routes along a main road is proposed. The structure of sterols is indicated by a number, a letter and a number. The first number represents a panel that gathers 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

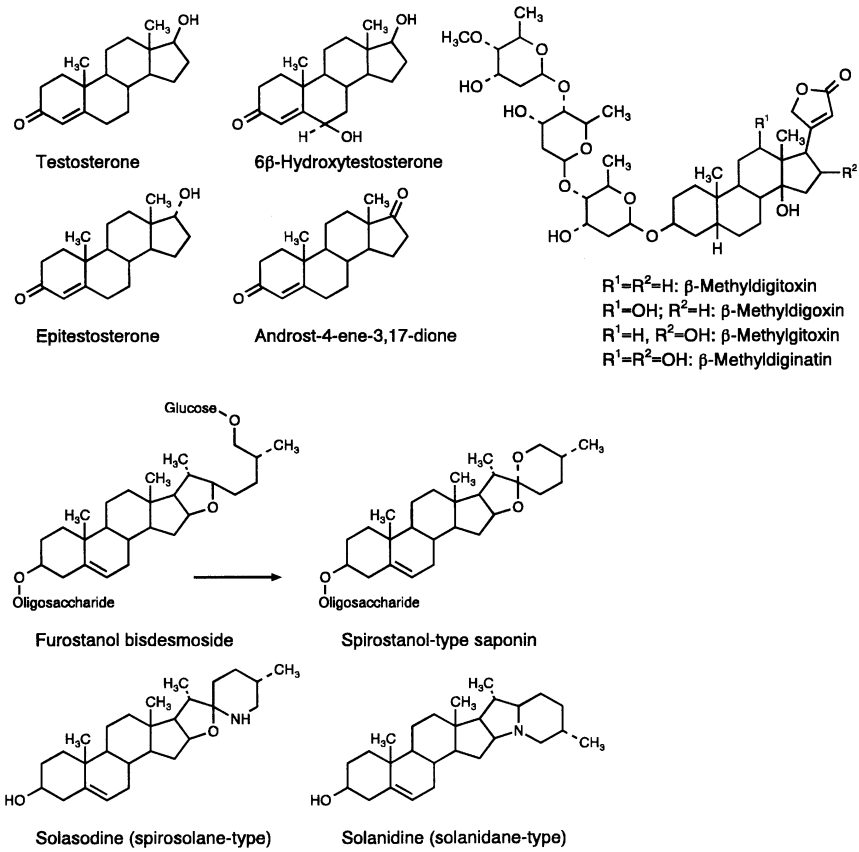


Figure 6.4 Chemical structures of estranes, androstanes, cardiac glycosides steroid saponins and steroid alkaloids mentioned in the text.

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).

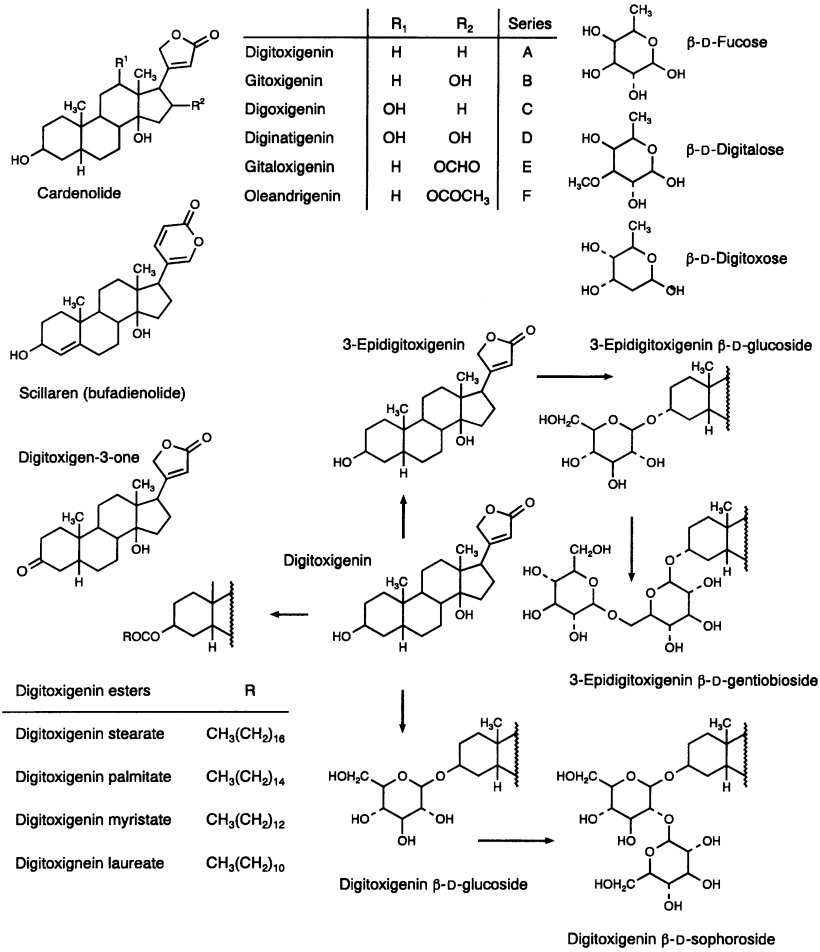
6.3 Cardiac glycosides

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

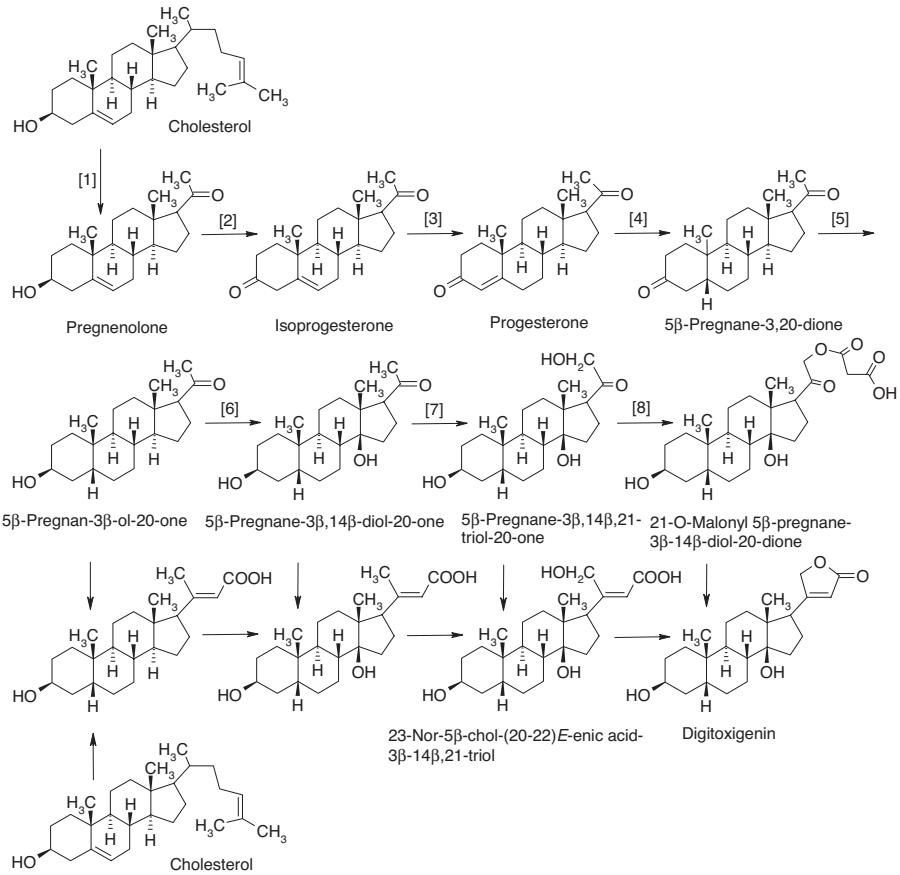


Figure 6.6 Routes for cardenolide genin formation in *Digitalis*. The 'classical' pathway is shown in the upper part, the alternative route via norcholenic acids is depicted in the lower part. Cholesterol (or another phytosterol) is assumed to be the starting point for both pathways.

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).

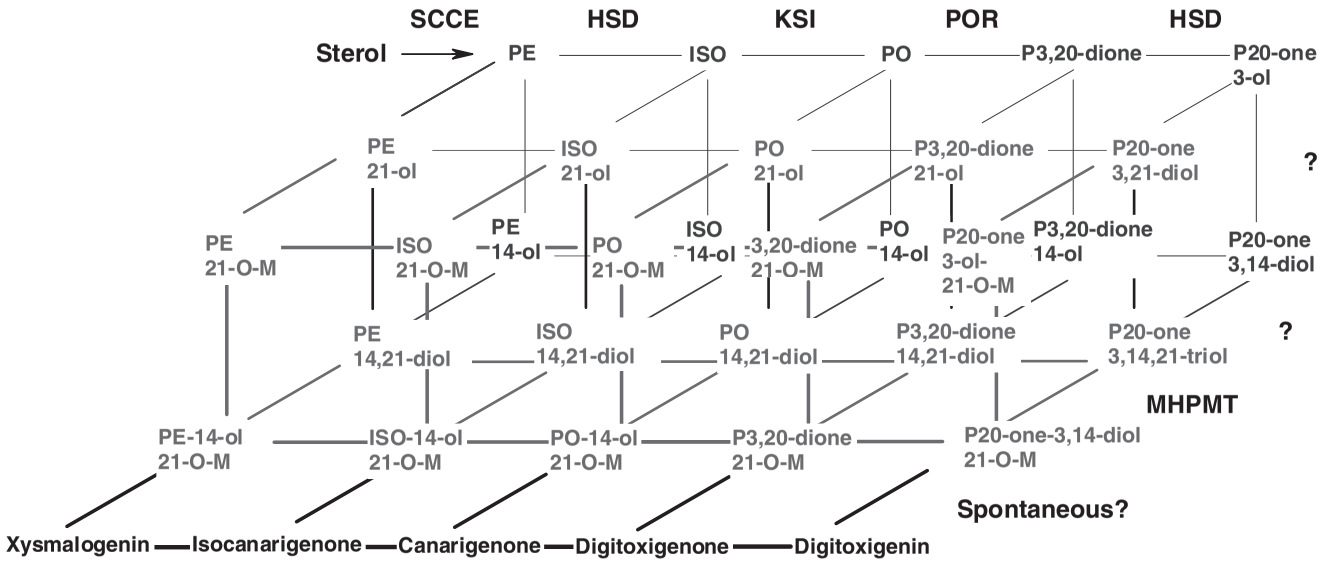


Figure 6.7 Biosynthesis of 5β-cardenolides and $\Delta^{5,6}$-cardenolides. A matrix of alternative routes in the 'classical' pathway is proposed. PE, pregnenolone; ISO, isoprogesterone; PO, progesterone; P, 5β-pregnane; M, malonyloxy residue. The numbers refer to the skeleton carbon to which substituents (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

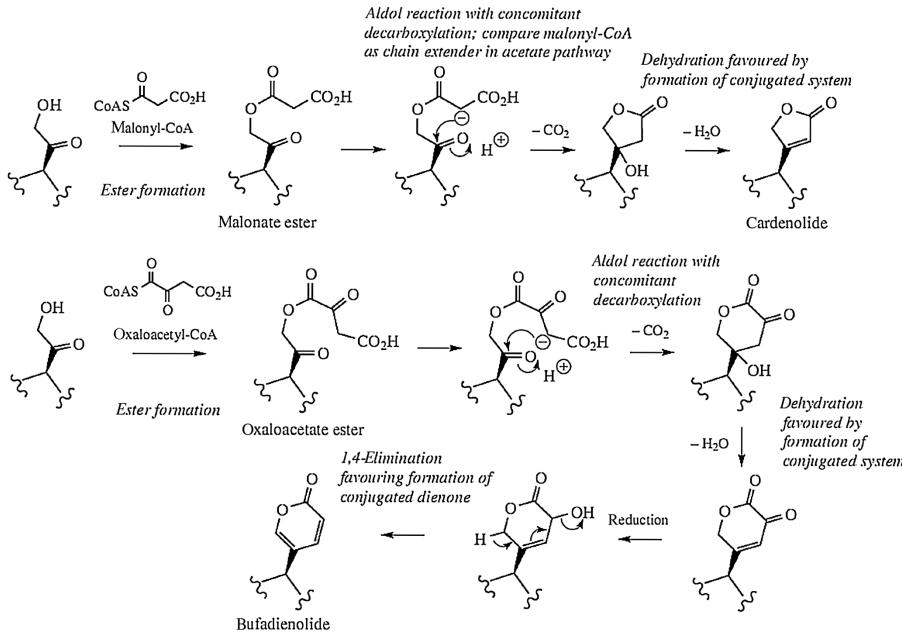


Figure 6.8 Proposed mechanism for lactone ring formation. Cardenolides: an intermediate malonate ester is involved, and ring formation probably occurs via an aldol addition process giving the cardenolide digitoxigenin, the carboxyl carbon of the malonate ester being lost by decarboxylation. Bufadienolides: 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.

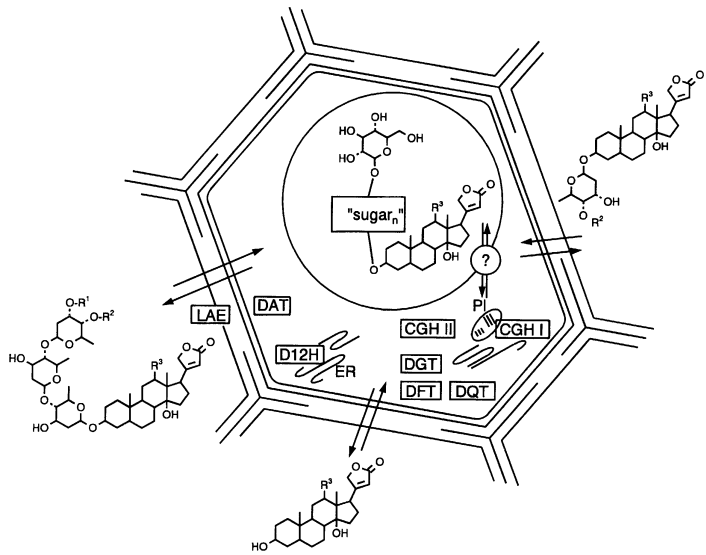


Figure 6.9 Cellular organization of cardenolides glycoside uptake, modification and storage. Exogenous cardenolides enter the cell by diffusion, after which they can be modified in several ways. Only those possessing a terminal glucose are stored in the vacuole, probably involving active transport across 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

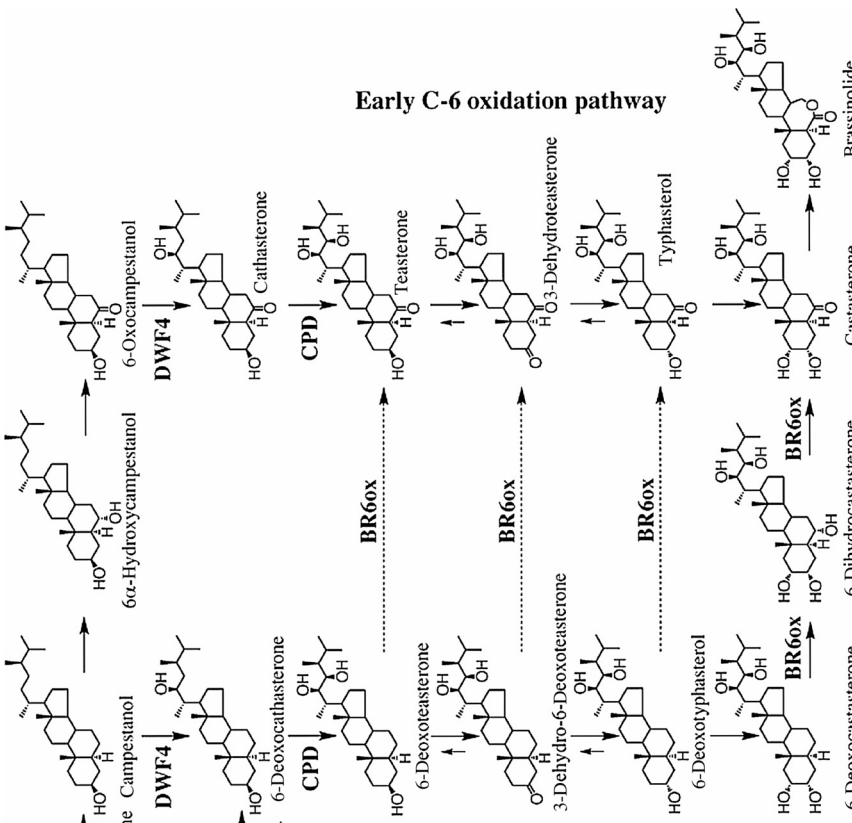


Figure 6.10 Biosynthesis of Brassinolide from campesterol. Enzymes identified from *Arabidopsis thaliana* are shown. Enzymes from other plants are identified in the text (Fujioka and Yokota, 2003).

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.

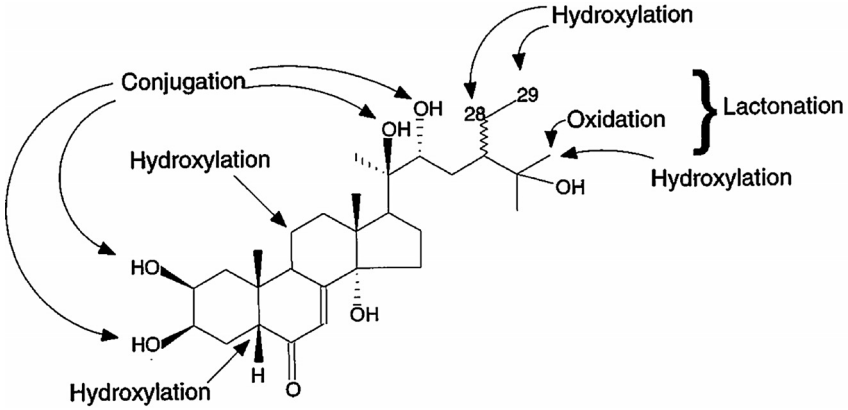


Figure 6.11 Structure and sites of biochemical modification of phytoecdysteroids. The structures of the most commonly reported phytoecdysteroid, 20-hydroxyecdysone, and a 24-alkylphytoecdysteroid, cyasterone. Some common sites of biochemical modifications reported for phytoecdysteroids. (From Adler and 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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Annual Plant Reviews (2010) **40**, 364–433
doi: 10.1002/9781444320503.ch7

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Chapter 7

CHEMOTAXONOMY SEEN FROM A PHYLOGENETIC PERSPECTIVE AND EVOLUTION OF SECONDARY METABOLISM

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Abstract: All plants produce secondary metabolites (SM); however, the structural types are often specific and restricted to taxonomically related plant groups. This observation was the base for the development of 'chemotaxonomy'. A closer look indicates that a number of SM have a taxonomically restricted distribution. Very often, we also find the same SM in other plant groups which are not related in a phylogenetic context. Examples are given for several groups of alkaloids (including pyrrolizidine and quinolizidine alkaloids) and for cardiac glycosides. How to explain the patchy distribution? Theoretically, the occurrence of SM in unrelated taxa may be due to convergent evolution. Alternatively, the genes encoding the enzymes of secondary metabolism might be widely distributed in the plant kingdom, but switched on or off in a certain context. The analysis of nucleotide and amino acid sequences, which were generated in numerous genome projects during the past decades, provides evidence that most of the genes which encode key enzymes of SM formation have indeed a wide distribution in the plant kingdom. Examples discussed are tryptophan decarboxylase, tyrosine decarboxylase, phenylalanine ammonia-lyase, chalcone synthase, strictosidine synthase, berberine bridge enzyme and codeine reductase. It is speculated that these genes were introduced into the plant genome by horizontal gene transfer, i.e. via bacteria that developed into mitochondria and chloroplasts. Evidence is presented that a patchy distribution can also be due to the presence of endophytic fungi, which are able to produce SM (e.g. ergot alkaloids in Convolvulaceae). The evolution of plant secondary metabolism is a complex process that took place over the past 500 million years.

 ISBN: 978-1-405-18397-0

Keywords: chemotaxonomy; patchy distribution; biosynthesis; genes; horizontal gene transfer; endophytes; evolution; tryptophan decarboxylase; tyrosine decarboxylase; phenylalanine ammonia-lyase; chalcone synthase; strictosidine synthase; berberine bridge enzyme; codeine reductase

7.1 Introduction

According to Wikipedia (http://en.wikipedia.org/wiki/Taxonomy), chemotaxonomy is defined as follows: 'Chemotaxonomy (from chemistry and taxonomy), also called chemosystematics, is the attempt to classify and identify organisms (originally plants), according to demonstrable differences and similarities in their biochemical compositions'. To some degree, man has been practising chemotaxonomy with respect to plants for a very long time. The senses of taste, smell and colour have all been used to gather information about the chemistry of an organism and, thus, to classify it in terms of importance and relevance. While it is a very difficult subject to research, it can now be stated unequivocally that the ability to classify is shared by many organisms, particularly in relation to selection of plants or plant parts for feeding and reproduction (Harborne, 1993). However, the outcome of such classification is entirely phenetic (i.e. based on overall similarity), associating plants on the basis of use, danger, etc., but usually telling us little, if anything, about the phylogenetic or evolutionary relationships between them. It should be noted that a true taxonomy should be based on phylogenetic relationships. Charles Darwin wrote to his friend T.H. Huxley in 1857, 'The time will come I believe, though I shall not live to see it, when we shall have fairly true genealogical trees of each kingdom of nature...'. Now, more than 150 years later, we have reached indeed a stage when fairly true 'genealogical' (we prefer the term 'phylogenetic' today) trees can be established for nearly every group of organisms by comparing DNA sequence data.

This chapter discusses the history and present state of chemotaxonomy in view of our knowledge of the molecular phylogeny of plants. In the second part of this chapter, the possible evolution of secondary metabolism will be elaborated (for a review, see Wink, 2003, 2007). This analysis helps to understand the discrepancies between chemotaxonomy and molecular phylogeny (for a review, see Wink, 2008).

7.2 Establishment of chemotaxonomy as a research discipline

While the potential value of plant secondary metabolites to taxonomy has been recognized for nearly 200 years (Candolle, 1804; Abbott, 1886), their practical application has been restricted to this century and predominantly to the past 45 years. The first extensive advocacy of chemical taxonomy came

from McNair (1935), who studied the distribution of volatile oils, fixed oils and alkaloids in the Angiospermae. At the same time, the first comparative analyses were being reported, most of which involved the volatile oils of the Myrtaceae, notably *Eucalyptus* (Penfold and Morrison, 1927). While these studies confirmed the distinctiveness of the chemistry of different taxa, even at this early stage, they illustrated the possibility for intraspecies variation in chemistry (which will be a common theme throughout this chapter).

The key technical development that allowed the establishment of chemotaxonomy was paper chromatography. This procedure allowed multiple samples to be extracted and compared for the presence or absence of specific metabolites (Bate-Smith, 1948; Alston and Turner, 1959). The flavonoids and related phenolic compounds proved to be particularly suitable for examination by paper and, subsequently, thin-layer chromatography (TLC). It was the distribution of some common phenolics that was first examined in some detail across the whole of the Angiospermae (Bate-Smith, 1958, 1962). Further methods, which were developed in the 1970s and 1980s and were essential for the advance of phytochemistry and chemotaxonomy, were capillary column (or high resolution) gas–liquid chromatography (GLC), high performance liquid chromatography (HPLC), mass spectrometry (MS, especially as GLC–MS, LC–MS) and nuclear magnetic resonance (^{1}H, ^{13}C- NMR).

The subject of chemotaxonomy really came of age in the early 1960s with the publication of several seminal works, notably those of Alston and Turner (1963) and Swain (1963, 1966), encompassing the discipline as a whole, and specialist works on flavonoids and other phenolics by Harborne (1964, 1967). Hegnauer had already embarked on his epic series, *Chemotaxonomie der Pflanzen*, in which, with great thoroughness, he compiled the current information on occurrence and distribution of metabolites within and between plant families (Hegnauer, 1962–1990; Hegnauer and Hegnauer, 1992–2001). By this time, sufficient data had been gathered concerning the occurrence of a wide range of secondary metabolites to allow for generalizations to be made on the taxonomic range of their distribution. This was accompanied by a rapidly growing library of experimental data dealing with the biosynthesis of these compounds (Geissmann and Crout, 1969), which allowed distribution to be placed in the context of a dynamic biosynthetic phylogeny. During this period, a number of very exciting discoveries were made which boded well for the impact that chemotaxonomy could have in unravelling Angiosperm evolution. These discoveries included the following.

7.2.1 Non-protein amino acids

Non-protein amino acids (NPAAs) are especially abundant in the family Fabaceae, but are also present in several monocots (families Alliaceae, Iridaceae, Liliaceae), Cucurbitaceae, Euphorbiaceae, Resedaceae, Sapindaceae and Cycadaceae. NPAAs are also toxic components of some fungi (e.g. coprine

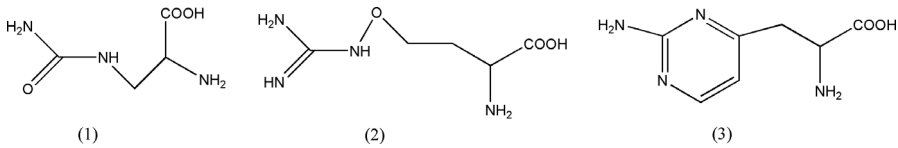


Figure 7.1 Structures of the non-protein amino acid albiziine (1), canavanine (2) and lathyrine (3).

in *Coprinus* species). Organs rich in these metabolites are seeds (Fabaceae) or rhizomes (monocots).

The Fabaceae are a major source of NPAAs, such as albiziine, canavanine and lathyrine (Fig. 7.1). These have proved to be useful taxonomic markers throughout the family, albiziine being a characteristic of the Mimosoideae, while lathyrine can be used to distinguish species of *Lathyrus* from *Vicia*. The distribution of canavanine in the Papilionoideae was examined very extensively and has been used in the compilation of phylogenies for that subfamily (Bell *et al.*, 1978; Polhill *et al.*, 1981a,b) (see Fig. 7.11c).

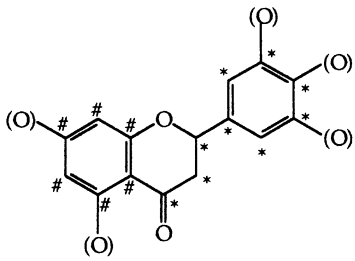
Concentrations in seeds can exceed 8% of dry weight and up to 50% of the nitrogen present can be attributed to them. Since NPAAs are often (at least partly) remobilized during germination, they certainly function as nitrogen storage compounds in addition to their role as defence chemicals against herbivores and microbial pathogens.

NPAAs often figure as antinutrients or antimetabolites (Rosenthal, 1982). Many NPAAs resemble protein amino acids and quite often can be considered to be their structural analogues and may interfere with the metabolism of humans, animals, even microbes and plants:

- In ribosomal protein biosynthesis, NPAAs can be accepted in place of the normal amino acid and incorporated into proteins, which thereby often become functionless.
- NPAAS may competitively inhibit uptake systems for amino acids in the gut.
- NPAAs can inhibit amino acid biosynthesis by substrate competition or by mimicking end product mediated feedback inhibition of earlier key enzymes in the pathway.
- NPAAs may affect other targets, such as DNA-, RNA-related processes (canavanine, mimosine), receptors of neurotransmitters, inhibit collagen biosynthesis (mimosine) or β-oxidation of lipids (L-hypoglycine).

7.2.2 Flavonoids

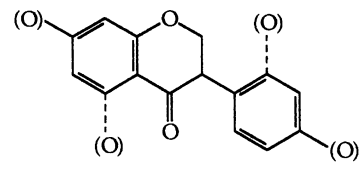
Flavonoids (Fig. 7.2) form one of the largest and most widespread groups of SM, being present from mosses, ferns, gymnosperms to angiosperms. An extensive range of structural variants has been found, based on the combination of a phenylpropenyl unit with three acetate units (a polyketide), including polymeric condensed tannins (e.g. procyanidins), ubiquitous



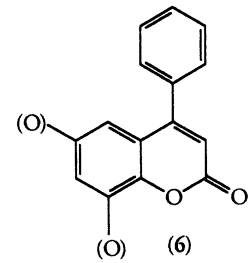
(4) Typical flavonoid

= Triketide component

* = Phenylpropene component



(5)



(6)

Figure 7.2 Structures of flavonoids; typical flavonoid (4), isoflavonoid (5) and neoflavonoid (6).

glycosidic forms, isoflavonoids and neoflavonoids. Flavonoids have received more attention from the chemotaxonomist than any other class of compounds because of their ubiquity and their accessibility through relatively simple methods of analysis (Mabry *et al.*, 1970). In his monumental survey of the plant kingdom, Bate-Smith (1962) revealed changes in substitution patterns that had broad correlation with taxonomic 'advancement', and suggested that a simplification in substitution patterns had occurred as part of the evolutionary process. Harborne (1966) proposed a series of primitive and advanced flavonoid characters that might be used to assess the phylogenetic position of a taxon.

The Fabaceae have also featured as a family with an interesting flavonoid distribution. The isoflavonoids (Fig 7.2 and 7.11f) are a particular feature of the Papilionoideae, where they occur as both constitutive metabolites and phytoalexins (Ingham, 1983). The neoflavonoids (Fig. 7.2) represent an alternative mode of cyclization of the phenylpropene and triketide precursor. They occur in the Papilionoideae (in the tribe Dalbergieae), but are also to be found in some genera of the Clusiaceae (formerly Guttiferae) (Donnelly, 1985). Isoflavones, which exhibit phytoestrogenic properties, have a limited distribution outside the Fabaceae in Asteraceae, Iridaceae (*Iris*), Myristicaceae (*Osteophleum, Virola*), Chenopodiaceae (*Spinacia*), Moraceae (*Maclura*) and Rosaceae (*Cotoneaster*).

7.2.3 Xanthones

Whereas flavonoids are the product of a C_6C_3 and a triketide precursor, the xanthones (Fig. 7.3) originate from a triketide linked with a C_6C_1 unit.

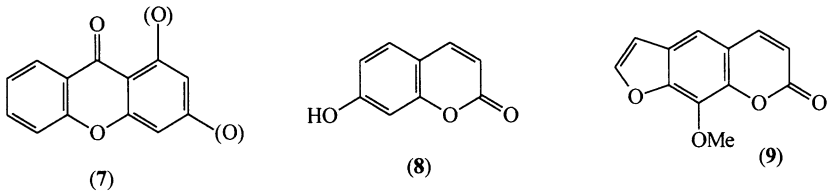


Figure 7.3 Structures of xanthones and coumarins: xanthone (7), coumarin (8) and furocoumarin (9).

There are two apparently unrelated centres of xanthone production in the Angiospermae: the Clusiaceae and the Gentianaceae. With respect to the former, the occurrence of xanthones in such genera as *Bonnetia* and *Archytaea* has been cited as powerful evidence for allying them to the Clusiaceae rather than the Theaceae (Kubitzki *et al.*, 1978).

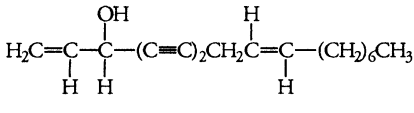
7.2.4 Coumarins

The simple coumarin nucleus (Fig 7.3), which is derived by lactone formation of an ortho-hydroxy-*cis* cinnamic acid, is a common metabolite in higher plants and is often found in glycosidic form. Coumarins are common in Apiaceae, in certain genera of Fabaceae (e.g. *Dipteryx odorata, Melilotus officinalis*), Poaceae (e.g. *Anthoxanthum odoratum*) and Rubiaceae (e.g. *Galium odoratum*). However, proliferation of coumarins to the status of major chemical markers occurs in only a few cases, most notably, but not exclusively, in the Apiaceae (subfamily Apioideae) and in the Rutaceae (Gray and Waterman, 1978; Murray *et al.*, 1982). In these cases, the coumarin nucleus has almost invariably been embellished by the addition of a prenyl unit leading to furocoumarin (Fig 7.3) and pyranocoumarin structures.

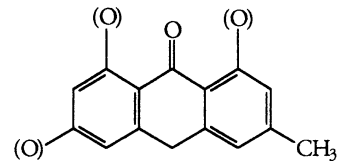
7.2.5 Fixed oils, fats and waxes

When gas chromatography became established as an analytical technique, certain classes of metabolite proved particularly amenable to study. These were volatile oils (see monoterpenes), the constituents of leaf surface waxes and, in the form of their methyl esters, the fatty acid components of fixed oils and fats, particularly those occurring in seeds. Structural variation within each of these types of compound proved to be somewhat less pronounced than with many other groups of metabolites. Nevertheless, the classification of plant families on the basis of the chain length and degree of unsaturation of the predominant fatty acids was proposed (Smith, 1976).

The polyacetylenes, which have a common origin with fatty acids, showed a far more restricted distribution (Bohlmann *et al.*, 1973). Falcarinol (Fig. 7.4) and allied structures were cited as being diagnostic of the Araliales (Araliaceae, Apiaceae, Pittosporaceae), while another major centre of production



(10)



(11)

Figure 7.4 Structures of the polyacetylenes, falcarinol (10), and the tricyclic polyketide, anthrone (11).

was found in the Asteraceae, where sulfur-containing polyacetylenes (i.e. thiophenes) occur.

7.2.6 Cyclic polyketides

The tricyclic polyketide, anthrone (Fig. 7.4), had been noted as a feature of *Aloe* and some related genera (*Asphodelus, Haworthia, Kniphofia, Eremurus*) in the monocot family Asphodelaceae and Liliaceae (*Xanthorrhoea*). They are also present in dicots such as Polygonaceae (*Rheum, Rumex, Polygonum, Fagopyrum, Oxygonum*), Rhamnaceae (*Rhamnus, Karwinskia, Maesopsis, Ventilago*), Fabaceae (*Andira, Cassia, Ferreira, Gleditsia, Vatairea*), Rubiaceae (*Asperula, Coprosma, Coelospermum, Galium, Hymenodictyon, Plocama, Relbunium, Rubia, Cinchona, Morinda, Sherardia*), Clusiaceae/Hypericaceae (*Hypericum, Harungana, Psorospermum*), Verbenaceae (*Tectona*), Scrophulariaceae (*Digitalis, Scrophularia*), Gesneriaceae (*Streptocarpus*) and Myrsinaceae (*Myrsine*). Anthraquinones are common defence compounds in lichens (*Nephroma, Lecidea, Caloplaca*).

7.2.7 Monoterpenes and sesquiterpenes (volatile oils)

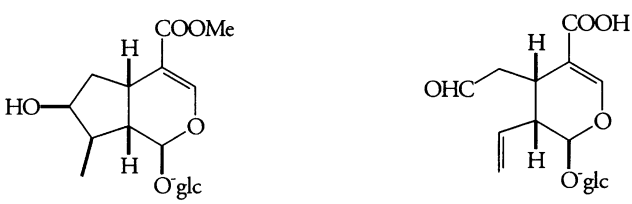
Because of the ease of qualitative analysis, first through distillation to isolate major components and, subsequently, through GLC, volatile oils have consistently attracted the attention of chemotaxonomists. These oils are almost invariably complex mixtures in which monoterpenes and/or sesquiterpenes usually predominate, although the biosynthetically unrelated phenylpropanes can also be important. Some of the earliest studies on the genetic control of SM involved the oils of mints, *Mentha* (Murray, 1960). Volatile oils yielded the first properly documented examples of chemical races (Penfold and Morrison, 1927; Sutherland and Park, 1967), while Zavarin and co-workers (1971) provided clear evidence for the impact of environmental factors on the composition of volatile oil. Because of the comparative nature of GLC analysis, volatile oils were among the first compounds to be extensively studied at the population level and to be subjected to numerical analysis. The work of Adams on *Juniperus* in south-eastern USA

and northern Mexico was an excellent early example of the exploitation of numerical techniques (Adams and Turner, 1970; Adams, 1972).

Among the sesquiterpenes, there are also some more highly oxidized non-volatile compounds. The best examples are the sesquiterpene lactones, which were found to be distributed quite widely in the Asteraceae, but were thought to be rare elsewhere (Herout and Sorm, 1969). Sesquiterpene lactones are widely distributed in Asteraceae (e.g. in the genera *Achillea, Ambrosia, Anaphalis, Anthemis, Arnica, Artemisia, Arctium, Arctotis, Baileya, Balduina, Baltimora, Cacalia, Calea, Calocephalus, Carpesia, Centaurea, Chaenactis, Chromolaena, Chrysanthemum, Cichorium, Cnicus, Cynara, Dicoma, Dugaldia, Elephantopus, Encelia, Enhydra, Eremanthus, Eriophyllum, Eupatorium, Gaillardia, Geigera, Grossheimia, Helenium, Helianthus, Homogyne, Hymenoxys, Inula, Isocarpha, Iva, Jurinea, Lactuca, Liatris, Ligularia, Lychnophora, Matricaria, Melampodium, Mikania, Moquinia, Onopordum, Oxylobus, Parthenium, Petasites, Podanthus, Psilostrophe, Saussurea, Senecio, Smallanthus, Stokesia, Tanacetum, Telekia, Tithonia, Ursinia, Vanillosmopsis, Vernonia, Viguiera, Wedelia, Xanthium, Xeranthemum, Zaluzania, Zexmenia, Zinnia*), but are also common in some Apiaceae (*Laser, Laserpitium, Thapsis*), Lamiaceae (*Glechoma*), Illiciaceae (*Illicium*), Coriariaceae (*Coriaria*), Magnoliaceae (*Liriodendron, Magnolia, Michelia*), Menispermaceae (*Anamirta*), Euphorbiaceae (*Toxicodendron, Hyaenanche*), Lauraceae (*Laurus nobilis, Lindera*), gymnosperms (*Cupressaceae*) and a few mosses (*Frullania*) (Wink and Van Wyk, 2008).

7.2.8 Iridoids

The iridoids are an atypical structural form of monoterpenes, exemplified by the two compounds, loganin and secologanin (Figs 7.5 and 7.14). Their relatively high level of oxidation and the regular occurrence of glycosides made these bitter-tasting compounds less tractable to study than the 'normal' volatile monoterpenes. However, it rapidly became obvious that their distribution was limited to a relatively small number of families, many of which were clearly of close affinity to one another. Iridoid glycosides (secoiridoids, secologanin derivates) with more than 200 structures are widely distributed in the related orders Gentianales (families Apocynaceae,



(12) (13)

Figure 7.5 Structures of loganin (12) and secologanin (13).

Gentianaceae, Loganiaceae, Menyanthaceae, Rubiaceae), Lamiales (Bignoniaceae, Buddlejaceae, Globulariaceae, Lamiaceae, Pedaliaceae, Plantaginaceae, Scrophulariaceae and Verbenaceae), Ericales (Monotropaceae), Cornales (Cornacaceae) and Dipsacales (Valerianaceae). Aucubin has been found in *Plantago* (Plantaginaceae), *Aucuba japonica* (Cornaceae), *Euphrasia, Rhinanthus, Veronica* (Scrophulariaceae) and *Ajuga* (Lamiaceae), catalpol in Catalpa (Bignoniaceae), *Veronica* (Scrophulariaceae), *Plantago* (Plantaginaceae) and *Buddleja* (Buddlejaceae). Harpagoside and harpagide have been found in *Harpagophytum produmbens* (Pedaliaceae), *Scrophularia* (Scrophulariaceae) and *Lamium* (Lamiaceae). Jensen and co-workers (1975) formerly proposed that the iridoid-producing families were a monophyletic group, which is only partly true when comparing the iridoid-producing taxa with the present Angiosperm Phylogeny Group (APG) phylogeny (Fig. 7.8).

7.2.9 Triterpenes, sterols and carotenoids

The common members of these classes, such as α-amyrin, β-sitosterol and β-carotene, occur very widely and were soon recognized to be of no taxonomic value. A number of rarer classes of triterpenes and sterols such as the withanolides, and the limonoids and quassinoids of the Rutales (Fig. 7.6), were noted for their limited distribution. Cardiac glycosides, which can be divided

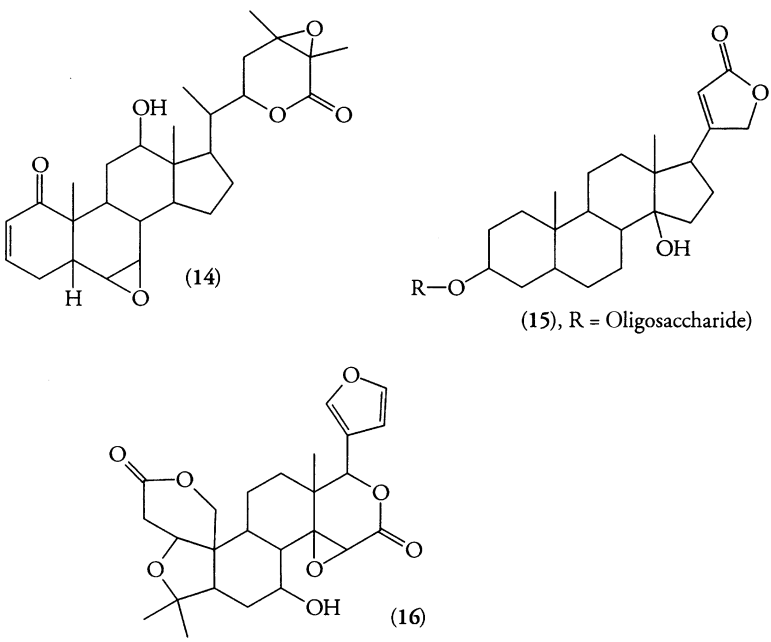


Figure 7.6 Structures of the withanolides (14), cardiac glycosides (15) and limonoids (16).

into cardenolides and bufadienolides, are common in some members of the Apocynaceae, Asclepidaceae and Scrophulariaceae, but also occur in other unrelated families (Fig. 7.8a): Cardenolides: *Digitalis* (Scrophulariaceae), *Convallaria* (Convallariaceae), *Acokanthera, Adenium, Alafia, Apocynum, Cerbera, Hunteria, Nerium, Strophanthus, Thevetia, Periploca, Xysmalobium* (Apocynaceae), *Asclepias, Calotropis, Cynanchum, Gomphocarpus, Sarcostemma, Cryptostegia* (Asclepiadaceae), *Adonis* (Ranunculaceae), *Euonymus*, *Lophopetalum* (Celastraceae), *Cheiranthus*, *Erysimum* (Brassicaceae), *Ornithogalum, Rhodea,* (Hyacynthaceae), *Coronilla/Securigera* (Fabaceae), *Antiaris, Castilloa, Naucleopsis, Maquira* (Moraceae), *Corchorus olitorius*, Tiliacae. Bufadienolides: *Bowiea, Drimia, Scilla, Urginea* (Hyacynthaceae), *Cotyledon, Kalanchoe, Tylecodon* (Crassulaceae), *Helleborus* (Ranunculaceae), *Homeria, Moraea* (Iridaceae), *Melianthus* (Melianthaceae) and *Thesium* (Santalaceae) (Wink and Van Wyk, 2008).

Some families, notably the Caryophyllaceae, Ranunculaceae, Phytolaccaceae, Chenopodiaceae, Styracaceae, Hippocastanaceae, Theaceae, Fabaceae, Apiaceae, Araliaceae, Asteraceae, Aquifoliaceae, Rosaceae, Polygalacdeae, Amaranthaceae/Chenopodiaceae, Cucurbitaceae, Rhamnaceae, Primulaceae, Poaceae and Sapotaceae, are able to produce triterpenes linked to several sugars to form a surfactant saponin, the presence of which could readily be detected by simple tests, such as blood cell haemolysis. Gymnosperms are apparently without saponins. Steroidal saponins are abundant in monocots of the families Dioscoreaceae, Trilliaceae, Liliaceae, Agavaceae, Asparagaceae, Convallariaceae, Zingiberaceae, Alliaceae, Poaceae and Smilacaceae, but also occur in some dicots (Fabaceae, Scrophulariaceae, Solanaceae).

7.2.10 Nitrogen-containing terpenes

Each class of terpene was found to associate with nitrogen to form alkaloid-like compounds, for which Hegnauer (1963) coined the term 'pseudoalkaloid'. The most interesting of these are 'diterpene alkaloids', which are found in *Delphinium* and *Aconitum* (Ranunculaceae), where they were recognized as supporting a close relationship between those genera (Jensen, 1968). 'Steroidal alkaloids' were identified as being significant markers in a number of families, notably the Apocynaceae, Asclepiadaceae, Buxaceae, Solanaceae and Liliaceae. Some of these families were also found to be major sources of true alkaloids.

7.2.11 Alkaloids

The alkaloids have long been recognized as an important group of metabolites because of their biological activity, but they, more than any other major group of metabolites, needed the technical revolutions in chromatography and spectroscopy to allow for an assessment of their distribution (Mothes *et al*., 1985; Wink, 1993a,c, 2000, 2007; Roberts and Wink, 1998). In chemotaxonomic terms, alkaloids were defined by Hegnauer (1963), who distinguished

'true alkaloids' from other nitrogen-containing metabolites on the basis of their origin from amino acids, their basic nature and their limited distribution. Hegnauer defined the major classes of alkaloid in terms of their biosynthesis from precursor amino acids rather than their final structure (see Chapter 2). For example, quinine was recognized as a monoterpene indole alkaloid arising from the same biosynthetic route as reserpine, but different from that leading to 6-methoxyflindersine, with which quinine shares a quinoline nucleus (Fig. 7.7). The major classes recognized are listed in Table 7.1 (see also Fig. 2.1, p. 22).

In the 1950s and 1960s, there were several notable successes involving the use of alkaloids as taxonomic markers. These included the acceptance of

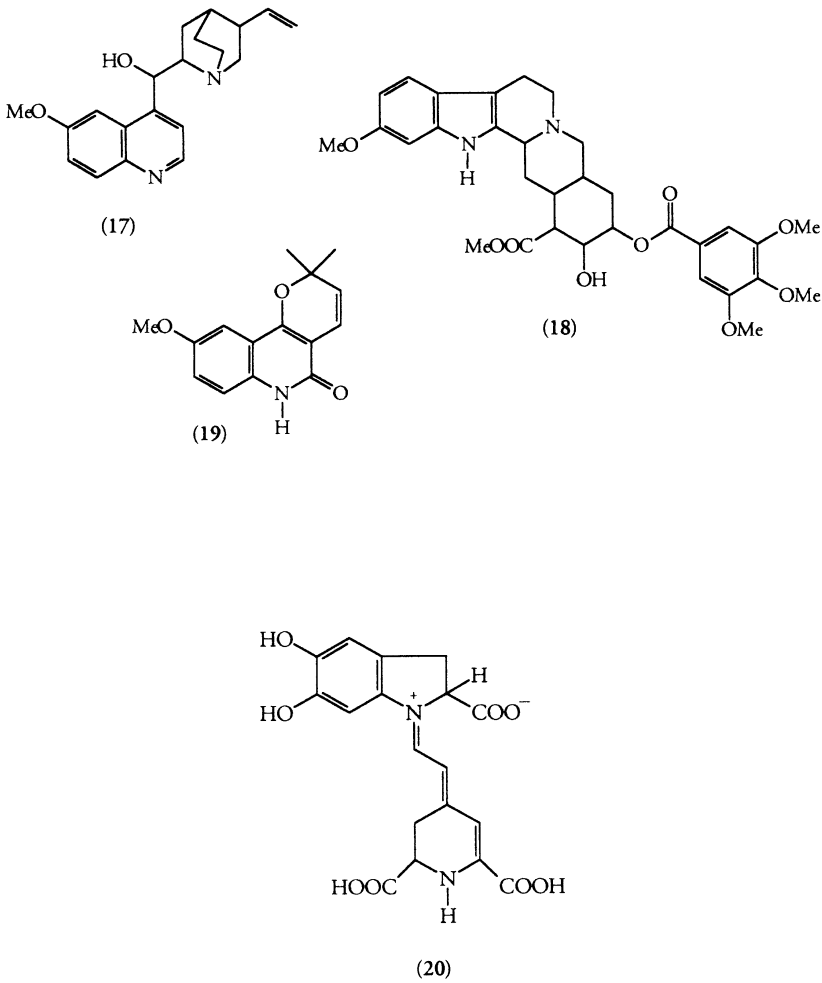


Figure 7.7 Structures of quinine (17), reserpine (18), 6-methoxyflindersine (19) and betanidin (20).

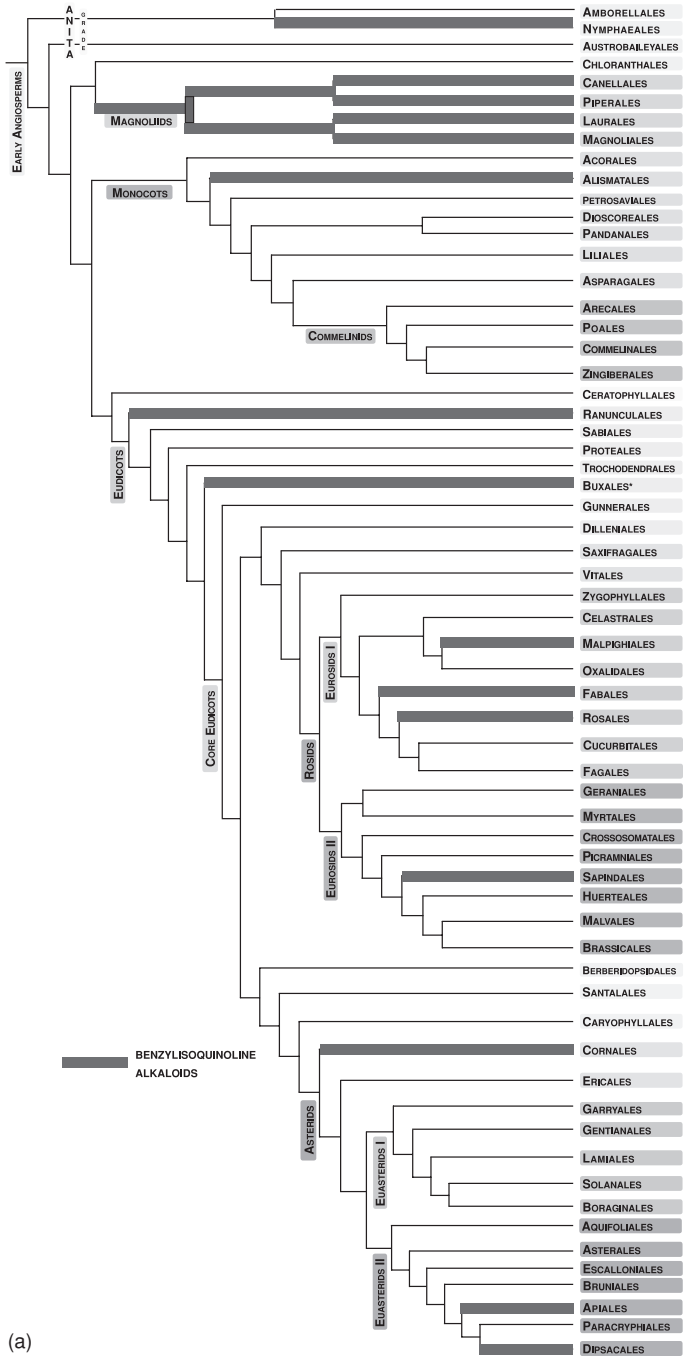
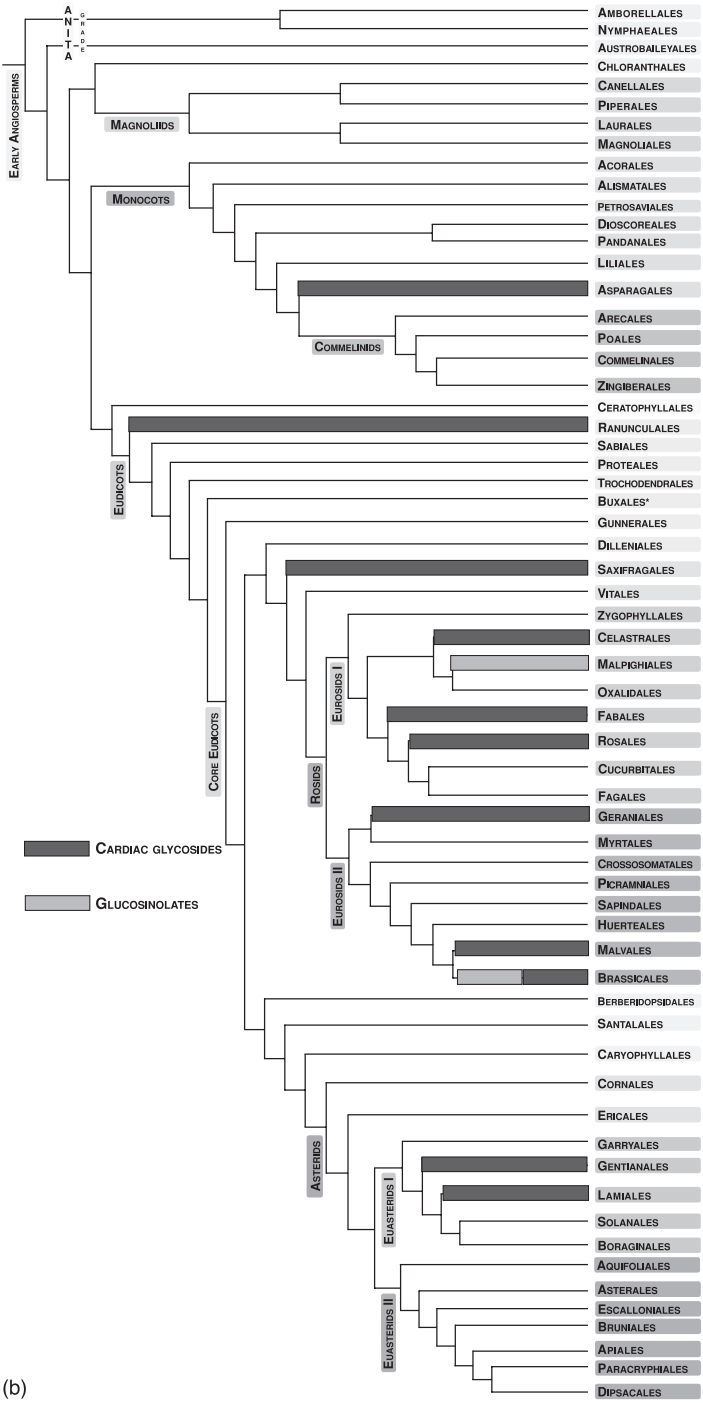
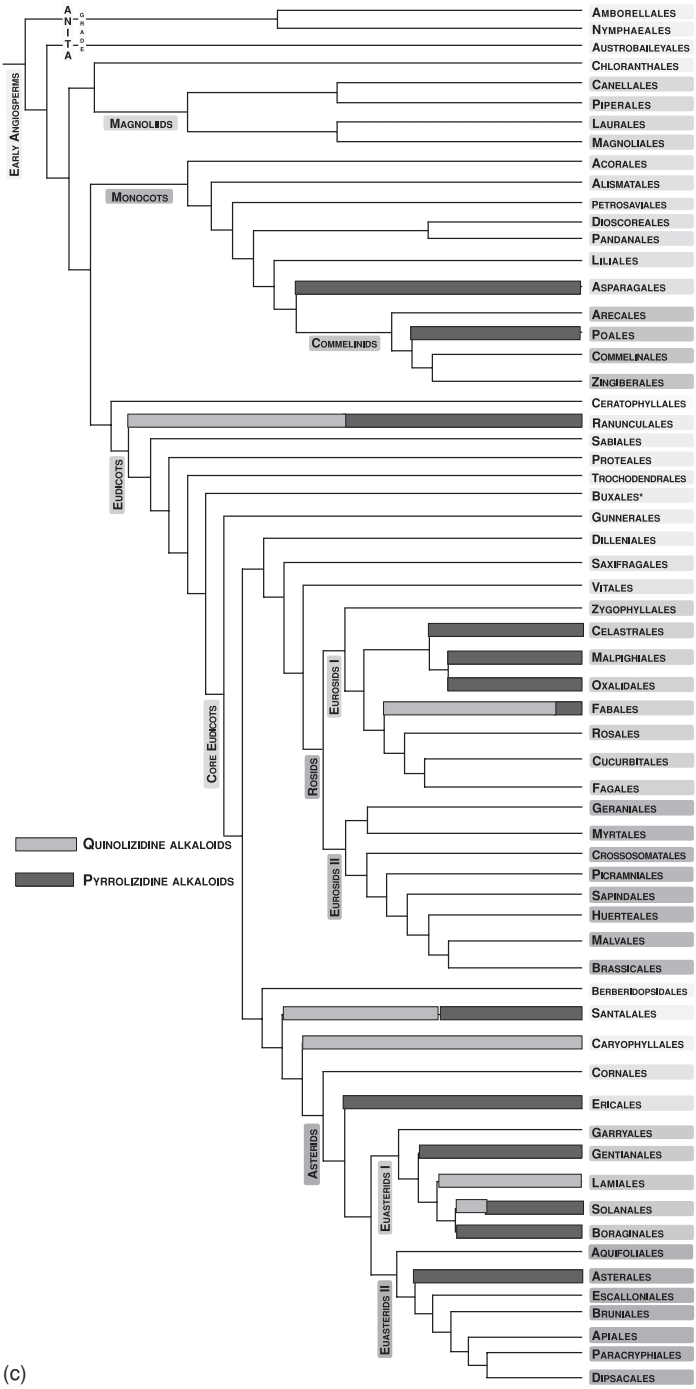


Figure 7.8 (a) Families and orders of higher plants, placed in a phylogenetic framework according to APG-III (2009). Branches leading to families, which accumulate benzylisoquinoline alkaloids



(b)

Figure 7.8 (*Continued*) (b), cardiac glycosides and glucosinolates



(c)

Figure 7.8 (*Continued*) (c) pyrrolizidine and quinolizidine alkaloids and are highlighted in colour. (See Plates 10–12 in colour plate section.)

Table 7.1 Biogenetic classification and principal centres of production of major alkaloid groups

Amino acid	Condensation group	Alkaloid type	Some major sources
Tyrosine or phenylalanine	Deaminated tyrosine or phenylalanine unit (C_6C_2)	1-Benzyltetrahydro-isoquinolines	Families of the former Ranales or Polycarpicae (Menispermaceae, Annonaceae, Lauraceae, Magnoliaceae, Monomiaceae), Berberidaceae, Papaveraceae, Fumariaceae, Rutaceae (in part), Fabaceae (in part)
	Deaminated tyrosine or phenylalanine unit (C_6C_1)		Monocotyledenous, families, notably Amaryllidaceae
	Tyrosine or proline	Betalains	Families of the Centrospermae (e.g. Cactaceae, Aizoaceae, Portulacaceae, Phytolaccaceae)
Anthranilic acid Tryptophan	Mono and triketides Secologanin	Quinolines Indole-monoterpene	Rutaceae Loganiaceae, Apocynaceae, Rubiaceae
Histidine	Acetate?	Imidazole	Rutaceae (in part), Fabaceae (in part)
Ornithine	Diketide	Tropane	Solanaceae, Erythroxylaceae, Convolvulaceae
Ornithine	Deaminated ornithine (putrescine)	Pyrrolizidine	Boraginaceae, Asteraceae (in part), Fabaceae (in part), Ranunculaceae
Lysine	Deaminated lysine (cadaverine)	Quinolizidine	Fabaceae

the proposition of Hegnauer (1961) to the effect that the Papaveraceae and Fumariaceae were misplaced in the Rhoedales *sensu* Wettstein and were better placed in or adjacent to the former Polycarpicae. Central to this argument was the co-occurrence of 1-benzyltetrahydroisoquinoline (1btiq) alkaloids in these two families and in many of the major families of the Polycarpicae (see Table 7.1). The Polycarpicae were themselves the focus of considerable attention because of the occurrence of 1-btiq alkaloids in many of the major

families (Fig. 7.8a). However, as noted by Hegnauer (1963), this distribution did not encompass all of the families of the former Polycarpicae and some were alkaloid free. This raised the question of whether the ability to produce these alkaloids was ancestral in the order or had arisen during its evolution. No satisfactory answer could be proposed.

Another fascinating class of compounds that originate from tyrosine are the betalains (see Chapter 2). These highly coloured substances, typified by betanidin (Fig. 7.7), were often referred to as pigments, but, biogenetically, they are alkaloids in every sense other than in the relative absence of pharmacological activity. Betalains were found to be restricted in distribution to several families that were placed together in the order Centrospermae (Mabry, 1966). However, as with the 1-btiq alkaloids in the Ranales, the distribution of betalains within the Centrospermae (now Caryophyllales) did not encompass all families, the Caryophyllaceae being the most notable exception. Once again, the dilemma of an ancestral or derived origin for these compounds became a major point of debate.

The large class of indole alkaloids, based on the combination of tryptamine and the monoterpene *seco*loganin (Fig. 7.5; 2.1), also offered considerable opportunities for the chemotaxonomist. Unravelling the biosynthesis and biogenetic relationships between these alkaloids offers ample evidence of the skills of those working in this area (Geissmann and Crout, 1969) and exemplifies the capacity of plants to generate extraordinary structural diversity from one set of precursors. As noted previously, *seco*loganin is an iridoid and it is among a subset of the iridoid-producing families that this group of alkaloids occur most widely, notably in the Apocynaceae, Loganiaceae and Rubiaceae. The Rubiaceae were often classified separately from the other two families, a course of action that was questioned on the basis of alkaloid distribution.

An interesting group of indole alkaloids are the ergot alkaloids. They are mainly produced by fungi: *Claviceps purpurea, C. microcephala, C. paspali* and more than 40 further members of this genus live as symbionts on grasses (tribes Festucaceae, Hordeae, Avenae, Agrosteae). Rye is especially affected among cereals. *Claviceps* is not a parasite but obviously a symbiotic organism. It takes nutrients from its host, but provides chemical defence against herbivores as compensation. Field experiments have shown that such a fungal infection is an ecological advantage for grasses in the wild. A related fungus *Epichloe* also produces ergot alkaloids. Ergot alkaloids (such as agroclavine, chanoclavine, ergine, ergosine and ergometrine) are also common SM of some genera of the Convolvulaceae (including *Argyreia, Ipomoea, Rivea corymbosa, Stictocardia tiliafolia*). It could be shown recently that the ergot alkaloid formation in the Convolvulaceae is due to an endophytic fungus that lives together with certain species in this plant family (Ahimsa-Müller *et al*., 2007; Markert *et al*., 2008). In this case, the isolated occurrence of ergot alkaloids is due to a symbiotic relationship. We may conclude that similar endophytic interactions could exist in other plants as well, which could explain the erratic appearance of some SM in the plant kingdom (Fig. 7.15).

Of all the alkaloid-producing families, one of the most prolific is the Rutaceae (Waterman, 1975). The alkaloids obtained included 1-benzyltetrahydroisoquinoline, simple tryptophan derivatives, imidazoles and, most commonly, quinoline alkaloids originating from anthranilic acid. The Rutaceae was the only family in which the direct use of anthranilic acid in alkaloid production occurs to any extent.

7.3 Developments in small molecule chemotaxonomy over the past 35 years

7.3.1 Phylogenetic frameworks derived from chemotaxonomy

By the beginning of the 1970s, chemotaxonomy had made a considerable impact on plant systematics and new systems of classification were being developed that took account of the distribution of secondary metabolites (Thorne, 1968, 1976; Dahlgren, 1980). This, in many respects, marked a high point for the use of low molecular weight secondary metabolites as taxonomic markers. In particular, the system produced by Dahlgren placed some emphasis on the distribution of these metabolites, and it was presented in a way that Dahlgren called a 'two-dimensional framework', in which the orders of plants were clustered to show proposed phylogenetic relationships (Wink and Waterman, 1999).

Dahlgren's framework allowed chemotaxonomists the opportunity to plot out known distribution patterns against a phylogenetic system of classification for the Angiospermae. The results of such analyses were very revealing and more than a little disconcerting for many chemotaxonomists as particular secondary metabolites could occur in apparently non-related groups. This is a pattern that would be repeated for almost all classes of metabolite mentioned in this chapter, with the notable exception of the betalains, which do appear to remain restricted to the Caryophyllilflorae and the glucosinolates in the Brassicales.

The inevitable conclusion drawn from these observations is that the expression of secondary metabolites of a given structural type has almost invariably arisen on a number of occasions. Consequently, the co-occurrence of a structural class of metabolite in two taxa cannot be taken to imply a monophyletic relationship. This means that the systematic value of chemical characters becomes a matter for interpretation by a systematicist in the same way as traditional morphological markers, despite the fact that they can be defined unambiguously in terms of both origin and structure. Given that the chemical record is usually only fragmentary for any taxon under investigation, this makes them of limited value as markers in studies at higher hierarchical levels.

7.3.2 Quantifying chemical data for numerical taxonomy

One advantage that secondary metabolites should have is that, when biosynthetic pathways are known, it is possible to identify events that have evolutionary implications. It has always been attractive, therefore, to consider the use of numerical methods in assessing the implications of chemical profiles. An early example of this was an analysis of the flavonoids of *Geranium* by Bate-Smith (1973), in which 'flavonoid scores' were produced for each species based on the presence or absence of individual flavonoids. These scores were used to identify the 'relative advancement' of individual species.

The major exponents of reducing chemical data to numbers have been Gottlieb and co-workers (Gottlieb, 1982). The approach adopted has been to identify structural skeletons and then, by recognizing modifying events, such as additional oxidation or substitution, to allocate scores, either positive or negative, that relate to the relevant advancement of each compound selected. Unfortunately, such interpretations are generally difficult to follow, particularly for the non-chemist. Selection of compounds to be included is based on the literature available rather than a consistent approach to data-gathering that is equivalent across the taxa under analysis, and this clearly causes bias in the results. This approach has not gained a wide level of acceptance among practising systematicists.

On the other hand, there has been an increasing employment of analytical methods to assess 'degrees of similarity' between comparable sets of chemical data produced from a series of taxa. Expansion of this approach has gone hand-in-hand with access to computing facilities. Some of the earliest examples came from work on volatile oils, an excellent example being the analysis of similarity between populations of *Juniperus ashei*, based on the comparison of 54 terpenoid characters (Adams, 1975). Such studies are today fairly commonplace and usually involve either volatile oils or flavonoids as it is relatively easy to establish data matrices on the presence, absence and abundance of individual compounds with little ambiguity. In the present literature, it is usual to see comprehensive cladistic analyses incorporating some chemical data.

7.3.3 What is the future of small molecule chemotaxonomy?

It is difficult not to conclude that we have now identified most, if not all, major insights that systematics will gain from studying the distribution of low molecular weight metabolites. The examples cited in reviews by Harborne and Turner (1993) and Waterman and Gray (1988) in the most recent volumes of *Chemotaxonomie der Pflanzen* (Hegnauer and Hegnauer, 1992–2001) and by Waterman (1997, 2007) all persist in emphasizing these early findings, largely because little of equal importance has happened since.

Life for the chemical taxonomist became more complicated as it emerged that the distribution map for almost every structural type of compound was

expanding as methods for detection and identification improved. Increasingly, new findings had to be rationalized in terms of parallel or convergent evolution, so making systematic relevance more difficult to establish. The advent and rapid development of molecular biology led to the recognition that the genetic infrastructure for the production of a given structure or structural skeleton was likely to be retained as part of the genome, even after expression ceased. This allowed for the option of re-expression of genes to be triggered at some latter point, so that the reappearance of a compound might well not even represent a 'reinvention' of a structure or the apparatus for its production. A further complication was the increasing recognition that there were considerable external pressures influencing the production of secondary metabolites, usually relating to the interaction of the producer with environmental factors, such as herbivores and pathogens (Waterman and Mole, 1989). For example, where two unrelated plant taxa were faced with similar problems in relation to seed dispersal, it was to be expected that the stratagem would evolve along similar lines, so involving the production of similar compounds for seed protection and the attraction of appropriate seed dispersal agents.

These confounding factors clearly have a greater impact at higher taxonomic levels. At lower taxonomic levels, the picture has partly been far more encouraging. The discipline remains bedevilled with practical problems of experimental design and practice that often fail to take account of aspects critical to taxonomic studies, even such elementary factors as adequate vouchering of material. Consequently, an appreciable amount of the body of literature, which purports to be of systematic value, has in fact no credibility. However, there remains a healthy flow of studies throwing light on relationships between taxa through the use of low molecular weight compounds.

7.4 Molecular biology and plant taxonomy

In the past two decades, the development of techniques to allow rapid sequencing of genetic material has opened up a whole new area of chemotaxonomic endeavour. There is now an opportunity to examine similarities and dissimilarities in the genetic material itself, with the generation of cladograms or phylograms expressing levels of comparability that are likely to have evolutionary significance. In addition, some of the genes for key enzymes in the biosynthesis of SM have been cloned and sequenced (Marasco and Schmidt-Dannert, 2007; Oksman-Caldentey *et al*. 2007; Sato *et al*. 2007; Verpoorte *et al*., 2007; Minami *et al*., 2008; Wu and Chappell, 2008). This allows the search for the occurrence of such genes throughout the plant kingdom and even to search for their origins in prokaryotes. These advances introduce possibilities for a re-analysis of micromolecular data, and in the rest of this chapter, these possibilities will be examined. Using DNA data the phylogeny of angiosperms has been reevaluated and presently the phylogeny shown in Fig. 7 replaces earlier attempts by Dahlgren (1980).

7.5 Comparison between patterns of secondary metabolites and molecular phylogeny

7.5.1 Use of molecular markers in plant systematics

Systematic and phylogenetic analyses are traditionally based on macroscopic and microscopic morphological characters (e.g. flower and pollen morphology, embryology or cytology), which are nowadays often evaluated phenetically or cladistically. Recent decades have seen the advent of chemical characters such as structures of secondary metabolites or of macromolecules, as additional systematic tools, as outlined in the first part of this review. Since the genome contains the basic information of the evolutionary past of all organisms, progress in molecular systematics depends on the ability to decipher the complexity of the corresponding genomes. This approach has profited tremendously from the rapid progress of molecular biology in general. Starting with chromosome analysis and serology of seed proteins, the field moved rapidly via DNA–DNA hybridization and restriction fragment length polymorphism (RFLP) analyses to the sequence analysis of marker genes.

The analysis of DNA sequences, among them chloroplast DNA or nuclear DNA (Soltis *et al.*, 1992, 1998; Doyle, 1993; Hillis *et al.*, 1996; APG-III, 2009), APG-III, 2009 has increasingly been employed to reconstruct the phylogeny both of higher and lower plants. This approach provides the best phylogenetic resolution so far and has been facilitated by

- rapid DNA amplification techniques, such as polymerase chain reaction (PCR)
- rapid DNA sequencing methods (automatic sequencing systems)
- powerful computation with software programs, such as phylogenetic analysis using parsimony (PAUP), phylogenetic interference package (PHYLIP) or molecular evolutionary genetics analysis (MEGA) or MrBayes.

Although in a strict sense, trees constructed from sequence data can only be gene trees, there is convincing evidence that gene trees very often reflect species trees (Doyle, 1992). Since phylogenetic relationships that are inferred from sequence data are not as much impaired by convergent traits as morphological characters, molecular phylogenies provide a valuable framework that allows the comparison and placement of many other experimental data in a phylogenetic or taxonomic context. The Angiosperm Phylogeny group (APG-III, 2009) has made a complete revision of Angiosperm taxonomy and proposed a new systematics (http://www.mobot.org/MOBOT/Research/APweb/), which is also used in this rest of this chapter.

Since many secondary metabolites show a restricted occurrence in apparently related groups of plants (as demonstrated in the first part of this review),

it is tempting to use the distribution of secondary metabolites as a systematic marker. The basic questions with regard to the distribution of secondary metabolites are as follows: If a group of species, genera, tribes or families shares common ancestry, should we expect that all members of such a monophyletic clade should share common apomorphic characters, such as a particular SM, and if secondary metabolites were non-adaptive traits, which is one of the basic assumptions made in using them as taxonomic markers, should we expect all members of such a clade to produce a particular metabolite?

7.5.2 Distribution of pyrrolizidine and quinolizidine alkaloids, cardiac glycosides and glucosinolates in the plant kingdom

Pyrrolizidine alkaloids (PAs), of which more than 370 structures are known, affect muscarinic and serotoninergic neuroreceptors (Schmeller *et al.*, 1997). In the liver of vertebrates, PAs are converted to toxic pyrrolic derivatives, which are alkylating compounds responsible for the long-term toxicity of PAs, and which through binding to proteins and DNA can cause mutations or even cancer (McLean, 1970; Mattocks, 1972; Roeder, 1995). PAs are produced as chemical defence compounds, mainly in the Asteraceae (tribes Eupatorieae, Senecioneae), Boraginaceae, Fabaceae (mainly genus *Crotalaria*) and Orchidaceae. Other families include the Apocynaceae, Celastraceae, Convolvulaceae, Elaeocarpaceae, Euphorbiaceae, Poaceae, Ranunculaceae, Rhizophoraceae, Santalaceae and Sapotaceae (Hartmann and Witte, 1995; Roeder, 1995) (Fig. 7.8c).

As can be seen from Fig 7.8c PA-producing families are distributed all over the plant kingdom and are apparently unrelated. This implies that PA formation in unrelated plant families could be a convergent trait and, thus, not useful as a taxonomic marker at the family level. Even within PA-producing families, PAs do not necessarily occur in all their member taxa. Although these members share common ancestry, the trait is probably either not evolved or biosynthetic processes have been turned off in these instances. Usually, other defence chemicals are then found instead of PAs.

Plant-derived PAs have also been detected in a number of specialized insects, which often advertise their unpalatability by aposematic coloration and/or pyrazines (Brown and Trigo, 1995; Hartmann and Witte, 1995; Rothschild *et al.*, 1979). Examples include: aphids, e.g. *Aphis jacobaeae*, *A. cacaliae*; beetles, e.g. *Oreina cacaliae*, *O. speciosissima* (Rowell-Rahier *et al.*, 1991); grasshoppers, e.g. *Zonocerus* (Bemays *et al.*, 1977); and many moths and butterflies, especially within the families Arctiidae and Nymphalidae (subfamilies Danainae and Ithomiinae) (Brown and Trigo, 1995; Hartmann and Witte, 1995; Rothschild *et al.*, 1979). Using sequences of the mitochondrial 16S ribosomal ribonucleic acid (rRNA), it has recently been shown that PA sequestration in insects also appears to be a convergent trait that has evolved independently in each order of insects (Fig. 7.9). Even within the Lepidoptera, PA

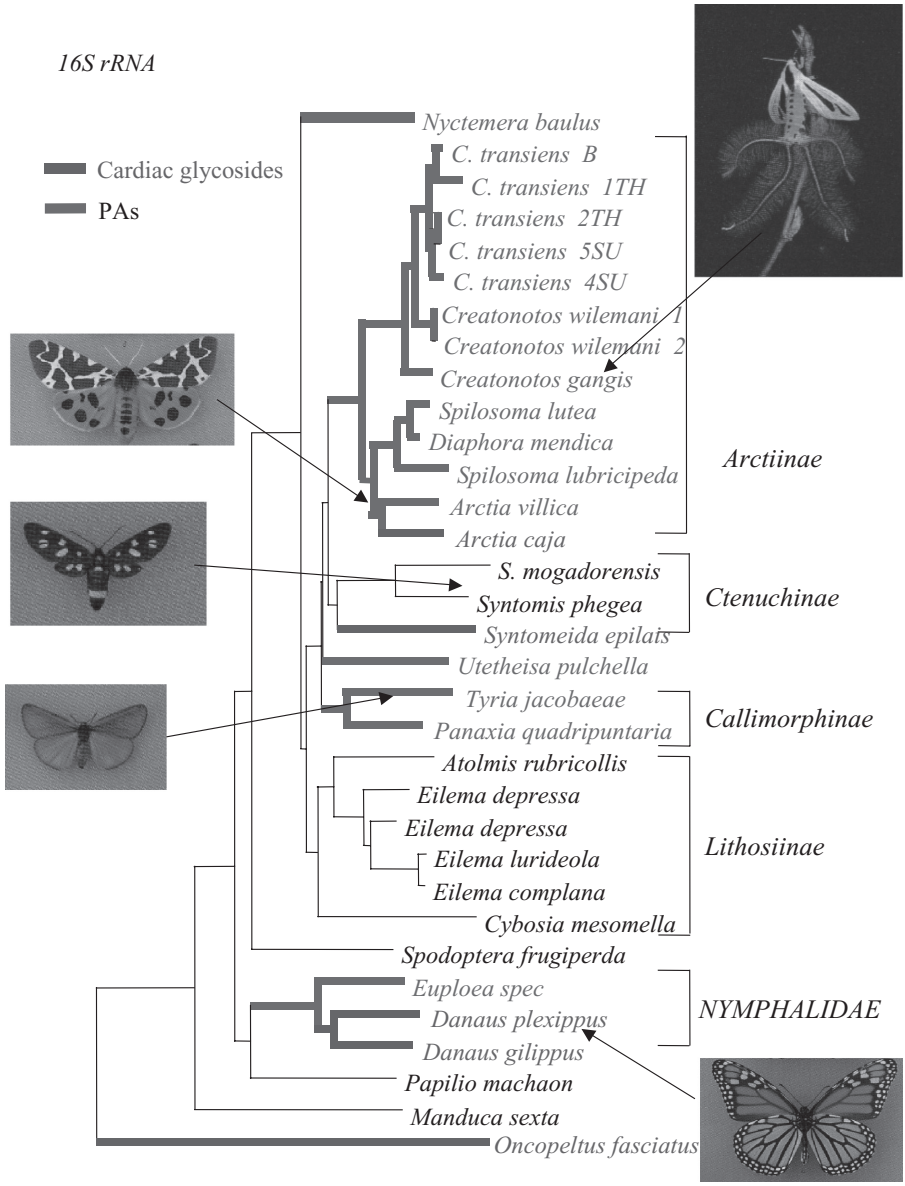


Figure 7.9 Sequestration of pyrrolizidine alkaloids and cardiac glycosides in Arctiidae, which use these SM as chemical defence against predators (after Wink and von Nickisch-Rosenegk, 1997). (See Plate 13 in colour plate section.)

sequestration evolved independently in Nymphalidae and Arctiidae (Wink and von Nickisch-Rosenegk, 1997). Thus, we find a similar theme both in plants and in herbivorous insects.

Cardiac glycosides (CGs) inhibit the Na^+, K^+-ATPase and thus destroy the ion gradients, which are necessary for many cellular functions, including neuronal activity, secondary active transport and muscle contraction. CGs therefore provide plants with potent chemical defence against herbivores. As can be seen from Fig. 7.8b, cardiac glycosides are produced in a limited number of genera in many unrelated plant families: Cardenolides in Plantaginaceae/Scrophulariaceae, Convallariaceae, Apocynaceae (including Asclepiadoideae), Ranunculaceae, Celastraceae, Brassicaceae, Hyacynthaceae, Fabaceae, Moraceae and Tiliacae; and Bufadienolides in Hyacynthaceae, Crassulaceae, Ranunculaceae, Iridaceae, Melianthaceae and Santalaceae (Fig. 7.8b). Even some animals, such as toads and beetles, can produce their own CGs. Most CG-producing plant families are unrelated, implying that cardiac glycosides are not a good phylogenetic marker at the family level, since they appear to have evolved independently on a number of occasions.

Analogous to the situation with PAs, a number of specialized insects (often aposematically coloured) are able to take up and store plant-derived CGs (Fig. 7.9). Examples include: grasshoppers, e.g. *Poekilocerus* and *Phytmaeteus* (Rothschild, 1966, 1972); aphids, e.g. *Aphis nerium* (Rothschild *et al.*, 1970b); lygaeid bugs, e.g. *Oncopeltus*, *Caenocoris*, *Spilostethus*, *Lygaeus*, *Apterola*, *Arocatus*, *Aspilocoryphus*, *Aulacopeltus*, *Graptostethus*, *Haemobaphus*, *Lygaeospilus*, *Melanerythrus*, *Microspilus* and *Horvathiolus* (Malcolm, 1990; Rothschild, 1972; Rothschild *et al.*, 1971); beetles, e.g. *Tetraopes* and *Epicauta* (Rothschild, 1972); *Diptera*, *Zenilla* (Rothschild, 1972); and again Lepidoptera, e.g. *Danaus*, *Syntomeida*, *Euchaetias*, *Arctia* and *Empyreuma* (Rothschild *et al.*, 1970a, 1973; Rothschild, 1972; Nickisch-Rosenegk von *et al.*, 1990). Insects sequestering CGs are usually protected from predators, such as birds (Rothschild, 1966; Brower *et al.*, 1975). Using 16S rRNA sequences (Fig. 7.9), it was shown that CG sequestration in Lepidoptera apparently evolved independently in Nymphalidae and Arctiidae (Wink and von Nickisch-Rosenegk, 1997), which corresponds to the situation in plants.

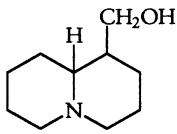
Glucosinolates are glycosides that are stored in the vacuole of plant cells. Upon wounding or infection, the cellular compartmentation breaks down, which brings together glucosinolates and corresponding glucosidases. As a result, mustard oils are released that show antimicrobial and herbivore-deterrent activities. Glucosinolates are produced by members of the Brassicaceae, Capparaceae, Resedaceae, Moringaceae, Tovariaceae, Limnanthaceae and Caricaceae. These plant families, of which some are traditionally grouped into the Brassicales (formerly Capparidales), are phylogenetically related and form a monophyletic clade (Fig. 7.8b). Interestingly, the Caricaceae, Gyrostemonaceae and Salvadoraceae, which had not been

placed in the Capparidales in classical systematics, are united with this group based both on molecular and phytochemical reasoning (Fig. 7.8b). It is therefore likely that the production of glucosinolates once evolved in an ancestor of this group and was maintained as a potent defence strategy by most of its members. This would be a good example of the usefulness of secondary molecules for taxonomy, if it were not for the fact that glucosinolates are also produced by members of the Euphorbiaceae (Teuscher and Lindequist, 1994), which are unrelated to the Capparidales. This provides a dilemma seen in most groups of secondary metabolites.

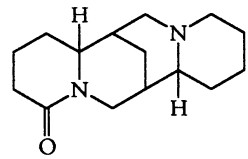
Summarizing the examples of Figs 7.8 and 7.9, it is apparent that most of these groups of compounds are of very limited value as a taxonomic marker at the higher hierarchical level. It has been argued above that the most likely explanation for the occurrence of PAs and CGs in unrelated families is convergent evolution. Since these metabolites appear to provide a strong selective advantage for the taxa producing them (as defence chemicals against microbes and/or herbivores) and since they affect important basic molecular targets in herbivores, they could have evolved randomly and been selected because of their biological activity (Wink, 1988; Wink and von Nickisch-Rosenegk, 1997; Wink *et al.*, 1998).

In the second part of this review, the phylogenetic framework provided by *rbc*L and other cpDNA sequences and nuclear ITS sequences (obtained in our Heidelberg lab) is used to discuss the distribution of a number of secondary metabolites within the plant kingdom on a lower taxonomic scale, especially within the Fabaceae, Solanaceae and Lamiaceae (Wink, 2003).

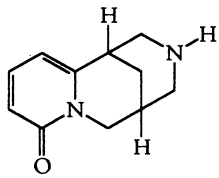
Quinolizidine alkaloids (QAs) (Fig. 7.10) are typical secondary metabolites in some phylogenetically related tribes of the Fabaceae (Fig. 7.11a), but they have also been found in other unrelated taxa, e.g. the families Chenopodiaceae, Berberidaceae, Ranunculaceae, Scrophulariaceae and Solanaceae (Teuscher and Lindequist, 1994) (Fig. 7.8c). Since traces of QAs can be detected in plants and cell cultures of even more taxa, it has been postulated (Wink and Witte, 1983) that the genes which encode the basic pathway leading to these alkaloids must have evolved early during evolution, that they are present but turned off or inactivated in most instances and that they are turned on again in plants that use the alkaloids as chemical defence substances (Wink, 1988, 1992). This hypothesis can be tested as soon as the genes that encode the biosynthesis of PAs, CGs, glucosinolates or QAs have been isolated. We suspect, however, that no single evolutionary scenario will be found for all groups of compounds but that convergent and phylogenetically conserved traits (which were inherited from an early ancestor) will co-occur (see distribution of glucosinolates in this section). As discussed in Section 7.6, some of the genes for key enzymes in the biosynthesis of SM have been identified and sequenced. This allows the search for the occurrence of such genes throughout the plant kingdom and a correlation with presence and actual SM production.



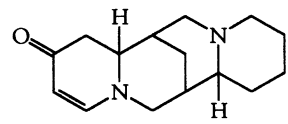
(21) Lupinine



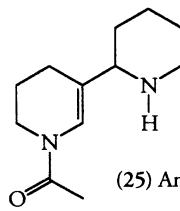
(22) Lupanine



(23) Cytisine



(24) Multiflorine



(25) Ammodendrine

Figure 7.10 Structures of the quinolizidine alkaloids, lupinine (21), lupanine (22), cytisine (23) and multiflorine (24) and of a dipiperidine alkaloid of the ammodendrine type (25).

7.5.3 Phytochemical traits of the Fabaceae

It might be argued that secondary metabolites are better and more reliable markers within families, tribes or genera than at the higher order level. The Fabaceae have been selected as an example to examine, since this very large plant family with 720 genera and more than 19,500 species has been extensively studied phytochemically. Several types of alkaloids, NPAAs, amines, flavonoids, isoflavones, coumarins, anthraquinones, di-, sesqui- and triterpenes, cyanogenic glycosides, protease inhibitors and lectins have been described in this family. Most of these compounds are thought to function as defence chemicals or as signal compounds (see reviews and compilations in Harborne *et al.*, 1971; Polhill *et al.*, 1981b; Kinghorn and Balandrin, 1984; Stirton, 1987; Wink, 1993c; Hegnauer and Hegnauer, 1994; Southon, 1994; Sprent and McKey, 1994; Wink *et al.*, 1995). Furthermore, *rbc*L sequences have been obtained for over 300 legumes (Käss and Wink, 1995, 1996, 1997a,b; Wink and Mohamed, 2003), so providing a DNA-based phylogenetic framework to analyse the distribution of secondary metabolites within the family.

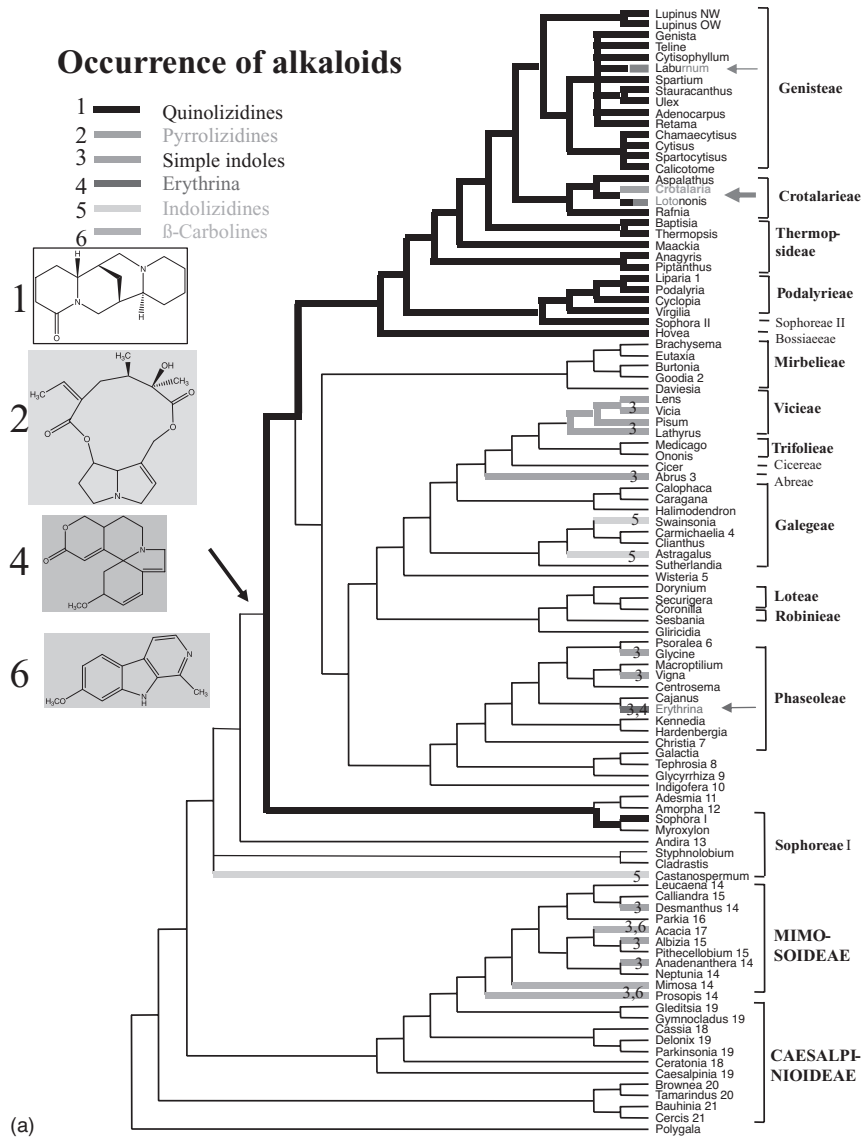
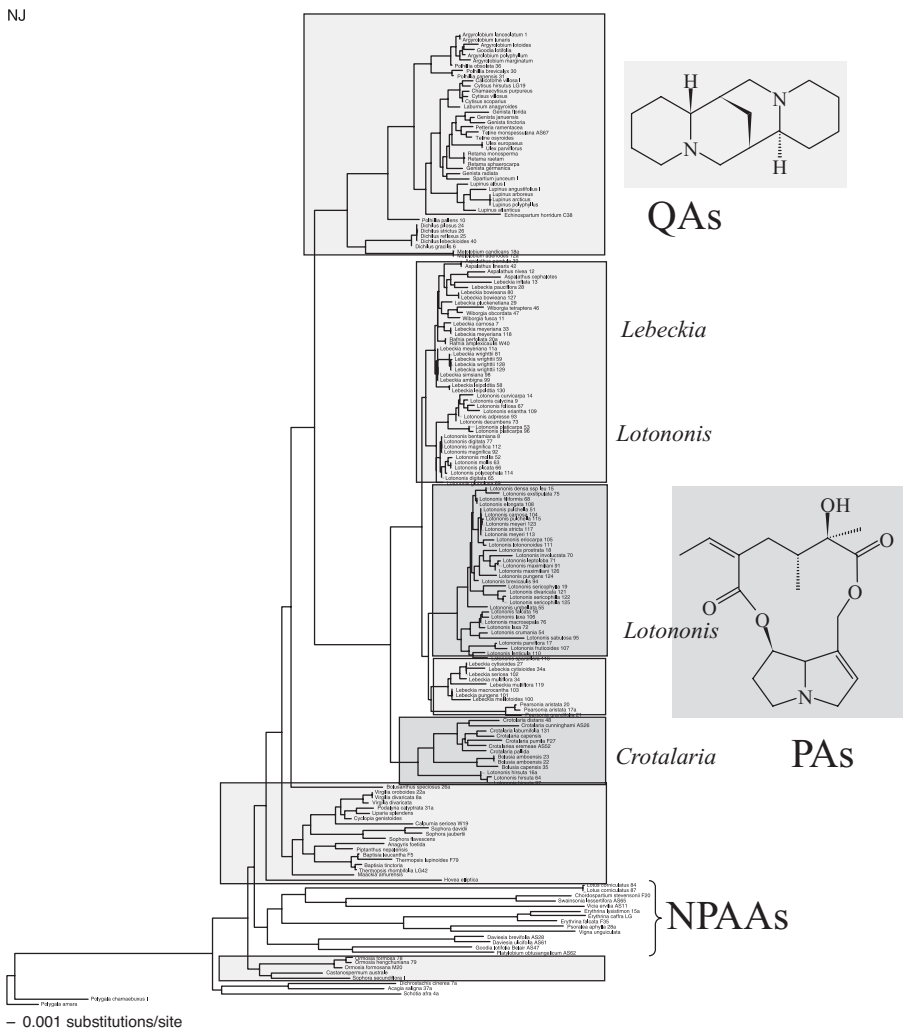


Figure 7.11 (a) Genera and tribes of the Fabaceae, placed in a phylogenetic framework reconstructed from nucleotide sequences of the *rbc*L gene. Illustrations (a)–(g) are presented as cladograms of a strict consensus of the six most parsimonious trees calculated by a heuristic search. Due to space limitations, a few tribal names are not listed in the figures, but are abbreviated by numbers after the genus name: 1 = Liparieae; 2 = Bossiaeeae; 3 = Abreae; 4 = Carmichaelieae; 5 = Millettieae; 6 = Psoraleae; 7 = Desmodieae; 8 = Tephrosieae (Millettieae); 9 = Galegeae; 10 = Indigofereae; 11 = Adesmieae; 12 = Amorpheae; 13 = Dalbergieae; 14 = Mimoseae; 15 = Ingeae; 16 = Parkieae; 17 = Acacieae; 18 = Cassieae; 19 = Caesalpinieae; 20 = Detarieae; 21 = Cercideae. (a) The occurrence of alkaloids. Key to branches leading to families that accumulate: quinolizidines, pyrrolizidines (No. 1; see arrows); *Erythrina* (No. 3); indolizidines (No. 4); β-carbolines (No. 5); or simple indoles (No. 2) are marked. The *rbc*L sequences used (1400 bp) derived from Käss and Wink, 1997a,b; Wink and Mohamed (2003). Trees were reconstructed with maximum parsimony.



(b)

Figure 7.11 (*Continued*) (b) Occurrence of QAs and PAs in the Papilionoideae, tribe Crotalarieae (reconstructed from ITS sequences).

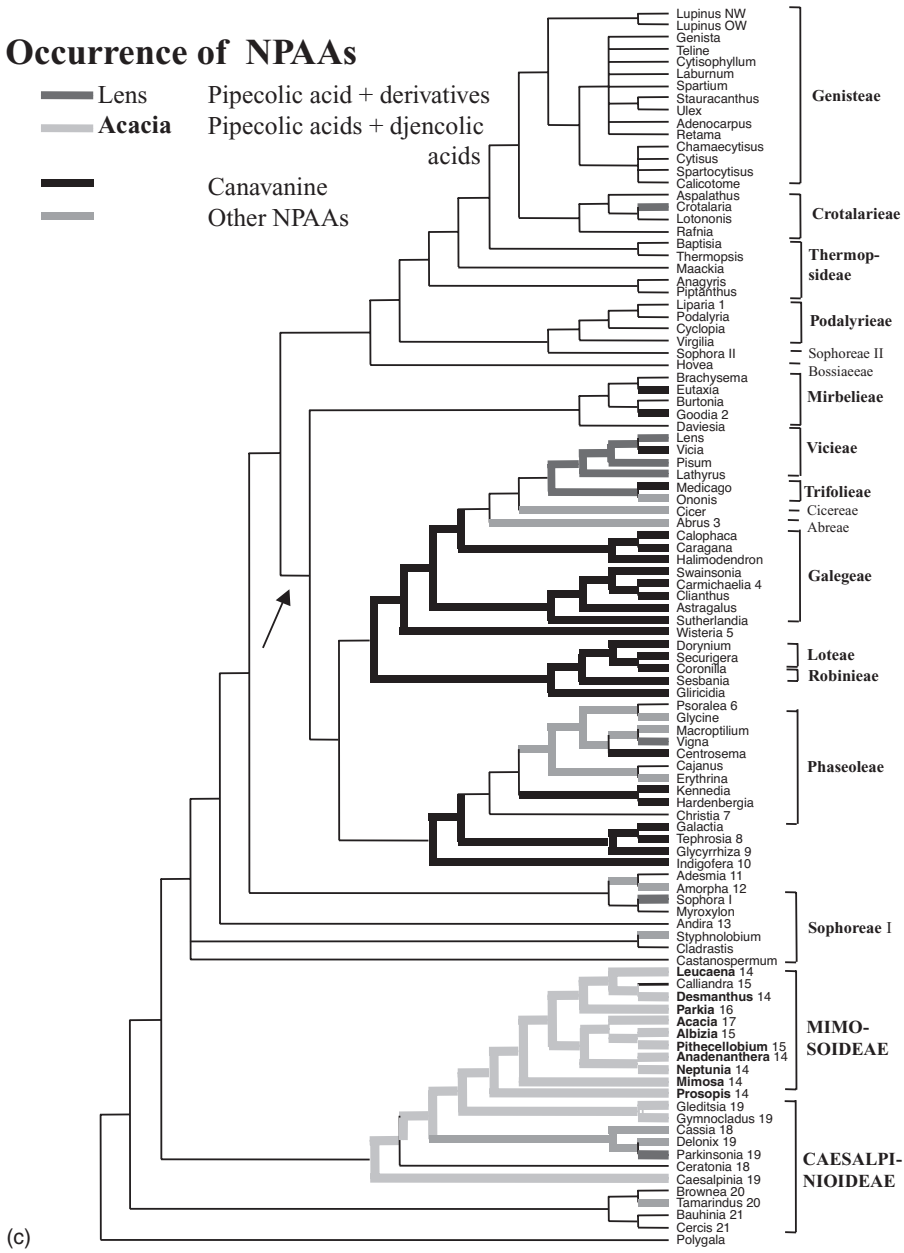
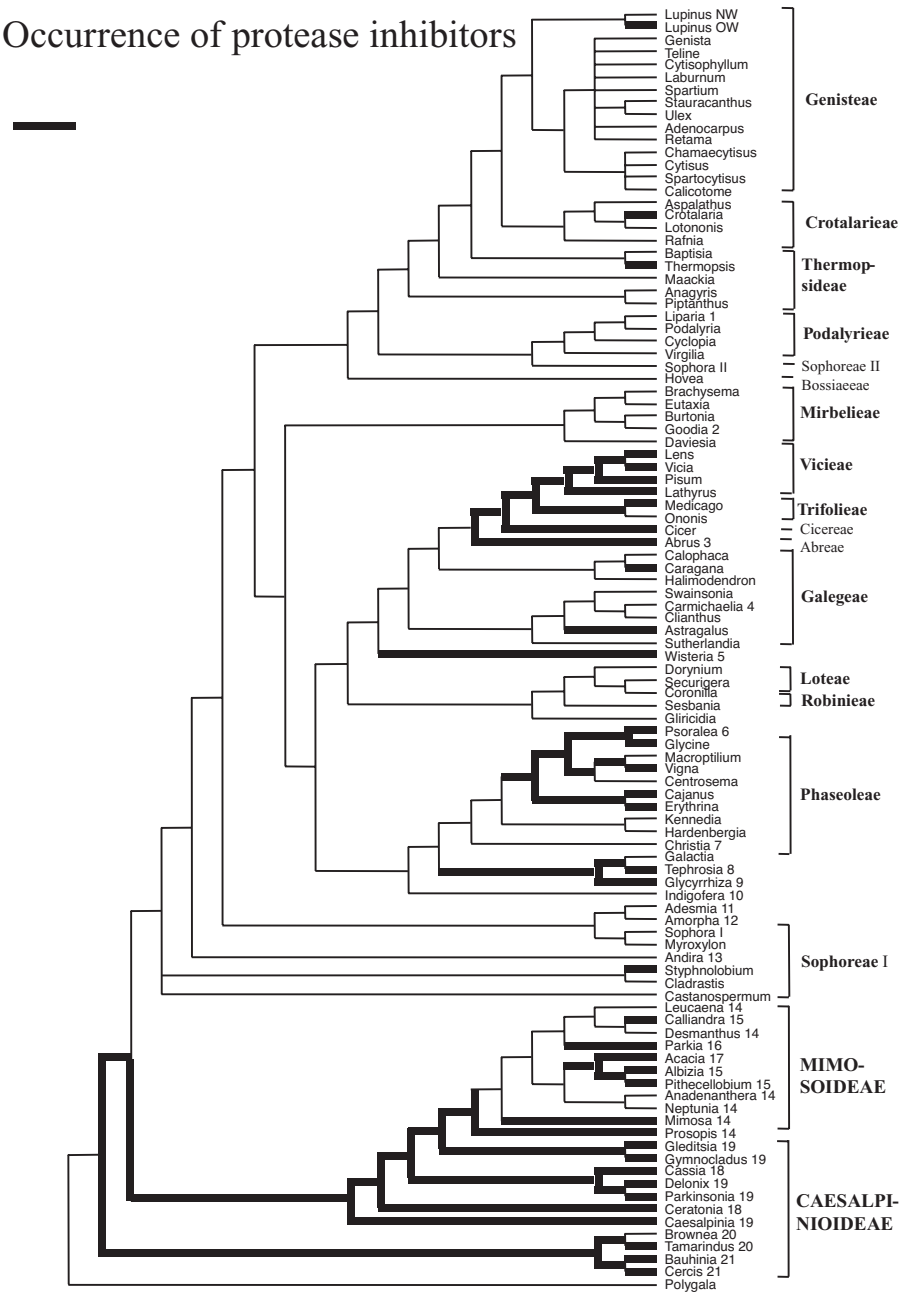


Figure 7.11 (*Continued*) (c) Occurrence of non-protein amino acids (NPAAs). Key to branches leading to families that accumulate: pipecolic acid and derivatives (*Lens*); pipecolic acid and djenkolic acids (*Acacia*); canavanine; others NPAAs. See also legend (a).



(d)

Figure 7.11 (*Continued*) (d) Occurrence of protease inhibitors. Key to branches leading to families that accumulate protease inhibitors. See also legend (a).

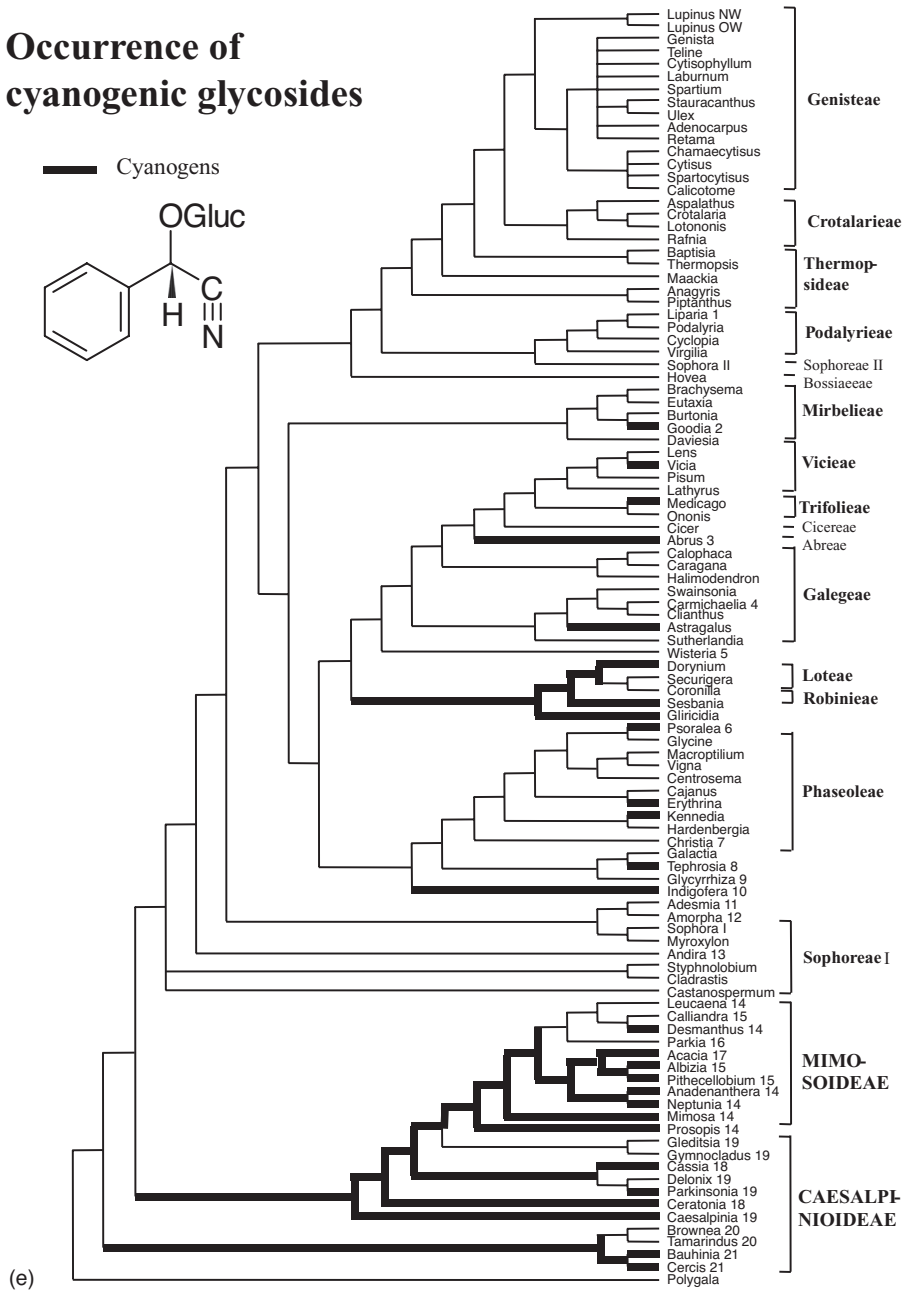
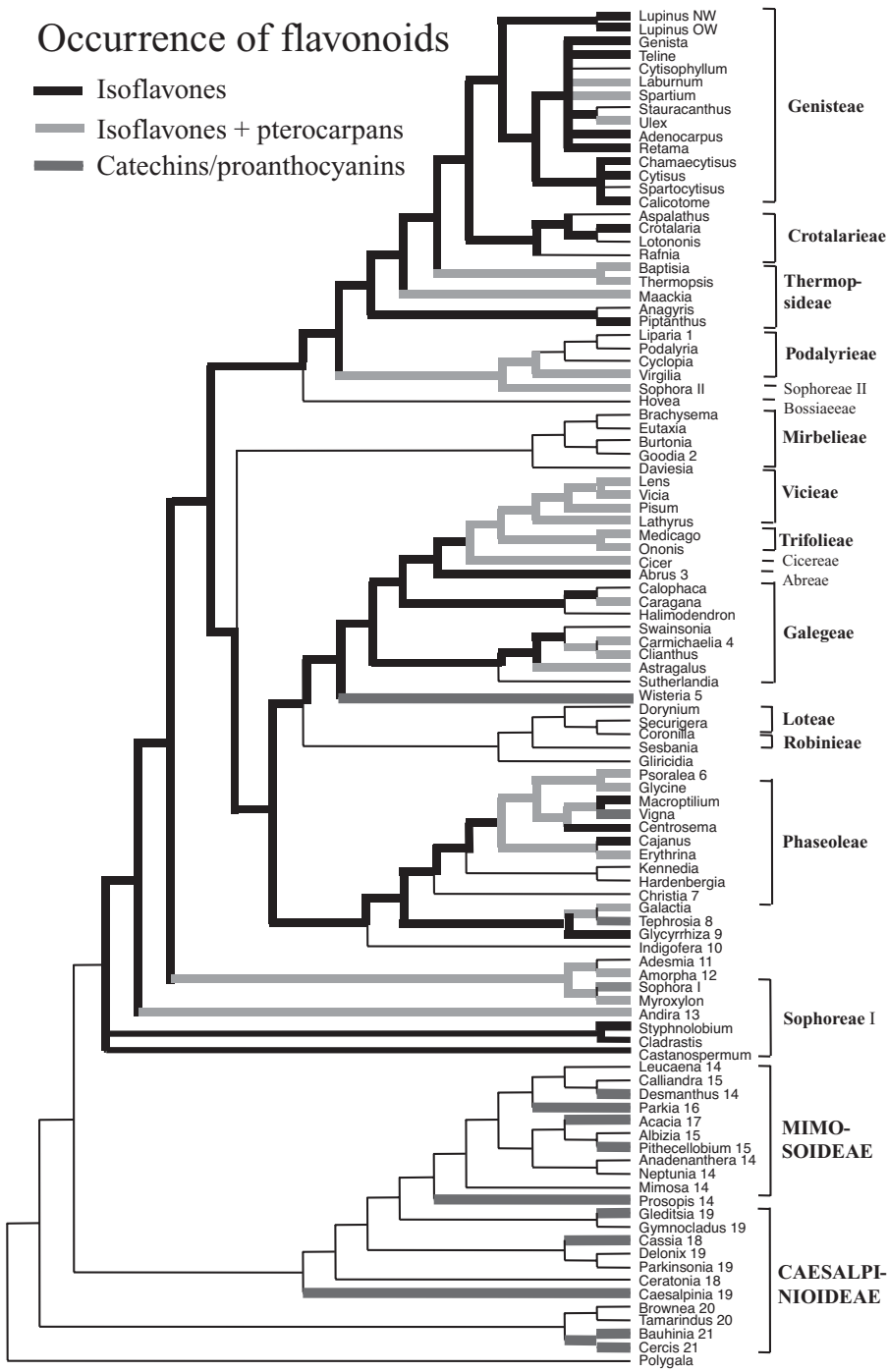
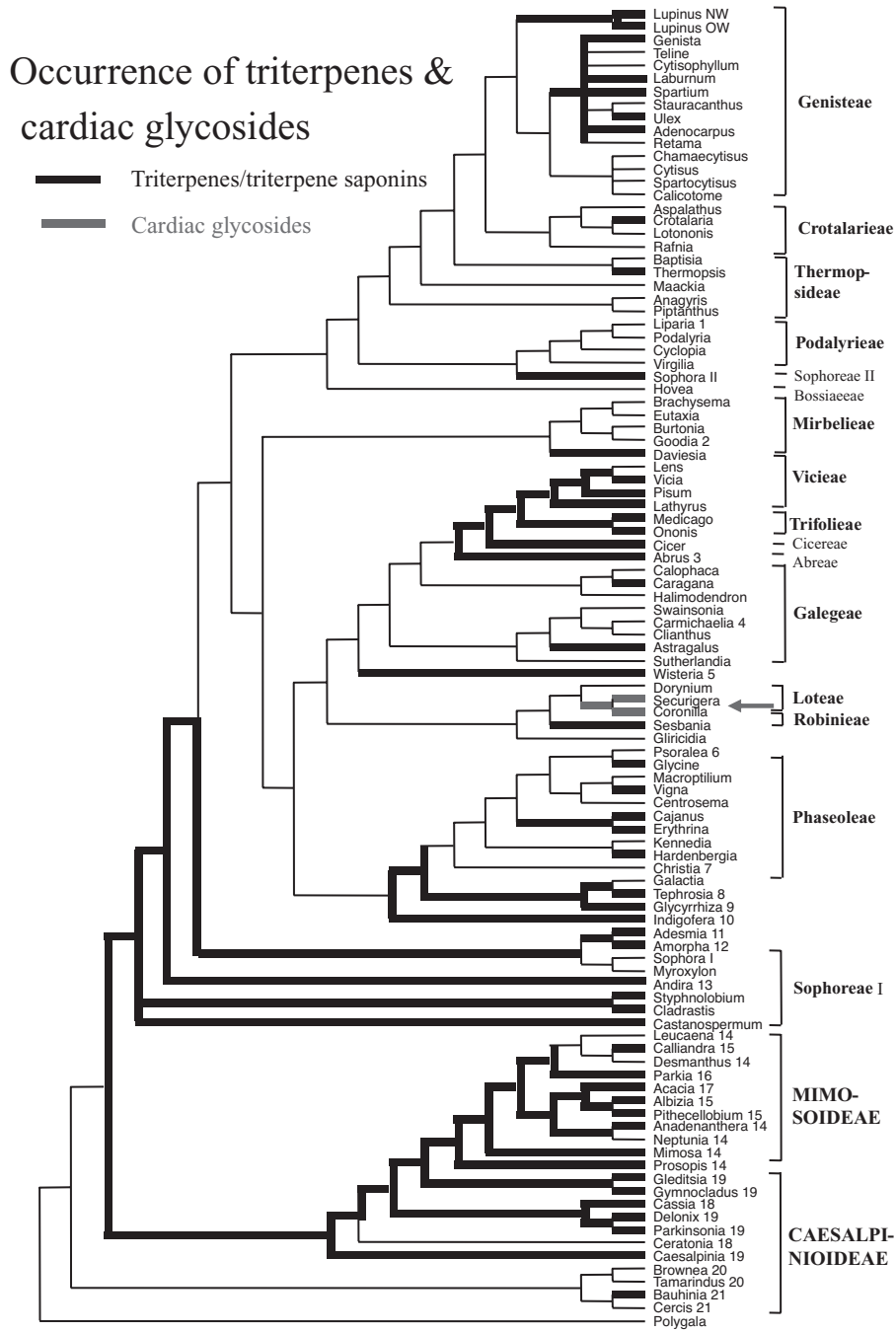


Figure 7.11 (*Continued*) (e) Occurrence of cyanogenic glycosides. Key to branches leading to families that accumulate cyanogens. See also legend (a).



(f)

Figure 7.11 (*Continued*) (f) Occurrence of flavonoids. Key to branches leading to families that accumulate: isoflavones; isoflavones and pterocarpans; catechins/proanthocyanins. See also legend (a).



(g)

Figure 7.11 (*Continued*) (g) Occurrence of triterpenes and cardiac glycosides. Key to branches leading to families that accumulate: triterpenes/triterpene saponins; cardiac glycosides. See also legend to (a). (See Plates 14–18 in colour plate section.)

About 100 genera that cover most tribes of the Fabaceae have been selected. In most cases, *rbc*L sequences of these genera cluster in a way which is consistent within their traditional grouping in tribes and subfamilies (Polhill, 1994). Members of the Caesalpinioideae cluster at the base of the legume tree, which is in agreement with the fossil record (Herendeen and Dilcher, 1992). Members of the Mimosoideae derive unambiguously from the Caesalpinioideae (Wink *et al.*, 1993; Doyle, 1994; Käss and Wink, 1995, 1996, 1997a) and are not ancestral, as had sometimes been assumed. Also, the groupings within the Papilionoideae, which form a monophyletic clade, are mostly congruent with traditional systematics (Polhill *et al.*, 1981a; Polhill, 1994), starting with Sophoreae at the base and leading to Genisteae as the more advanced tribes.

7.5.4 Nitrogen-containing secondary metabolites

QAs (Fig. 7.10) are the most prominent group of alkaloids in legumes, being present in members of the subfamily Papilionoideae in the tribes Genisteae, Crotalarieae, Podalyrieae, Thermopsideae, Liparieae, Euchresteae, Bossiaeeae and Sophoreae (Kinghorn and Balandrin, 1984; Wink, 1993a). Dipiperidine alkaloids (DPAs) of the ammodendrine type (Fig. 7.10), which also derive from lysine as a precursor, exhibit a comparable distribution pattern. As can be seen from Fig. 7.11a, the QA-producing tribes, with the exception of the Sophoreae, are apparently monophyletic and nearly all taxa in this assemblage accumulate QAs. Obvious exceptions are members of the large genus *Crotalaria*, which sequester either PAs (Figs. 7.11a and 7.11b) or NPAAs (Fig. 7.11c). In *Lotononis*, a genus closely allied to *Crotalaria*, some taxa produce QAs and others PAs (Fig. 7.11b). *Crotalaria* and *Lotononis* derive from ancestors that definitely produced QAs but not PAs; therefore, the general ability to make QAs must have been present, but the corresponding genes are either lost or completely turned off in *Crotalaria* and partially turned off in *Lotononis*. The formation of PAs rather than QAs appears to be a new acquisition for chemical defence, which could have evolved convergently (compare Fig. 7.8c). Because the PA structures in Crotalarieae (a member of the rosids) are similar or even identical as PAs in Asteraceae (a member of the asterids) (Fig. 7.8c), a common ancestral biosynthetic PA pathway in the plants also needs to be taken into consideration.

In a few taxa that cluster within QA-accumulating genera, QAs are hardly detectable or levels are very low, such as in *Ulex*, *Calicotome* or *Spartocytisus*. These taxa have in common extensive spines that have apparently supplanted chemical defence. In such cases, the presence or absence of QAs is clearly a trait reflecting different ecological strategies rather than taxonomic relationships.

The Sophoreae, and in particular the genus *Sophora*, appear to be polyphyletic and need thorough revision (Polhill *et al.*, 1981b; Stirton, 1987; Käss and Wink, 1995, 1996, 1997a). Part of the QA-producing genera *Sophora* (here

Sophora II) and *Maackia* always cluster outside the Sophoreae, as part of the Podalyrieae and Liparieae or Thermopsideae (Fig. 7.11a). More ancestral Sophoreae include *Sophora secundiflora* (*Sophora* I) and related taxa, which cluster as a sister taxon to *Myroxylon*, while *Sophora japonica* is related to *Cladrastis* and *Castanospermum*. *S. japonica* has recently been removed from the genus *Sophora* into the genus *Styphnolobium*, thus recognizing this obvious discrepancy. *Sophora* I accumulate QAs as secondary constituents, respectively, indicating that the genetic capacity to make QAs must be present in the very early members of the Papilionoideae. An alternative explanation of a parallel evolution of QAs in early and later Papilionoideae appears less likely. As with *Crotalaria*, which no longer accumulates QAs, we can assume that all the other tribes of the Papilionoideae that diverge at the branch that is indicated by an arrow (Fig. 7.11a) had, at that point, the capacity to synthesise QAs. This ability has subsequently been lost or the corresponding genes have simply been turned off. As shown shortly, these QA-deficient tribes accumulate other defence compounds instead.

In addition to QAs, legumes accumulate a wide range of other alkaloids, deriving from different precursors. Most of them have distributions that are restricted to a few, often non-related taxa. For example, *Erythrina* alkaloids, which derive from tyrosine as a precursor, are typical of members of the large genus *Erythrina* and have not been found elsewhere in the plant kingdom. Indolizidine alkaloids, which inhibit hydrolytic enzymes, have been reported in *Swainsonia*, *Astragalus* (tribe Galegeae) and *Castanospermum* (Sophoreae). β-Carboline alkaloids have been detected in a few mimosoid taxa of the tribes Mimoseae and Acacieae. A number of simple phenylethylamine or simple indole alkaloids have been found, usually in taxa that do not accumulate QAs (Fig. 7.11a). Interestingly, the occurrence of quinolizidines and other alkaloids is usually mutually exclusive, indicating the parsimonious utilization of chemical defence strategies.

The distribution of protease inhibitors (PIs) (Fig. 7.11d) (i.e. trypsin and chymotrypsin inhibitors) exhibits an almost complementary pattern to QAs. Most Caesalpinoideae and many Mimosoideae accumulate PIs in their seeds, where they serve concomitantly as chemical defence and nitrogen storage compounds. It is unclear whether some genera of the Mimosoideae have secondarily lost this trait or whether they have not been studied in sufficient detail. Within the Papilionoideae, PIs are prominent in the tribes Vicieae, Trifolieae, Cicereae, Abreae, Galegeae, Loteae, Phaseoleae and Tephrosieae, but have not been detected in the Mirbelieae. According to Fig. 7.11d, PI formation in Caesalpinioideae/Mimosoideae and Papilionoideae could be based on common ancestry. This would mean, however, that the trait has been turned off in a number of papilionoid tribes, which produce QAs and other secondary metabolites instead. Alternatively, PI formation could have evolved independently in these legume subfamilies. Since the genes for PIs are known, it would be challenging to analyse whether PI genes are present or absent in non-PI-producing taxa.

Another prominent group of SM in Fabaceae are NPAAs. When all NPAAs with different structures and activities are grouped together (Fig. 7.11c), the pattern of NPAA accumulation is again almost complementary to the distribution of QAs (Fig. 7.11a). Like PIs and QAs, NPAAs are thought to serve at least two purposes: as chemical defence compounds and as mobile nitrogen storage compounds of seeds, which are used as a nitrogen source for the seedling. Considering different structural types of NPAAs, however, a more differentiated picture becomes apparent. At least three groups of NPAAs are common in legumes, mainly canavanine, pipecolic acid and derivatives, and the sulfur-containing djencolic acids. Canavanine is common in the tribes Galegeae, Loteae, Tephrosieae, Robinieae and in some Phaseoleae, and it might be assumed that the trait of canavanine accumulation was acquired by an ancestor (see arrow in Fig. 7.11c), from which all the other tribes derived. If this were so, then the canavanine genes are turned off in the Vicieae, Trifolieae, Cicereae and Abreae, which produce pipecolic acids instead. Whether pipecolic acid biosynthesis was independently invented in Caesalpinioideae/Mimosoideae and in the papilionoid tribes, Vicieae and Trifolieae, or whether the canavanine genes were only inactivated in Vicieae and Trifolieae is open to debate, analogous to the situation of PIs (Fig. 7.11d). As strict taxonomic markers, both canavanine and pipecolic acid derivatives are of limited value, since they would place the wrong groups together in several instances. By contrast, djencolic acids appear more appropriate as a taxonomic marker, since taxa that accumulate them all belong to the Mimosoideae. Several other NPAAs have been described from legumes (Harborne *et al.*, 1971; Polhill *et al.*, 1981b; Stirton, 1987; Hegnauer and Hegnauer, 1994; Southon, 1994; Sprent and McKey, 1994), most of which have a more restricted occurrence; i.e. presence or absence in phylogenetically related taxa is a common theme.

Cyanogenic glycosides appear to be more common in the ancestral legume tribes (Fig. 7.11e). Whether the occurrence of cyanogenic glycosides is based on common genes that are turned off in most instances and turned on in a few cannot yet be answered; both convergent and independent evolution are plausible scenarios.

In summary, the numerous nitrogen-containing metabolites seem to function both as chemical defence and nitrogen storage compounds in legumes, and are thus open to natural selection. Although they appear as plausible taxonomic markers in a few parts of the legume tree, they fail to do so in others. Their occurrence appears to reflect different evolutionary and life strategies, rather than taxonomic stringency.

7.5.5 Nitrogen-free secondary metabolites

Are non-nitrogenous secondary metabolites better taxonomic markers? Whereas flavonoids are found in all three subfamilies, and are thus of limited value at the family/tribal level, isoflavones are obviously restricted to the

subfamily Papilionoideae (Fig. 7.11f). With the exception of a few tribes and genera, among which are several Australian taxa, all Papilionoideae accumulate isoflavones and derivatives, including phytoalexines of the pterocarpan type (Fig. 7.11f). It remains an open question as to whether the Australian taxa have not been studied appropriately to identify these compounds or whether they are absent due to the fact that a loss of biosynthetic capacity occurred in ancestors when colonizing Australia. Catechins and proanthocyanins or galloylcatechins occur in all three subfamilies; their occurrence reflects life style, i.e. growth as trees, rather than taxonomic relatedness. In the Caesalpinioideae and Mimosoideae, both traits are almost congruent, since woody life style dominates in both subfamilies.

Coumarins and furanocoumarins, which serve as potent defence compounds in the Apiaceae and Rutaceae, occur in a few, mostly unrelated, species. Only in the genus *Psoralea* do they have a wide distribution. Anthraquinones, which are potent Na^+, K^+-ATPase inhibitors and strong purgatives, occur widely in the genus *Cassia (Senna)*, but otherwise only occasionally in *Andira* and *Abrus*.

All classes of terpenoids have been found in legumes. The known distribution of triterpenes and triterpene saponins and steroidal saponins (including cardiac glycosides in *Securigera* and *Coronilla*, both Loteae) is illustrated in Fig. 7.11g. Triterpenes and saponins, which are again considered to be powerful defence compounds against microbes and herbivores, are more common in the ancestral Caesalpinioideae/Mimosoideae and in the basal tribes of the Papilionoideae, but are also important in the Vicieae, Trifolieae, Cicereae and Phaseoleae. Whether they have arisen independently in different taxa, which seems probable as there is no clear nodal link, or whether the genes evolved at the beginning of legume evolution but have switched on or switched off according to ecological needs, cannot be answered with certainty. The wide distribution of triterpenes and triterpene saponins in the plant kingdom and their common basic structures favour a wide presence of corresponding biosynthesis genes.

As seen in Figs. 7.11a and 7.11g, a particular group of secondary metabolites is often confined to a systematically unrelated group of species or genera. Are, therefore, patterns of secondary metabolites better markers at the genus level? To assess this possibility, the occurrence of QAs in the genus *Lupinus* has been analysed.

7.5.6 Quinolizidine alkaloids in the genus *Lupinus*

For a finer phylogenetic resolution, the nuclear ITS1 and ITS2 regions have been chosen to reconstruct the phylogeny of Genisteae and some other papilionid tribes (Käss and Wink, 1997a,b). Overall, tree topology is congruent between ITS and *rbc*L trees, indicating that reticulate evolution is not a major problem in this part of the Fabaceae (Käss and Wink, 1997a).

The genus *Lupinus* comprises several hundred more or less well-defined species, 12 in the Old World and the others in the New World of North, Central and South America. Sequence data indicate that New World lupins apparently derived from Old World species. Long distance dispersal from Old World origin seems to have led to the colonization of the Atlantic part of South America (clade with *L. aureonitens*, *L. albescens* and *L. paraguarensis*) and of North America (see Fig. 7.12a) (Käss and Wink, 1997b).

The biosynthesis of QAs proceeds from lysine via cadaverine to tetracyclic alkaloids, such as lupanine or sparteine. Lupanine/sparteine is a precursor for tricyclic alkaloids, such as angustifoline, or the alpha-pyridone alkaloids, such as anagyrine or cytisine. Most species of lupins exhibit typical profiles of QA that could, potentially, work as taxonomic markers. In Figs 7.12b and 7.14, the branches leading to taxa, which accumulate a certain structural type of QA, have been highlighted. All taxa show alkaloids of the sparteine/lupanine type, at least as minor alkaloids; their occurrence would be congruent to the picture shown in Fig. 7.12a. Alpha-pyridone alkaloids are apparently present in the more ancestral tribes of the Papilionoideae, but they also occur in the more advanced *Cytisus/Genista* complex of the Genisteae. This suggests that the ancestors of lupins, which represent a sister clade to the modern Genisteae, must have possessed the biosynthetic capacity to produce these alkaloids. However, anagyrine and related alkaloids have been found in comparably few lupins of North America, and they are definitely absent from Old World lupins (Fig. 7.12a). The occurrence of alpha-pyridones in North American taxa is sporadic and apparently not helpful as a taxonomic marker. The alkaloid 5,6-dehydrolupanine is an intermediate between lupanine and alpha-pyridone alkaloids. Surprisingly, many more lupins have been detected that accumulate this alkaloid, at least as a minor component. As can be seen from Fig. 7.12a, two Old World and most North and Central American taxa show this trait. This suggests that the biosynthetic pathway leading to alpha-pyridone alkaloids is present at the genomic level, but is not expressed in most lupins. Since alpha-pyridone alkaloids, such as cytisine and *N*-methylcytisine, are strong agonists at nicotinic acetylcholine receptors (Schmeller *et al.*, 1994) or even induce mutations (anagyrine), it is surprising that more lupins fail to express these defence compounds rather than the tetracyclic alkaloids and their esters.

Bicyclic QAs, such as lupinine and derivatives, already occur in the more ancestral tribes such as Thermopsideae and Podalyrieae. They are rarely found in members of the *Cytisus/Genista* complex, but are typical for lupins of the subgroup, Scabrispermae (*L. atlanticus*, *L. digitatus*, *L. princei*, *L. pilosus*, *L. cosentinii*), and of the closely related *L. luteus/L. hispanicus* pair. In North American lupins, bicyclic QAs occur only sporadically as minor components.

Alkaloids of the multiflorine type (multiflorine, albine) have been recorded only from lupins. They are major constituents of Old World species of the subgroup, Scabrispermae, and of *L. albus/L. micranthus* (Fig. 7.12b). They

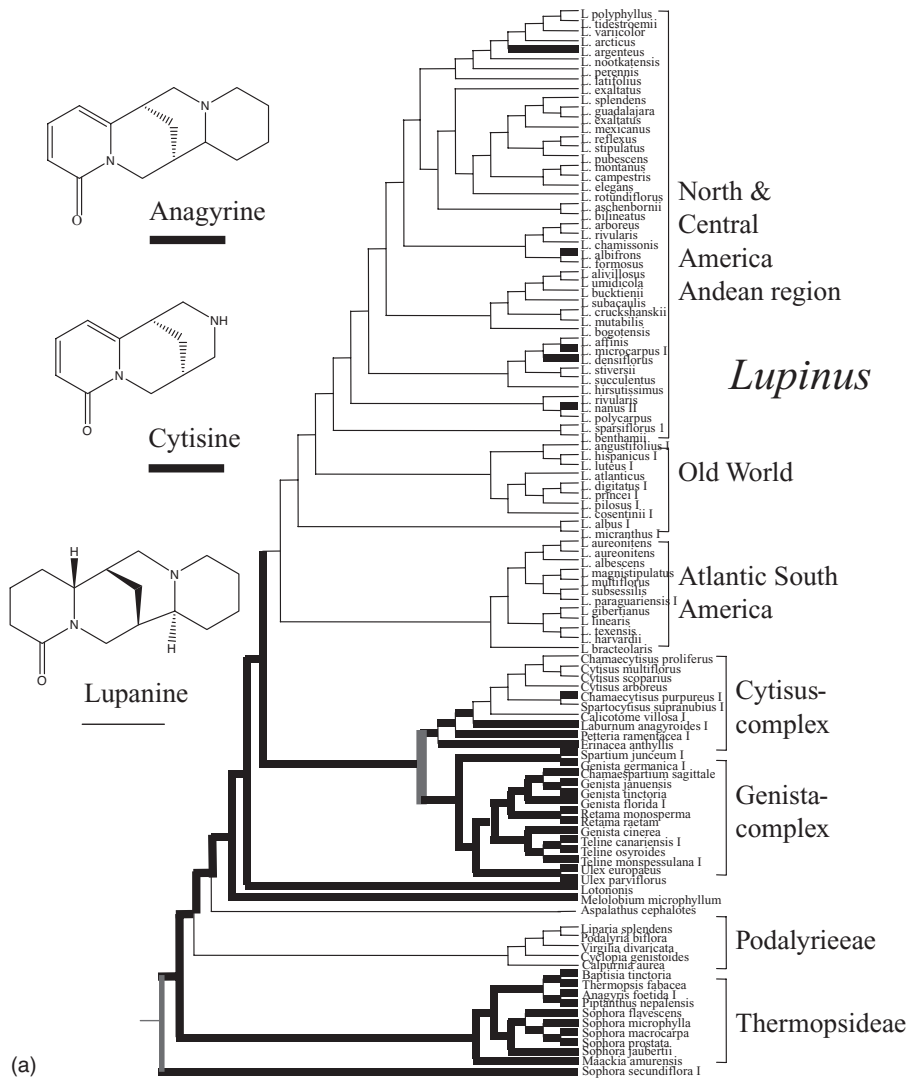


Figure 7.12 (a) Occurrence of quinolizidine alkaloids in the genus *Lupinus*, and some other papilionoid tribes. Genera and species of the Papilionoideae were placed in a phylogenetic framework reconstructed from nucleotide sequences of the ITS1 and ITS2 regions. The illustrations are presented as cladograms of a strict consensus of the 20 most parsimonious trees calculated by a heuristic search. This panel shows the distribution of lupanine and alpha-pyridone alkaloids (such as anagyrine and cytisine).

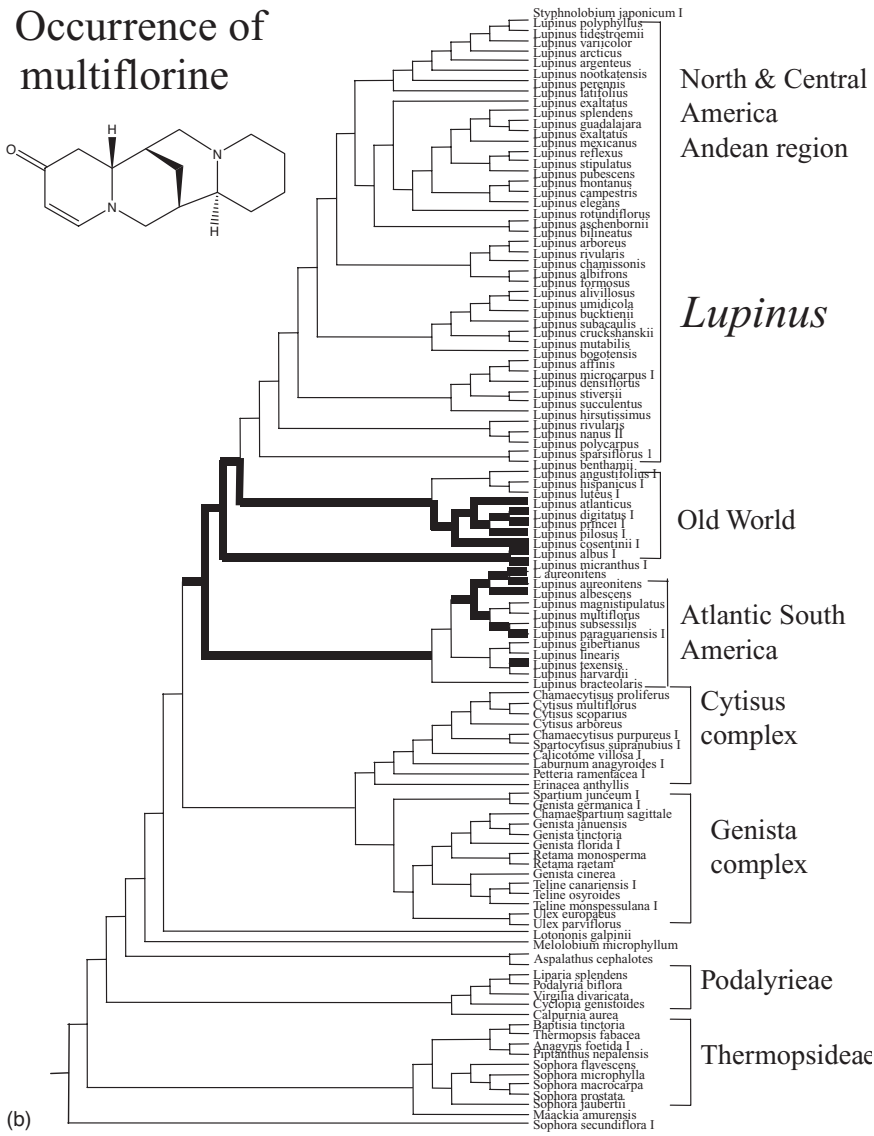


Figure 7.12 (*Continued*) (b) Distribution of tetracyclic multiflorine type alkaloids. Key to branches leading to species that accumulate: major constituent; minor constituent. See also legend to Figure 7.14.

also occur as major alkaloids in South American lupins with an Atlantic distribution, which cluster as a sister taxon to the Old World Scabrispermae. Multiflorine has been sporadically recorded as a minor component in North American lupins. DPAs, such as ammodendrine and derivatives, derive from lysine, as do QAs. The distribution of DPAs resembles that of QAs, and

ammodendrine is a minor component of most QA plants. In a few lupins, such as in *Lupinus sulphureus*, DPAs figure as major constituents.

In conclusion, when analysing the alkaloid profiles within a genus, we observe the same phenomenon as found for other secondary metabolites at the tribal or family level. In some instances, all members of a monophyletic clade share a chemical characteristic (this would favour their use as a taxonomic marker); in other instances they do not. Since a good marker should work in all instances, the main question is: which were the selective forces to activate the corresponding genes in one taxon and to turn them off in another? Since secondary metabolites play a vital role as defence or signal compounds, their occurrence apparently reflects adaptations and particular life strategies rather then taxonomic relationships. Studying the distribution of secondary metabolites in plants, thus, offers information on the underlying evolutionary, ecological and systematic processes and strategies, but their value as taxonomic markers is constrained by the reticulate nature of their metabolic expression.

7.5.7 Distribution of tropane and steroidal alkaloids in the Solanaceae

Tropane alkaloids, such as hyoscyamine, are common in Solanaceae, especially in the genera *Anthocercis, Atropa, Datura, Duboisia, Hyoscyamus, Latua, Mandragora, Physalis, Physoclaina, Salpichroa, Scopolia* and *Schizanthus*. Cocaine, which is formally a tropane alkaloid, and the related alkaloid occur in *Erythroxylum* (Erythroxylaceae) (Woolley, 1993). Alkaloids with tropane structures have also been detected in unrelated families, such as Convolvulaceae (*Convolvulus*), Brassicaceae, Dioscoreaceae, Elaeocarpaceae, Euphorbiaceae, Orchidaceae, Proteaceae (*Bellendena, Darlingia, Knightia*) and Rhizophoraceae (*Crossostylis ebertii, Bruguiera sexangula*) (Gemeinholzer and Wink, 2001; Wink and van Wyk, 2008). Within the Solanaceae, tropane alkaloids occur in more than ten clades, which are apparently not closely related (Fig. 7.13). On the other hand, taxa with steroidal glycoalkaloids, such as solanine, share common ancestry. There is no overlap between tropane and GA-producing taxa, these alkaloids are mutually exclusive.

7.5.8 Distribution of iridoid glycosides in the Lamiaceae

Iridoid glycosides (secoiridoids, secologanin derivates) with more than 200 structures are widely distributed in partly related orders (Fig. 7.10), such as Lamiales (Bignoniaceae, Buddlejaceae, Globulariaceae, Lamiaceae, Pedaliaceae, Plantaginaceae, Scrophulariaceae, Verbenaceae), Gentianales (Apocynaceae, Gentianaceae, Loganiaceae, Menyanthaceae, Rubiaceae), Dipsacales (Valerianaceae), Cornales (Cornaceae) and Ericales (Monotropaceae). Iridoid

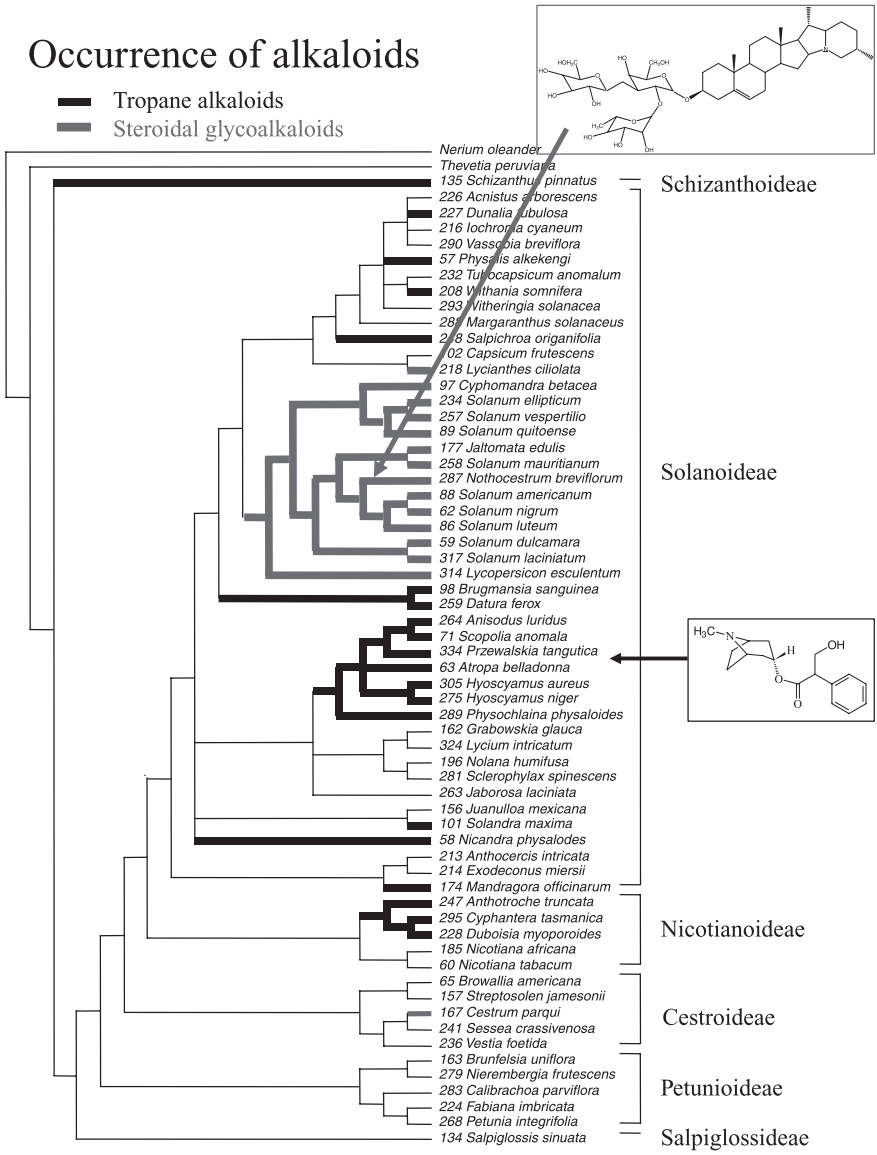


Figure 7.13 Distribution of tropane and steroidal glycoalkaloids in the family Solanaceae. (After Wink, 2003.) (See Plate 19 in colour plate section.)

glucosides, such as aucubin and harpagoside, are cleaved by β-glucosidase into an unstable aglycon. As can be seen from Fig. 7.14, iridoid glucosides are typical for members of the subfamily Lamioideae, but are widely absent in the Nepetoideae, although the Nepetoideae represent a derived clade (Wink, 2003).

Occurrence of iridoid glycosides in Lamiaceae

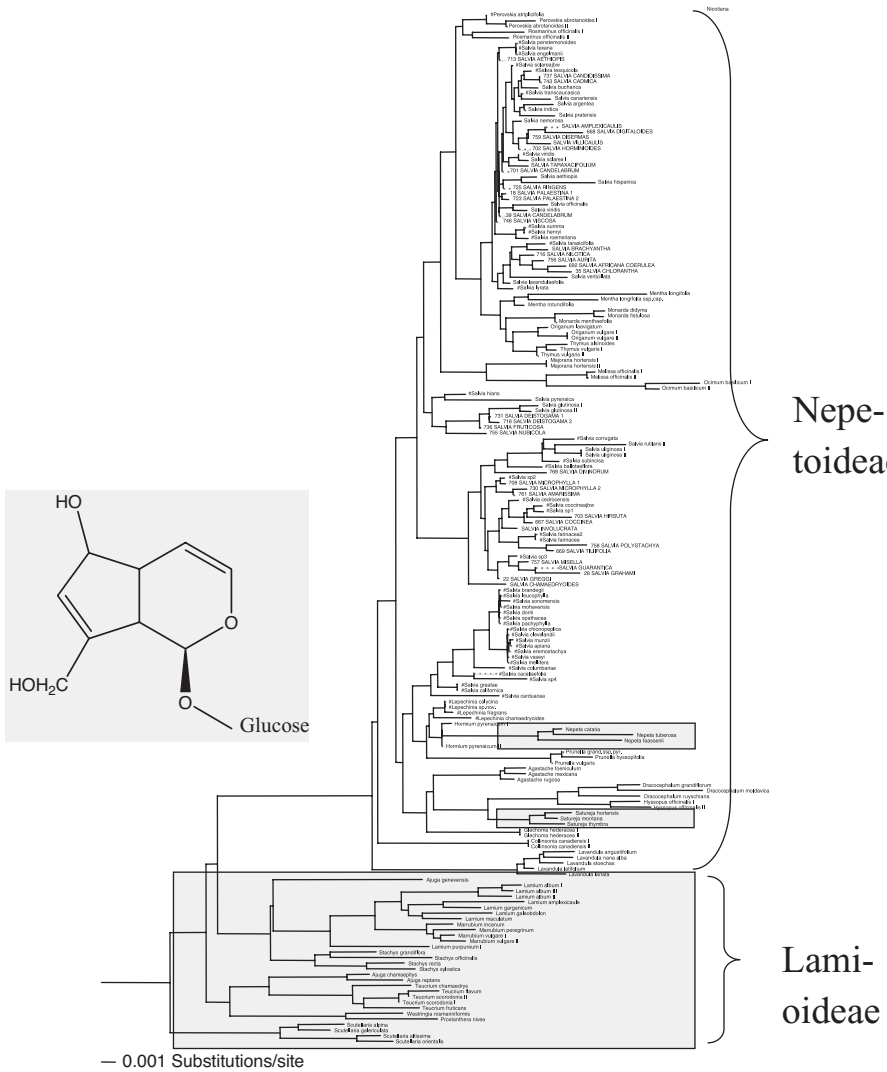


Figure 7.14 Distribution of iridoid glycosides in the family Lamiaceae, reconstructed from a *rbcL* data set. (After Wink and Kaufmann, 1996.) (See Plate 20 in colour plate section.)

7.6 Evolution of plant secondary metabolism

7.6.1 Occurrence of genes encoding key enzymes of secondary metabolism

In Fig. 7.15, the contradictory findings of the first two parts of this review are summarized schematically. This helps to define scientific evidence for convergence or shared ancestry of genes involved in secondary metabolism. During the past 10–15 years quite a large number of genomes have been sequenced (more than 400 at present), among them several eukaryotes, such as fungi, plants and animals. In addition, some of the important key enzymes in pathways leading to SM have been identified and the corresponding genes have been cloned (Marasco and Schmidt-Dannert, 2007; Oksman-Caldentey *et al*. 2007; Sato *et al*. 2007; Verpoorte *et al*., 2007; Minami *et al*., 2008; Wu and Chappell, 2008). We have analysed the sequences deposited from a number of selected genes/proteins in the sequence databases (NCBI, EMBL) and explored their distribution among plants, other eukaryotes, but also in prokaryotes. We have selected key enzymes at the start of SM pathways (see Figs 1.2, 1.3 [p. 7–8] and 2.1 [p. 22]), such as

- ornithine decarboxylase (leading to tropane, pyrrolidine and PAs, and putrescine)
- tryptophan decarboxylase (leading to indole alkaloids)
- tyrosine decarboxylase (leading to isoquinoline alkaloids)
- phenylalanine ammonia-lyase (leading to phenylpropanoids and flavonoids).

Ornithine decarboxylase is apparently present in pro- and eukaryotes (Fig. 7.16a; Table 7.2). It catalyses the decarboxylation of ornithine to

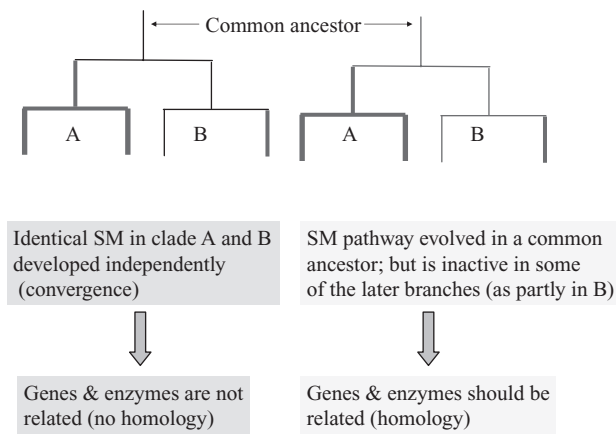


Figure 7.15 Schematical outline of the genetic base of SM distribution profiles.

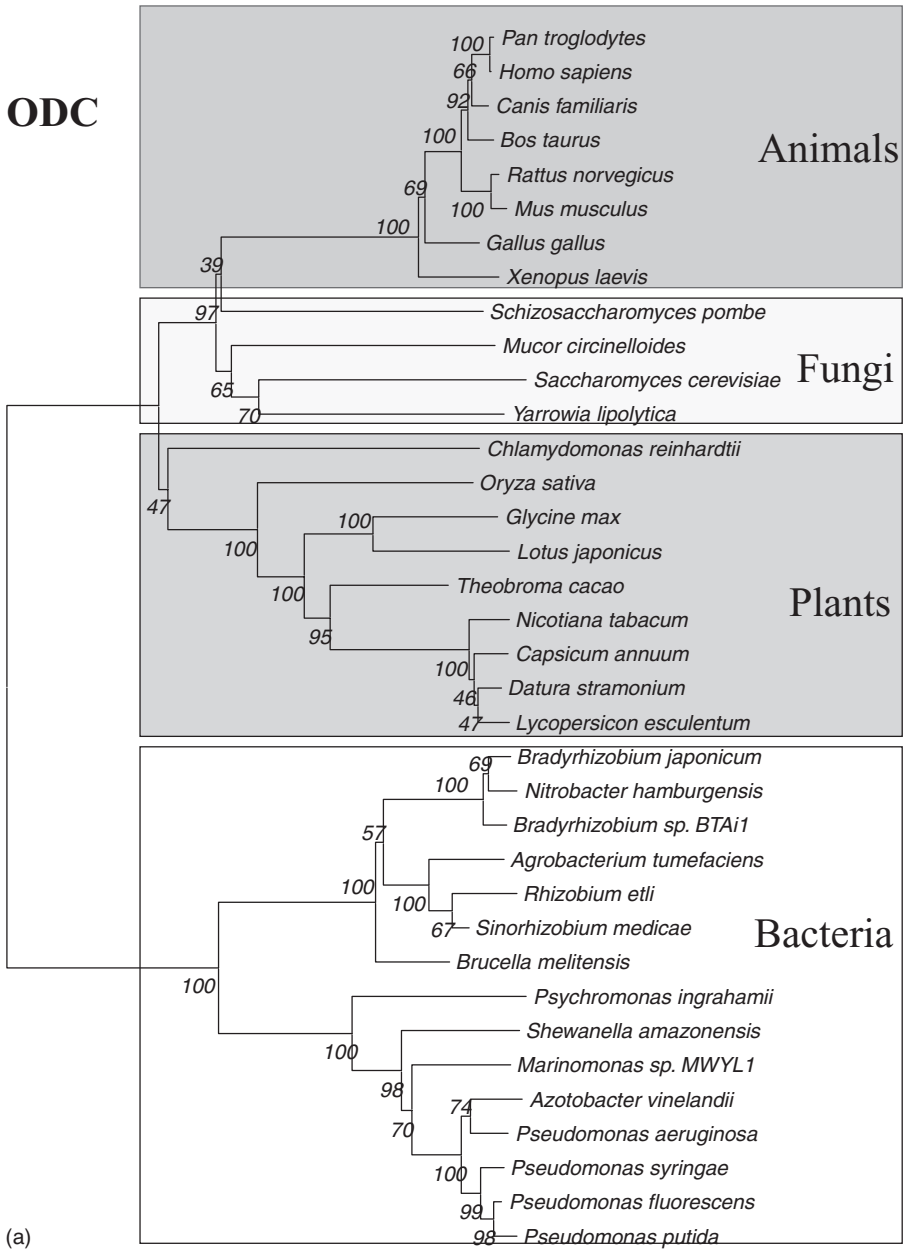
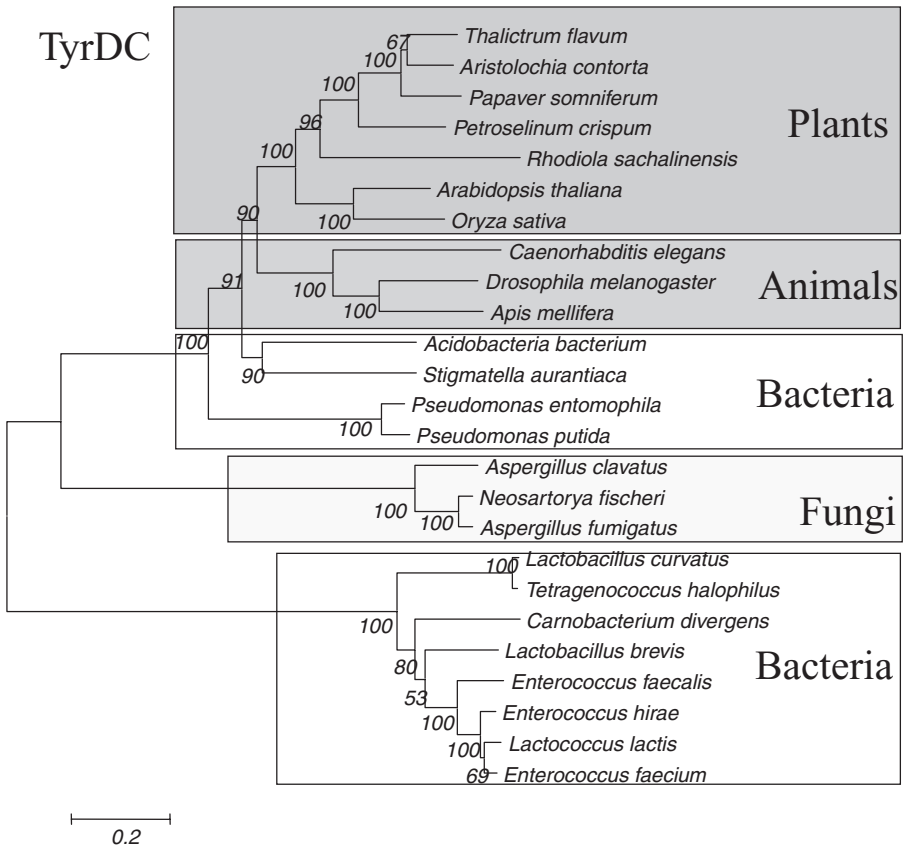


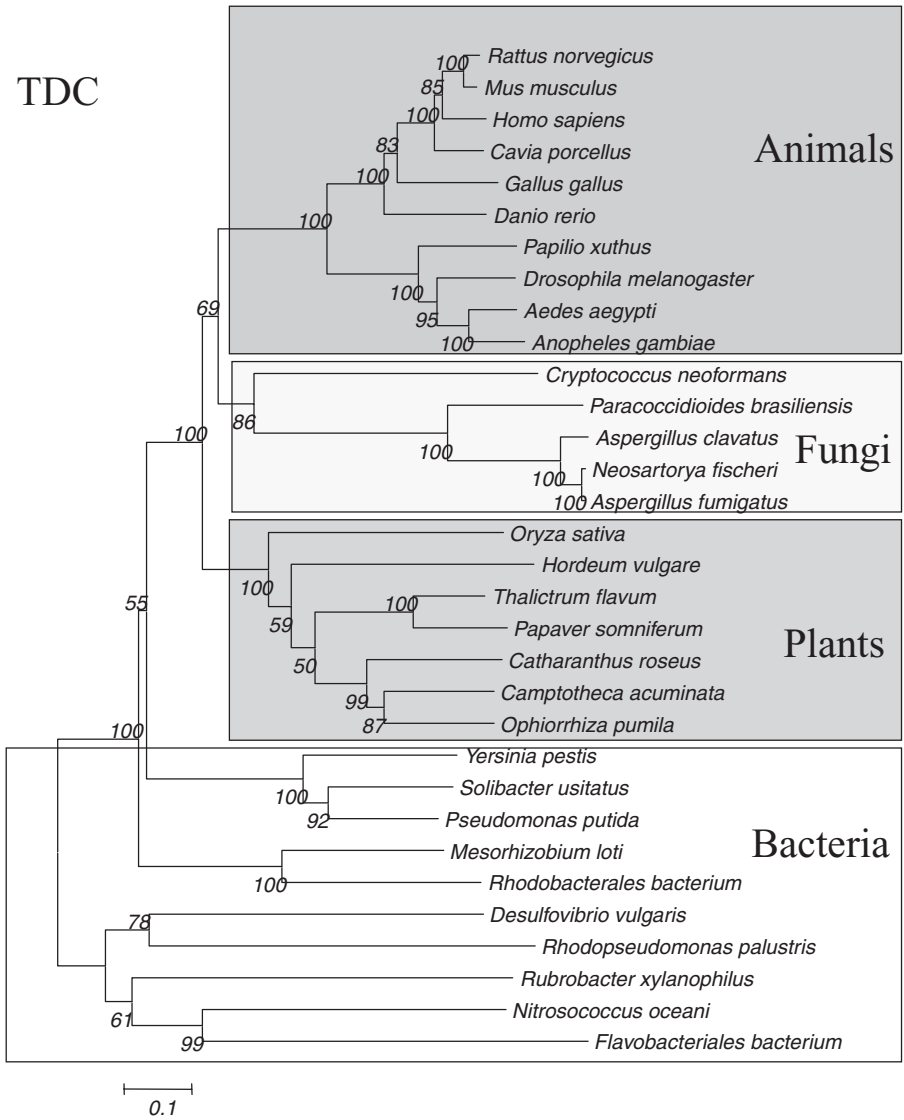
Figure 7.16 Phylogenetic relationships in key enzymes of pathways leading to SM, based on amino acid sequences. (a) Ornithine decarboxylase (ODC). (b) Tyrosine decarboxylase (TyrDC). (c) Tryptophan decarboxylase (TDC). (d) Phenylalanine ammonia-lyase (PAL). Numbers at nodes are bootstrap values.



(b)

Figure 7.16 (*Continued*)

putrescine, which is a compound of its own but also a precursor for tropane, *Nicotiana* and PAs. In the phylogram, reconstructed from amino acid data, ODC of plants, fungi, animals and bacteria clusters in monophyletic clades. ODC from animals and fungi forms a sister group, which agrees with the general tree of life. The fungal/animal clades share common ancestry with ODC from plants, indicating that an ancestral ODC must have been present. Also ODC from bacteria clusters in a monophyletic clade, in which ODC from Gram-positive and Gram-negative bacteria groups together. In a sequence alignment of representative species (Table 7.2), it becomes apparent that ODC from pro- and eucaryote shares a significant number of common conserved sites. This finding suggests that ODC is an old enzyme, which probably evolved in prokaryotes and which was imported into eukaryotes probably via the endosymbionts (the later mitochondria). ODC is functional in alkaloid biosynthesis in *Nicotiana* and *Datura*, but apparently not in

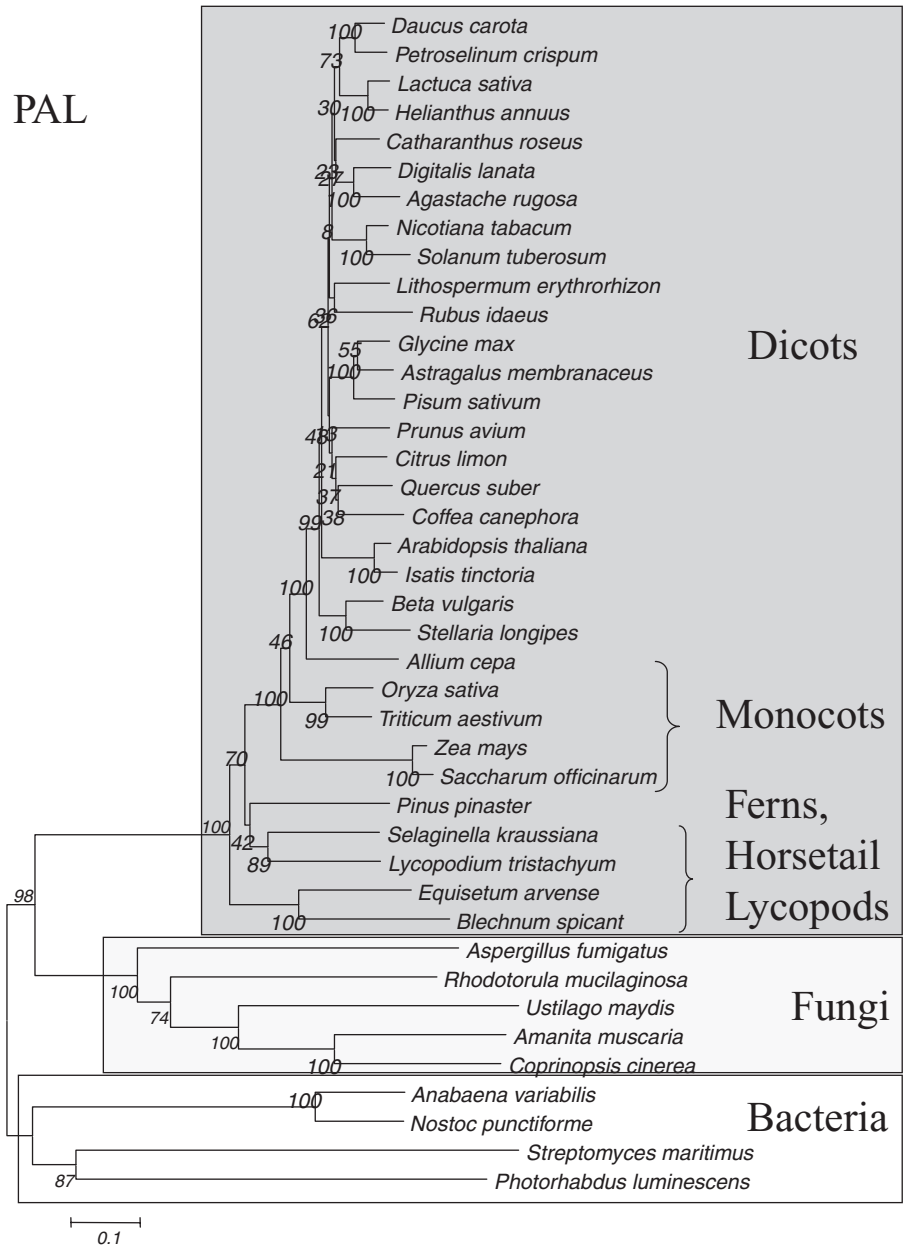


(c)

Figure 7.16 (*Continued*)

Capsicum and *Lycopersicon*; the corresponding ODC gene is present in all four taxa and has a very similar structure. This analysis would favour hypothesis II in Fig. 7.15.

Tyrosine decarboxylase, which is a key enzyme in the biosynthesis of isoquinoline alkaloids in plants, also occurs in all kingdoms of life (Fig. 7.16b, Table 7.3). The enzyme catalyses the decarboxylation of tyrosine



(d)

Figure 7.16 (*Continued*)

Table 7.2 Sequence alignment (amino acids) of ornithine decarboxylase (ODC) of selected taxa from plants, animals, fungi and bacteria. Conserved sites are marked by 'x'

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Nicotiana_tabacum          PFYVLDLGEV VSLMDQWKSA LIRPFYAVKC NPEPSFLSIL SAMGSNFDCA SRAEIEYVLS GISPIVFANP CKPESDIIFA
Datura_stramonium          .......... .......NAG .......... ........M. .......... .......... .......... ..........
Lycopersicon_esculentum    .......... ....E..N.. .......... ........M. .......... .......... .......... ..........
Theobroma_cacao            ........V. MA.F.K.ARN .AQ....... ..N.AL.GA. ATL..G.... .K....S... .V...I.... ..A..H.KY.
Glycine_max                ..S.....V. MG.....ACK .VQ....... ..NL.LIGA. A.L..S.... .K....S... .V...IY... ..S..H.RY.
Oryza_sativa               A.H.F..AK. .D.HRG.RR. .V..C..... ..DGAM.AA. A.L.AG.... ......A..A .VR...Y... ....AHLEY.
Saccharomyces_cerevisiae   S.FIC..... KR.FNN.VKE ..K....... ..DTKV..L. AEL.V..... .KV..DR... N.....Y... ..VA.F.RY.
Mucor_circinelloides       A.F.G..... .RQHIR..L ..E....I.. ..D.MVVQL. ASL.CG.... .KQ..QQ..D .VD..IY... ..QA.F.RYS
Gallus_gallus              A...A...DI .KKHMR.HK. .VT....... .DSEAVVKT. AVL.AG.... .KT..QL.Q. .VP..IY... ..QL.Q.KH.
Xenopus_laevis             A...A.F.DI .KKHVR.FK. .VT....... .DGKAIVKT. .IL.AG.... .KT..QL.Q. .V...IY... ..QV.Q.KY.
Homo_sapiens               A...A...DI LKKHLR.LK. .VT....... .DSKAIVKT. A.T.TG.... .KT..QL.Q. .VP..IY... ..QV.Q.KY.
Mus_musculus               A...A...DI LKKHLR.LK. .VT....... .DSRAIV.T. A.I.TG.... .KT..QL.QG .VPAVIY... ..QV.Q.KY.
Bradyrhizobium_japonicum   .CL.V..EV. RDNYQTFSLP DS.V.....A ..A.EV.AL. AS...C..T. TV....MA.A ATPD.S.G.T I.K.R..AR.
Nitrobacter_hamburgensis   .CL.V..DV. RDNYQTFALP DS.V.....A ..A.EV..L. ASL..C..T. TV....MA.A ATPDVS.G.T I.K.R..AR.
Bradyrhizobium_sp._BTAi1   .CL.V..DV. RDNYMNFALP DS.V.....A ..A.EV..L. ASL..S..T. TV....MA.A ATPD.SYG.T I.K.R..AR.
Azotobacter_vinelandii     ..V.I.TEII ANAYEELGFP FA.IY....A ..AVEIIRL. RDK.....I. ..Y.LDK.MA VGPEVSYG.T I.KAK..RYF
Shewanella_amazonensis     ..V.I.TNII AKQY.DMVFP FANVY....A ..AKEI.TL. KDK.....I. .IY.LDM.MA VTADVS.G.T I.KRK.VRAF
Conserved sites                  x                        xx x  x        x    x  xx x    x                   x   x
Conserved sites (plants)   x x xx  x   x   x    x  x xxxxx xx       x    x  xxxx x  xx  xx  x  xx  xxx xx  x    x

Nicotiana_tabacum          AKVGVNLTTY DSEDEVYKIR KHHPKSELLL RIKDNARCPM GPKYGALPEE VDPLLRAAQA ARLTVSGVSF HIGSGDADSN
Datura_stramonium          .........F .......... .....C.... .......... .......... .E....T... .......... .........K
Lycopersicon_esculentum    E.I....... .......... .....C.... .......... .......... IE....T... .......... ..........
Theobroma_cacao            .T......F ..KE.LE..K .W...CA..I .V..G....L .......... .T...Q...T .....T.... .....AMQFR
Glycine_max                .S....V... ..I...E... .C..TC.... ....G..TSL .L........ ..E..Q..HE .G.K.T.... .....G..TR
Oryza_sativa               .E........ ...E..A.V. RC..RC.... ...SD.KVDL .L....N.D. .L.......R EGVA.A.... .V...ASRAD
Saccharomyces_cerevisiae   .SKN.MKS.F .NVE.LH..K .F..E.Q... ..A.T.Q.RL ST...CEM.N ..V..K.IKE LG.NLA.... .V...AS.FT
Mucor_circinelloides       .QQN.SRM.F .NAE.LF..K .YY.DA..V. ..L.MSL.QL .L.F..PLHT .QH..QL.KE LN.N.I.... .V...CL.E.
Gallus_gallus              .NS..RMM.F ...V.LM..A RP...AK... ..T.K.V.RL SV.F..TLKT SRL..ER.KE LD.AIV.... .V...CT.PE
Xenopus_laevis             .SC..EKM.F ...V.LM.VA RN..NAK.V. ..A.K.V.RL SV.F..TLKT SRL..ER.KE LNVDII.... .V...CT.PQ
Homo_sapiens               .NN..QMM.F ...V.LM.VA RA...AK.V. ..A.K.V.RL SV.F..TLRT SRL..ER.KE LNID.V.... .V...CT.PE
Mus_musculus               .SN..QMM.F ...I.LM.VA RA...AK.V. ..A.K.V.RL SV.F..TLKT SRL..ER.KE LNID.I.... .V...CT.PD
Bradyrhizobium_japonicum   FAL.IR.FAV .CAA..E..A RAA.GAVFCR ILYCG.EW.L SR.F.CD..M AVDV.DV.KR LG.EPC.I.. .V..QQRKVK
Nitrobacter_hamburgensis   YAL.IR.FAV .CTA..E..A RAA.GAVFCR ILYCG.EW.L SR.F.CD..M AVDV.DL.KR LS.EPV.I.. .V..QQRKVK
Bradyrhizobium_sp._BTAi1   YAL.IR.FAV .CAA..E..S RAA.GAVFCR ILYCG.EW.L SR.F.CD..M AVEV.DL.KR LG.EPC.I.. .V..QQRKVK
Azotobacter_vinelandii     YEK..R.FAT ...ADLRN.A .AA.G.VVVR ILTTS.DW.L SR.F.CQ.DM ALD.MIL.RQ LG.VPY.L.. .V..QQR.ID
Shewanella_amazonensis     YER..R.FAS ...ADLRM.A EEA.G.VYVR ILTST.DW.L SR.F.CQN.M AYE..AL.KE LG.DPY.I.. .V..QQR.IG
Conserved sites                x       x              x                  x x                    x xx x xx
Conserved sites (plants)     xx  xx  xx  x  x     xx   xx  x x  x     x xxxx x x    xx  x       x xxxx x xxx

Nicotiana_tabacum          AYLGAIAAAK EVFETAAKMS MTVLDVGGGF TQFTAAVAVK SALKQHFDDE LTIIAEPGRF FAETAFTLAT TIIGKRVELR
Datura_stramonium          .......... G......R.. .....I.... .....SA..R ...E...H.. .......... .......... ..........
Lycopersicon_esculentum    .......... Q......Q.P .....I.... ......P... ...ET..H.F .......... .......... .........K
Theobroma_cacao            ..RE...... T......R.P .H..NI.... ...A..ST.. A..QAY.PN. ..V....... ...S...... N......D..
Glycine_max                ..H...S... N...S.SRLP .G...I.... .S.E..LKIN A.IEGS.GK. .VV.G....Y .......... RV.....DV.
Oryza_sativa               V.R...E..R .A.DA..A.P .R...I.... MT.D..AVIN R..ER..G.L VEV.G....Y .........A RV....T...
Saccharomyces_cerevisiae   SLYK.VRD.R T..DK..ELP LKI..... QS.KSTAVLR L..EEF.PVG VD......Y .VA......S HV.A..K.NE
Mucor_circinelloides       .FGD.VVR.. N..DQ.KAYD FKF..... PT.EV.AVLG PIVDRL.PKD VRV.....Y YVAS..N..V NV..R.TSPE
Gallus_gallus              TFVQ..SD.R C..DMG.EFN .YL..I.... PK.EITSVIN P..DKY.PLD V.......Y YVAS....V N..A.KIDKT
Xenopus_laevis             T.VQ.VSD.R C..DMG.EFN .HL..I.... PK.EITSVIN P..DKY.PAD VK......Y YVASS...V N..A.K.DKT
Homo_sapiens               TFVQ..SD.R C..DMG.EF. .YL..I.... PK.EITGVIN P..DKY.PSD VR......Y YVAS....V N..A.KI.QT
Mus_musculus               TFVQ.VSD.R C..DM.TEF. .HL..I.... PK.EITSVIN P..DKY.PSD VR......Y YVAS....V N..A.KT.QT
Bradyrhizobium_japonicum   .WDR.L.M.S Q..RDC.RGI LSMVNM.... PYLKPV.TYG RSIFRALRKH PET.I....G MVGN.GIIES EVVLISKDEV
Nitrobacter_hamburgensis   .WDR.L.MVS Q..RDC.RGI L.MVNM.... PYLRPV.QYG RSIFRALRKH PET.I....G MVGN.GVIE. EVVLISRDEN
Bradyrhizobium_sp._BTAi1   .WDR.L.M.S T..RDC.RGI LSMVNM.... PYLKPV.QYG RSIFRALRKH PET.I....G MVGN.GIIES EVVLISRDEL
Azotobacter_vinelandii     VWDAV..KV. VI..RLKEGI LKMINM.... PYI.SLQTYA EEIIRFLKED PE..L....S LIAN.GI.VS EVVLVSRAVE
Shewanella_amazonensis     .WDA..GKV. VL.DRLRDGI LKMINM.... PYIDQLGVYA QQI.HFLKED PE..L....S LISN.GV.IS EVVLISRA.E
Conserved sites                       x              xxxx                    x xxxx
Conserved sites (plants)     xx xx    x  x     x xx  xxxx   x xx      x   x      x xxxx  xxx xxxxx    xxxx

Nicotiana_tabacum          EYWINDGLYG SMNCVLYDHA TVVLPTTVFG PTCDALDTVL RDYQLPELQV NDWLVFPNMG AYTKAAGSNF NGFNSAIVTH
Datura_stramonium          .......... .......... ..CM.S.... .......... .......... ....I..... .......... ..........
Lycopersicon_esculentum    .......... .......... ..CM.S.... .......... .......... ....I..... .......... ..........
Theobroma_cacao            .......I.. ....I..... V.RADS.... .......... KV.P...... .......... ...A...... .......L.Y
Glycine_max                ....D..I.. TL.NIVF.Y. ..CT.S.... ....SI.... .......... .......... ...TSS.T.. ...S..KSIF
Oryza_sativa               ....D..... .L..I.M..Y VPAAAS.... ....S....V TG.....MS. G.....DD.. ...T...... ...A...KI.
Saccharomyces_cerevisiae   MIYT...V.. N...I.F..Q EPHNKVSIW. ....G..CIA KE.YMKDVI. G..FY...AL. ...SS.ATQ. ...ET.DIVY
Mucor_circinelloides       M.YV...M.. .V..IIF..Q V.KNESS.W. ....SI.CLN KSAR..K.EP G...Y.E... .H.IC.A.Q. ...RKSE.LY
Gallus_gallus              M.YV...V.. .F..I..... H.RPSCSIW. ....G..RIV ERCNM..... G..IL.E... ...V..A.T. ...QRPTIHY
Xenopus_laevis             M.YV...V.. .F..I.F... H.KPSSSIW. ....G..RIV ERFE...... G..ML.E... ...V..A.T. ...QRPTLYY
Homo_sapiens               M.YV...V.. .F..I..... H.RPSSSIW. ....G..RIV ERCD...MH. G..ML.E... ...V..A.T. ...QRPTIYY
Mus_musculus               M.YV...V.. .F..I..... H.RPSSSIW. ....G..RIV ERCN...MH. G..ML.E... ...V..A.T. ...QRPNIYY
Bradyrhizobium_japonicum   WVYLDI.KF. GLAETMDESI RYDG.CVLA. ....SA.VLK NP.P..T.EI G.KVLIEGT. ...TYSSVA. ..IPLKTYHI
Nitrobacter_hamburgensis   WVYLDI.KFS GLAETMDESI RYDG.CVLA. ....SA.VLK QP.P..T.EI G.K.LIEGT. ...TYSSVA. ..IPLKTYHI
Bradyrhizobium_sp._BTAi1   WVYLDI.KF. GLAETMDESI RYDG.CVLA. ....SA.VMK LP.P..T.SI G.KVLIEGT. ...TYSSVA. ..IPLKTYHI
Azotobacter_vinelandii     WVYTDV.KFS GLIETMDEAI KYKGEVVIA. ....SA.IMN YK.G..N.AS G.R.YWLST. ...SYSAVE. ...PLKSYYL
Shewanella_amazonensis     WVYTDV.KFS GLIETMDEAI KYKGKCVIA. ....SA.IMH YA.G..D.AI G.R.YWLTA. ...TYSAVC. ...PLKDYYL
Conserved sites                  x                        x xxxx  x              x       x x x      x xx
Conserved sites (plants)   xxxx xx xx   x    x         xxxx xxxx  xxx    x xxxxxx  xxx    xx xxx   x xx xxx xx
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Table 7.3 Sequence alignment (amino acids) of tyrosine decarboxylase (TyrDC) of selected taxa from plants, animals, fungi and bacteria. Conserved sites are marked by 'x'

```
Arabidopsis_thaliana    MDSEQLREYG HLMVFIADYY KTIEDFPVLS QVQPGYLHKL LPDSAPDHPE TLDQVLDDVR AKILPGVTHW QSSFFAYYPS
Oryza_sativa            ..A.....C. .R...V.... .S..A..... .......KEV ......RQ.D ...SLF..IQ Q..I...... ..NY......
Thalictrum_flavum       L.P.EF.RQ. .M.I.L.... RD..KY..R. ..E....R.E I.....YN.. SIETI.E..H KQ.I..I... ..NY...F..
Papaver_somniferum      L.P.EF.RQ. .MII.L.... RDV.KY..R. ..E....R.R ..ET..YN.. SIETI.Q..T TE.I..L.... ..NYY..F..
Aristolochia_contorta   L.P.EF.RQ. .M.I.L.... RDV.KY..R. ..E....R.R ..E...YN.. PIESIIQ..Q SH.V..I... ..NY...F..
Petroselinum_crispum    LEP.EF.RQ. ...I.L.... RKV.NY..R. ..S....REI ..E...YN.. S.ETI.Q..Q T..I..I... ..N....F..
Rhodiola_sachalinensis  ..LTE.STES R.VT..TQ.. Q.L.TR..QP L.K..F.TSQ ..ED..FYG. SMEEI.S..N E..V..L... ..N.H..F.A
Stigmatella_aurantiaca  LAA.EF.QL. YR..W..G.W DRL.S...RA P.A..DVAAR ..PHP.EQGL DGEK.FQ.LE QVV...T... .....G.F.A
Pseudomonas_entomophila .TP..F.Q.. .QLIL....R Q.VAER..MA ..E....KAA ...M..QQA. PFEAI.K..D QLLM..LS.. .HD.YG.F..
Pseudomonas_putida      VTP..F.Q.. .QLIL....R Q.VGER..MA ..E....KAA ..AT..QQG. PFAAI....N NLVM..LS.. .HD.YG.F..
Aspergillus_clavatus    DINST.E.QT N.QKLWSITQ TPW.SGVLP. ADHLARARAS ..VKLANDGL GFES.RQHIL DD.V.ALNSS I.NYYGFVTG
Neosartorya_fischeri    EVDTT.EKQQ DFQQLWKIAQ SEWKSDILP. ANDLARARAS ..K.LSNAGM GYEASKQHIL DD.I..LNSS I.NYYGFVTG
Drosophila_melanogaster ...TEF.KR. ME..Y.CN.L E.LNERR.TP S.E....RH. ..PE..QE.. DW..IMR..E D..M..... .HR.H..F.A
Apis_mellifera          ..IQEF.VR. KE..Y.CEFM SN.HNRR.TP D.G....RP. ..SE..QQ.. PWENIMR..E S..M..I... .HR.H..F.A
Conserved sites                                                     x                    x
Conserved sites (plants)                  xx   x  xx    x xx x    x  xx           xx    x xxxxx  xx   xx x

Arabidopsis_thaliana    NSSVAGFLGE MLSAGLGIVG FSWVTSPAAT ELEMIVLDWV AKLLNLPGNG GGVIQGSASE AVLVVLIAAR DKVLRSVGEK
Oryza_sativa            ...T...... ....AFN... ...I...... ...V.....F ..M.Q..AL. ......T... ....A.L... .RA.KKH...
Thalictrum_flavum       SG........ ...T.FNV.. .N.MS..... ...S..M..L G.M.K..... ...L..TTC. .I.CT.T... .RM.NKI.C.
Papaver_somniferum      SG........ ...T.FNV.. .N.MS..... ...SV.M..F G.M.....S. ...L..TSC. .I.CT.T... .RK.NKI.GR
Aristolochia_contorta   SG.T..... ...T.FNV.. .N.MS..... ...S..M..L G.M.R...SE ...L..TTC. .I.CT.T... .RA.CEI.GR
Petroselinum_crispum    SG.T..... ...T.FNV.. .N.MV..... ...NV.T..F G.M.Q...G. ...L..TTC. .I.CT.V... ..N..QH.G.
Rhodiola_sachalinensis  S..N..LM.. L.CS..SVI. .T.SS..... ...NV.V..M .M.....G. ...LHSNTC. ...CT.A... ..TMER..N.
Stigmatella_aurantiaca  .V.GPAV... L......VQ. ML.S.G..C. ...AR.M..L VE..G..PT. .......... .T..AMV... ARIR.MSPAP
Pseudomonas_entomophila .GTLSSV..D F..T...VL. L..QS...LS ...ETT...L RQ..G.SSQW S....DT..T ST..A..C.. ERTTDYALRP
Pseudomonas_putida      .GTLSSV..D F..T...VL. L..QS...LS ...ETT...L RQ..G.S.QW S....DT..T ST..A..S.. ERATDYALKP
Aspergillus_clavatus    GVTP.ALFAD NIVSAYDN.Q VHLPDHSV.. DV.YNA.GLL VD..Q.D.AT ASN.L.L.CG REFILRH..K KRGVTLQSIS
Neosartorya_fischeri    GVTP.ALFAD NVVSAYDN.Q VHLPEHSV.. DV.YSA.GLL LD..Q.D.AT ASN.L.L.CG REFA.EA..K SRGAALQSLS
Drosophila_melanogaster GN.FPSI..D ..GD.I.CI. ...AA...C. ...T.....L G.AIG..ST. .....T.... C...TML... AQA.KRLKS.
Apis_mellifera          GN.FPSI..D ...DAI.CI. ...AA...C. ...T..C..F G.AIG..SK. .......... CI..CML... AQAIARLKG.
Conserved sites                                             x           x                    x
Conserved sites (plants)  x xx  xx  x       x x x  xxx x xxx  x xx     x xx    xxx      x x x  x xxx x      x

Arabidopsis_thaliana    LVVYSSDQTH SALQKACQIA GIHRVLTTST NYALRPESLQ EAVSDLEAGL IPFFLCANVG TTSSTAVDPL AALGKIANSN
Oryza_sativa            ....A..... .......... ..F..VIANK ...VA..AVS ..L...SS.. ....I..T.. ....S..... PE..Q..K..
Thalictrum_flavum       ....G..... C.....A... ....AVP.AN D.G.SASA.R STIL.I.... V.L....T.. .........I GP.C.V.SDY
Papaver_somniferum      ....G..... C.....A.V. ..N.AIK.EN SFG.SAAT.R .VIL.I.... ..L.V.PT.. .........I SPICEV.KEY
Aristolochia_contorta   ....G..... C.....A... ..D.AVK..D .FGMSAAA.R A..E.TAR.. V.L.V..T.. .......... GP.CEV.REH
Petroselinum_crispum    ....C..... .......AK.. ..D.AIE..S .FK.C.KR.E S.IL..QN.. ..LY....T. .....T.... P..TEV.KKY
Rhodiola_sachalinensis  ....C..... FTIH.GAKLI ..RKS...EN E.G.C.ND.R N.IE.MK... V..Y..GTI. ..ALG....I KE...VVREY
Stigmatella_aurantiaca  ..A.A.T.V. .S.L..AMLC .VA.TIA.DA T.G.N..A.E R.IT..A..K R...V..TL. ....G...R. GPI.EVLART
Pseudomonas_entomophila ....V.AHA. .SVD..ALL. .FG.LIP.DE QF.M...A.R A.ID..A..N Q.CAVV.TT. ..TT..L... R.I.E..QA.
Pseudomonas_putida      .I..V.AHA. .SVD..ALL. .FG.LIP.DD R......A.. A.IE..A..N Q.CAVV.TT. ..TT..L... R.V.E..QA.
Aspergillus_clavatus    GLQVL.TLP. .S.I..AG.L ..GKNVCRDN SLMFDLQK.E AELAPDK.TI VAVSCGEIT. QFATAGLEEM REIRRLCDKY
Neosartorya_fischeri    GLQVL.TLP. .S.V..AG.L ..GKNICRDN PLLFDLAR.E AELASEK.TI VAVSCGEVT. HFATAGLEEM HEIRRLCDKY
Drosophila_melanogaster .MA.C.KEA. .CVE..AM.C FVK.I.EPDD DAS..GQTIY ..ME.ELQ.. V...VSTTL. ..G.C.F.N. PEI..QLQRF
Apis_mellifera          .MA.C.RES. .SVE.DAM.C FVK.I.EPDD KSV..G.T.R Q.IE.TAE.Y ....VSTTL. ..ACCSF.N. KEI.PVCKKY
Conserved sites             x   x      x
Conserved sites (plants)xxxx xxxxx     x      xx                          x   xx  x   x  xx   xxx

Arabidopsis_thaliana    GIWFHVDAAY AGSACICPEY RQYIDGVETA DSFNMNAHKW FLTNFDCSLL WVKDQDLTLA LSTNPEFLKS QANLVVDYKD
Oryza_sativa            DM...I.... .......... .HHLN...E. .......... .......... ....RS.IQS .......... ...S...F..
Thalictrum_flavum       S..V...... .........F .HF.....N. ...SL..... .F.TL..CC. ...EPS.IK. ......Y.RT ESHQ......
Papaver_somniferum      EM.V...... .........F .HF.....E. ...SL..... .F.TL..CC. ....PS.VK. ......Y.RT ESRQ......
Aristolochia_contorta   .M.V...... .........F .HF.....E. ...SL..... LF.IL..CC. ....PG.VK. ......Y.RT ESRQ......
Petroselinum_crispum    DL.V...... .........F ...L....N. ...SL..... ...TL..CC. ..R.PS.IKS ...Y...... ET.K......
Rhodiola_sachalinensis  DL.....G.. .........F QH.L..I.L. ..IS...... L.S.L..CFM .LRSPK.IQS .AAEGT...G GSEMM.....
Stigmatella_aurantiaca  .G.L....W ..A.LV...F .EGLA.M.AV ...CFDP... L......DAF FTR.RG.LE. ..VM..Y.R. ASGS.T..R.
Pseudomonas_entomophila QL.L...S.M ....M.L..C .WMW..I.L. ..VVV..... LGVA....IY Y.R.PQ.IRV M....SY.QA VDGE.KNLR.
Pseudomonas_putida      .L.L...S.M ....M.L..C .WMW..I.L. ..VVV..... LGVA....IY Y.R.PQ.IRV M....SY.QA VDGE.KNLR.
Aspergillus_clavatus    .A.V.A.G.F GIFGR.LDDI KKGC..I.L. ..ITGDG..L LNVPY..GFF LCRHS.AQNV FQA.AAY.TA GGPSIPSPLN
Neosartorya_fischeri    .A.L...G.F GIFGRVLDDI KKGCQ.I.L. ..ITGDG..L LNVPY..GFF LCRHATAHDV FQA.AAY.TT DGPSIPSPLN
Drosophila_melanogaster PV.L...... ..NSF....L KPLLK.I.Y. ....T.PN.. L.......T. ..R.RI..S. .VVD.LY..G YSDAAI..RH
Apis_mellifera          PV.L...... ..NSF....L KYLMA.I.Y. ....T.TN.F L.......C. ..R.RF..S. .VVD.LY.QT H.DTAI..RH
Conserved sites           x x x x                 x x   xx      x       xx                    x
Conserved sites (plants)  x x x xx xx x   xx      xx x x xx   xxxxx     xx    x    x    x       x       xxxxx

Arabidopsis_thaliana    WQIPLGRRFR SLKLWMVLRL YGSETLKSYI RNHIKLAKEF EQLVSQDPNF EIVTPRIFAL VCFRLVKCNN RNRELLDAVN
Oryza_sativa            .......... .......... ..VDN.Q... .K..H..EH. ...LLS.SR. .V....T.S. ......NGRK L.YDMM.G..
Thalictrum_flavum       ...A.S.... AM...L...S ..VAN.RNFL .S.V.M..N. .GFIAL.KR. ...V..T..M .....LRA.E L..R..ESI.
Papaver_somniferum      ...A.S.... .........S ..VTN.RNFL .S.V.M..T. .G.ICM.GR. ...TV..T..M .....LLA.K L.QVY.ET..
Aristolochia_contorta   ...A.S.... A....L...S ..VSN.RNFL .T.V.M..T. .G.LAM.KR. .V.V..T..M .....LAV.E L..K..ESI.
Petroselinum_crispum    ...M.S.... A....F...S ..VGQ.REF. .G.VGM..Y. .G..GL.KR. .V.A..L.SM ....IKEV.E I..K..ES..
Rhodiola_sachalinensis  ...S.S.... AI.M.V.I.R ..VSN.IEH. .SDVSM.AR. .EM.AASDR. ...F..K.S. ...K.RRDSE LT...MEK..
Stigmatella_aurantiaca  ..V..... A....L...H ..RQG.QA.. .E.VR..QR. VGW.A..AR. .LAV..SL.. .....AATDA ...A..ERL.
Pseudomonas_entomophila .G..... A....FM..S E.V.N.QQRL .RDLDN.RWL AEQ.QASGEW .LLA.VQLQT L.I.HRALDA HT.GWA.RL.
Pseudomonas_putida      .G..... A....FM..S E.VDA.QARL .RDLDN.QWL AGQ.EAAAEW .VLA.VQLQT L.I.HRALDA HTKGWAERL.
Aspergillus_clavatus    IG.ENS.... A.PVYAS.VA ..RAGYQTVL QRQ.R..RKV AAW..DH.AY .ALPEMGSTE ELLDKT.KDS L.QR.ASTI.
Neosartorya_fischeri    IG.ENS.... A.PVYTS.LA ..RAGYQNML QRQ.R..RKI VGW.FDH.AY .VLPEA.NKE DVLEKT.DDV L.QR.ASTI.
Drosophila_melanogaster .GV..S.... .....F...S ..ISG.QH.. .H.....R. .E..LK.KR. ..CNQVKLG. .....KGSDK L.EK..SII.
Apis_mellifera          .S...S.... .....F.M.S ..ISG.QA.. ...VQ...R. .A..RK.TR. .LCNEVVLG. ....AKGSDK L.QK..STI.
Conserved sites             xxxx            x              x                 x                          x
Conserved sites (plants)xxx x xxxx   x x x x  xx   x     x     x  x         x x   xx x   xxx              x
```

to tyramine. TyrDC in plants, animals and Gram-negative bacteria cluster in a common clade (100% bootstrap support), whereas the proteins of fungi and Gram-positive bacteria are still related but differ to a higher degree (Table 7.3). Similar to the situation in ODC, we can postulate a common origin for TyrDC; its presence in plants and animals might be due to import from Gram-negative bacteria (known to be the progenitors of the mitochondria). Among the plant taxa in the plant clade, only *Thalictrum, Aristolochia* and *Papaver* use a TyrDC as a key enzyme in alkaloid biosynthesis. Whether the enzyme is active in taxa, not producing alkaloids, such as *Rhodiola, Arabidopsis* or *Oryza*, is less likely, but needs to be explored. This analysis would again favour hypothesis II in Fig. 7.15.

Tryptophan decarboxylase (TDC), which is a member of the aromatic amino acid DC, is a key enzyme in the biosynthesis of simple indole alkaloids and monoterpene indole alkaloids. It catalyses the decarboxylation of tryptophan to tryptamine. The phylogenetic pattern of TDC shows strong similarities to that of ODC (Fig. 7.16c). TDC from plants shares ancestry with TDC from fungi and animals, which form a sister group relationship. TDC is also present in bacteria and Gram-negative bacteria (proteobacteria), and shares many similarities with TDC from eukaryotes (Table 7.4), indicating that TDC in eukaryotes is of bacterial (e.g. endosymbiotic) origin. TDC is functionally expressed in *Catharanthus, Camptotheca* and *Ophiorrhiza*, which produce monoterpene indole alkaloids. *Thalictrum* and *Papaver* produce isoquinoline alkaloids and need TyrDC, but not TDC, as a key enzyme. Poaceae produce gramine, a simple indole for which TDC could be required, which might explain RDC in *Oryza* and *Hordeum*. Again this analysis would provide evidence for hypothesis II in Fig. 7.15.

Phenylalanine ammonia-lyase (PAL) is the key enzyme for the conversion of alanine to cinnamic acid, which is a precursor for coumarins, lignin, other phenylpropanoids and flavonoids (Fig. 1.3). PAL genes can be found throughout the land plants, including early ferns, lycopods and horsetails. It is also present in fungi, bacteria, but not in animals. Within bacteria, we find PAL in Cyanobacteria (*Anabaena, Nostoc*), but also Gram-positive (*Streptomyces*) and Gram-negative bacteria (*Photorhabdus*) (Fig. 7.16d). PAL proteins share many conserved sites in pro- and eukaryotes, indicating their origin from a common ancestral protein (Table 7.5). It is remarkable that PAL is absent in animals. This suggests that PAL in plants might have been imported by cyanobacteria (progenitors of chloroplasts), but not Gram-negative bacteria; otherwise we might have also detected PAL in animals. PAL appears to be functional in all land plants, indicating that the pathway leading to phenolic compounds started very early during the evolution of plants.

In a second approach we have selected key enzymes which catalyse later steps in SM pathways, such as

- chalcone synthase (CHS) (combining malonyl-CoA and p-coumaryl-CoA to chalcone, which later cyclisises to flavonoids)

Table 7.4 Sequence alignment (amino acids) of tryptophan decarboxylase (TDC) of selected taxa from plants, animals, fungi and bacteria. Conserved sites are marked by 'x'

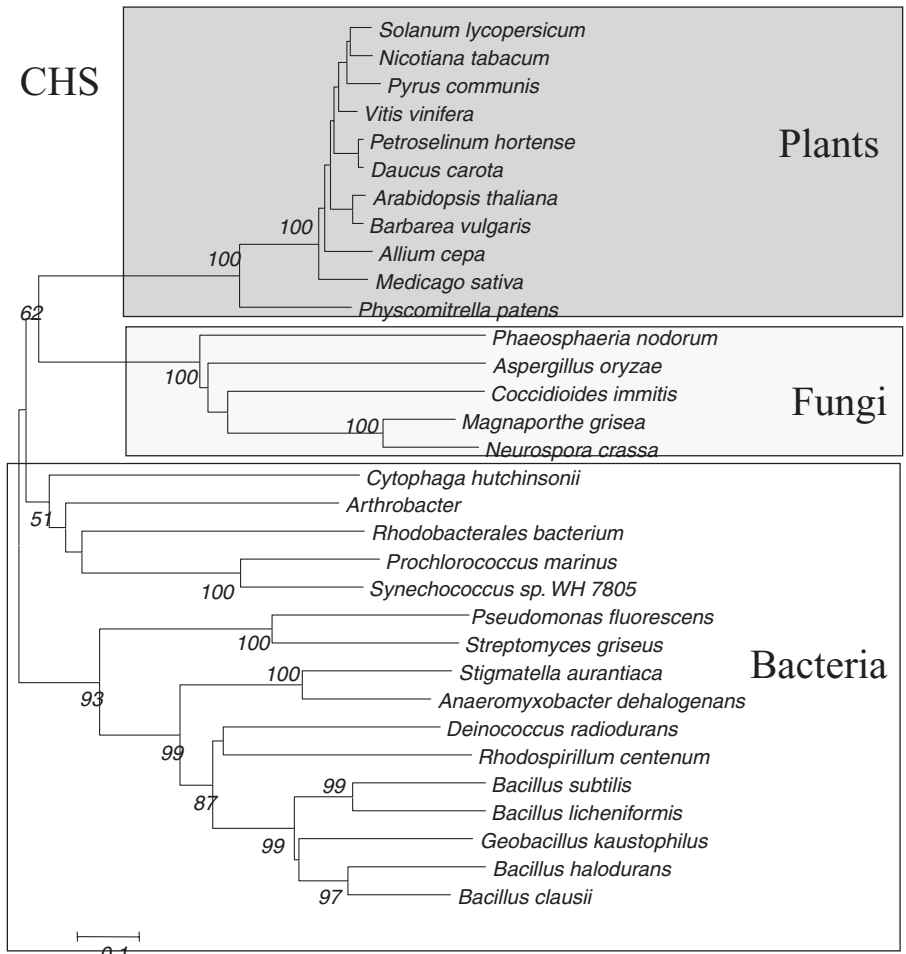
```
Catharanthus_roseus   LEAEEFRKQA HRMVDFIADY YKNVETYPVL SEVEPGYLRK RIPETAPYLP EPLDDIMKDI QKDIIPGMTN WMSPNFYAFF
Camptotheca_acuminata .DP.....  .CI.....  ...I.S....  .Q.D...RHS .LGKN...RS ..FES.L..V .........H ......F.H.
Ophiorrhiza_pumila    ..P.....  .V.....  ...I.N....  .Q......KN .L.....H..  .SFET.L...  K...V.....  .L....F.Y.
Thalictrum_flavum     .DP....R.G .M.I..L...  .RDI.K...R .Q.....  E..DS...N.  .SIET.LE.V H.Q....I.H .Q...YF.Y.
Papaver_somniferum    .DPD...R.G .MII..L...  .....K...R TQ.D....K.  .L..S...N.  .SIET.LE.V TN.....L.H .Q...YF.Y.
Hordeum_vulgare       .NP.DV.AYL .KA....S..  .T...SM...  PN.K....QD ELTASP.THS A.F.VT..EL RTSVV....H .A....F...
Oryza_sativa          .D.D...RLG .QV.....  .AGLGD...H PS.T..F..R QL.AD..SR.  ..FAAALR.V RDL.L..V.H .Q..RHF.H.
Pseudomonas_putida    MTP.Q..QYG .QLI.L....  RQT.GER..M AQ......KA AL.AA..QQG  ..FEA.LD.V NQLVM..LSH .QH.D..GY.
Mesorhizobium_loti    MNND...QWS R.AA.WG...  RNSLRER..R PL....DIFR S.DASP.EDA  ..M.R.FA.F EEK.V....H .QH.R.F.Y.
Yersinia_pestis       MTP....RHG YA.I.L....  RQ.I.LRG.N PTTA..EIKS .LSLN..EKA  ..FEH.IS..  EEL.M..LLH .QH.D.FGY.
Aspergillus_clavatus  MDR.Q..AA.  .AAI.E.V..  FDGLPSQR..  PTI.....P L...SP.DE.  .QWSQ.QA..  ETK.K..L.H .Q....M...
Neosartorya_fischeri  MDRDQ..AA.  .AAI.E.I..  FDGLPSQR.V PTI.....P L...NP.EE.  .QWSQ.QA..  ETK.K..L.H .Q....M...
Homo_sapiens          MN.S...RRG KE...YV.N.  MEGI.GRQ.Y PD.....P L..AA..QE.  DTFE..IN.V E.I.M..V.H .H..Y.F.Y.
Papilio_xuthus        M..GD.KDF. KA.T.Y..E.  LE.IRDR..V PL.K.....P LV..Q..DKA ..WTAV.A..  ERVVMS.V.H .H..R.H.Y.
Conserved sites                  x   x        x      xx          x                    x   x x     x
Conserved sites (pl)  x     x   x   xx  xx x      xx    x xx      x                    xx x  x xx   x x

Catharanthus_roseus   PATVSSAAFL GEMLSTALNS VGPTWVSSPA ATELEMIVMD WLAQILKLPK SFMGVIQNTT SESILCTIIA ARERALEKLG
Camptotheca_acuminata .........V ....C.CF..  ...N.LA...  ......V.I.  ...NM.....  .....L.G..  ..A....L..  .SPMHF.IV.
Ophiorrhiza_pumila    .........V ....C.GF..  ...N.LA...  S.....V.I.  ...NM.....  .......G..  ..A....L..  ..DG....I.
Thalictrum_flavum     .SSG.V.G..  ......GF.V  ...N.M....  .....S....  ..GKM.....  .L..L.G..  C.A....LT.  ..D.M.N.I.
Papaver_somniferum    .SSG.I.G..  ......GF.V  ...N.M....  .....S...N  ..G.M.T...  .L..L.G..  C.A....LT.  ..DKM.N.I.
Hordeum_vulgare       .S.N.A..IA .DLIAS.M.T  .....QA...  ...M.VLAL.  ....L.H..T T.....LG..  ..AM.V.LV.  ..DA..RRV.
Oryza_sativa          ..SS.TVGA. ..A.AAGI.V .P...AA...  ......V.V.  ..GRA.H..E .LL.T.LG.S C.AV..ALV.  ..D.K.AEI.
Pseudomonas_putida    .SNGTLSSV. .DC...G.GV I.LS.Q....  L..I.EVATN ..LKM.G.SV AWS....DSA .TAT.VAL.S G...SNYAK.
Mesorhizobium_loti    ..NAAPVSVV A.Y.VS.MAA QCML.QT...  .....TRTV. .MR.A.G..E G.S....DSA .SAT.NAVLT M...LDWQK.
Yersinia_pestis       .SN.ELSSV. .DC...G.GV I.LS.Q....  L..I.EVATN ..LKM.G.SV AWS....DSA .TAT.VAL.S G...SNYAK.
Aspergillus_clavatus  ..G.TYPSI. ...Y.A.FTA PA.N.LC...  C....T....  .M..A.G..Q C.Y....VSA .DAVATVM..  ....EGLTAE
Neosartorya_fischeri  ..G.TYPSI. ...Y.A.FTA PA.N.LC...  C....T....  .M.KA.G..E C.L....V.A .DAVATVM..  ....EGL.VE
Homo_sapiens          .TAS.YP.M. AD..CG.IGC I..S.AA...  C....TVM..  ..GKM.E...  A.L....GSA ..AT.VALL.  ..TKHRLQPE
Papilio_xuthus        .TAN.YP.IV AD...G.IAC I....IA...  C....VVML.  ..G.M.G..E C.L....G.A ..AT.VALLG .KS.QRV.PE
Conserved sites       x                      x  xxx   x x       x    x x      x
Conserved sites (pl)  x   x      x        x  x x x  xxx  xx x      xx   x xx     xx   x   x  x    x x    x   x

Catharanthus_roseus   PDSIGKLVCY GSDQTHTMFP KTCKLAGINI RLIPTTGISP QVLRKMVEDD VAAGYVPLFL CATLGTTSTT ATDPVDSLSE
Camptotheca_acuminata VKTSTSF.V. .....STYA .A........  .S....SV..  LL..RAI.A. K...M...YI ...V.....  .I..LS..AD
Ophiorrhiza_pumila    MENV....V. .....SF.Q ....V.....  K.....SM..  IA..EQI.A. ..D.L..I..  .T.V.....A .I...SEVAK
Thalictrum_flavum     REN.C...V. .....CALQ .AAQI....F  .AV....L.A SA..STILE. IE..L.....  ...V....S. .V..IGP.CK
Papaver_somniferum    REN.N...V. A....LSALQ .AAQI....F LA.A.S.L.. NS.QSTILA. IES.L.....  ...V....S. .V..IGP.CA
Hordeum_vulgare       VSD.PR.AV. AA....ST.F .A.R...F.. .S...G.LD. AK.LEVMQA. AD..L..TYV ...V....SN .V...GAVAD
Oryza_sativa          ARR..D..V. C.....FA.R .AARF...HC .E...CAL.. TA.HAAMQA. .D..L..... ...V...Q.. .V...RE.CA
Pseudomonas_putida    LQAAKP.IV. V.AHA.SSVD .AAL...F.. .....DALRS EA.QAAI.Q. L...NQ.CAV V..T...T.. .L..LRPIG.
Mesorhizobium_loti    LAGQ.Q.RI. S...V..SID RAIWVS...L VR..VGSMDT AA.EAAIVA. R...ML.AGI IGSV.G..IG G..DIAAVAG
Yersinia_pestis       MQNDAP.IV. T.SEA.SGVN .AAL...F.T .V..DAM.. LA.K.IIQQ. LVK.NR.CIV V..T...AA. .M..L.AIGD
Aspergillus_clavatus  YEDRPR..AL ..N.A.SSTA .GAL...TRY .SVTAREMTG PR..EVL.QC DKD.LT.YYI TLGM...N.C .L.RFAEIKA
Neosartorya_fischeri  YEDRPR..AL ..N.A.SSTA .GAL...TRY .SVTAREMTG PR..EVL.QC DKD.LT.YYI TLGM...N.C .L.RFAEIKA
Homo_sapiens          LTQME...A. S...A.SSVE RAGLIG.VKL KA..SDAMRA SA.QEAL.R. K...LI.F.M V......TCC SF.NLLEVGP
Papilio_xuthus        WTDLS...G. CNK.A.SSVE RAGL.G.VKM .SLKPDRLRG DI.QEAIDE. IKK.LI.FYV V.......SC TF.AL.EIG.
Conserved sites             x                      x                 x          x  x
Conserved sites (pl)        x x   xxx        x    xxx          x        x      x  xx     x x xxx    x xx

Catharanthus_roseus   IANEFGIWIH VDAAYAGSAC ICPEPHYLDG IERVDSLSLS PHKWLLAYLD CTCLWVKQPH LLLRALTTNP EYLKQSDLVV
Camptotheca_acuminata V..DY.V.F. .......... .......... ...A..... .....S.... .C.....S.S ..VK..S.D. ...QP.ES..
Ophiorrhiza_pumila    V..D.N..V. .......... .....Q.... ..L...F... ......CF.. .C...L.K.. .MVK..S... ....R.EF..
Thalictrum_flavum     V.SDYS..V. .......... ......FI.. V.NA..F..N A...FFTT.. .C.....E.S A.IK..S... ....ATES..
Papaver_somniferum    V.KLH...V. I......... ......FI.. V.DA..F..N A...FFTT.. .C.....DSD S.VK..S.S. ....AT.S.I
Hordeum_vulgare       V.AM.NA.V. .......... .....H... V.....I.M. ......TC.. ....Y.RDA. R.SDS.E... ...DVT.S.T
Oryza_sativa          V.ARH.V.V. .N......L V....DVIA. A.A.....MN A......NN. .CAV..AA.S A.VA..G.EQ ...AAAEG..
Pseudomonas_putida    ..QAH.L.L. ..S.M....M .L..CWMW.. ..LA..VVVN A....GVAF. .SIYY.RD.Q H.I.VMS... S..AVDGE.K
Mesorhizobium_loti    V.RRH.LYF. ....W....M ....Y.FWA. A.QA..VVFN .....G.QF. .SIQFLR..E D.V.T.AIK. .F.HGR.GII
Yersinia_pestis       .TQRY.L.L. ..S.M....M .L..C.LWC. ..KA..VVIN .....G.AF. .SVYC.QDSE H.I.IMS... SF.DADGK.R
Aspergillus_clavatus  VLK.KP..V. I......A.L VAD.WIAK.F A.G...FNVN M.....VNF. AS..YIRNRF D.TD..DIT. A..PY.ET.I
Neosartorya_fischeri  VLK.KP..V. I......A.L VAD.WIAK.F A.G...FNMN M.....VNF. AS..Y.RNRF D.TD..DIT. A..PY.ET.I
Homo_sapiens          .C.KED..L. .........F ......L.N. V.FA..FNFN ......VNF. .SAM...KRT D.TG.FRLD. T..SHQ.SIT
Papilio_xuthus        VCAARDV.L. .........F ....YYLMK. V.KA..FNFN ....M.VNF. .SAM.L...R WIVD.FNVD. L...HDQQAP
Conserved sites             x x       xx x      x       x  xx      xxx    x                    x
Conserved sites (pl)  x     x x   xxxxxxx    xxxx    x  x  xx      xxx    xx x                x x   xxx     x

Catharanthus_roseus   DFKNWQIATG RKFRSLKLWL ILRSYGVVNL QSHIRSDVAM GKMFEEWVRS DSRFEIVVPR NFSLVCFRLK EEVNKKLLDM
Camptotheca_acuminata .Y.D..VG.. .R.KA.R..F VM.....A.. .....T..Q. A....GF.K. .P....L... V........N .AL.R....W
Ophiorrhiza_pumila    ...D...G.. .R.KA.R... VM.....E.. KR..L...Q. A....GL.K. .P....I... A.A.....N .IL..E...L
Thalictrum_flavum     .Y.D....LS .R..AM.... V......A.. RNFL..H.K. A.N..GFIAL .K........ T.AM.....L N.L.RR..ES
Papaver_somniferum    .Y.D....LS .R...M.... V......A.. RTFL..H.K. A.H.QGLIGM .N..... T.AM.....  N...A...ES
Hordeum_vulgare       .L.DM.VGV. .R..G....M VM.T..TAK. .E........ A....DS..A .N...V.... ..A....I. D...RL.MEN
Oryza_sativa          .Y.D.GTTLT .R..A..V.. V..C...EG. R..V..H... AAA..AM..G .A...V.A.. R.A.....R N.L.RR..EE
Pseudomonas_putida    NLRD.G.PL. .R..A....F M...E..DA. .ARL.R.LDN AQWLAGQ.GA KAEW.VLA.V QLQTL.I.HR DAHT.GWAER
Mesorhizobium_loti    NYSE.SVPL. .R..A....F L..AH.LE.. RAMV.NH.TW SEGLAARLAK EPD....SEP ML..FS..HR D.H.LR.VNA
Yersinia_pestis       NYRD.G.PL. .R..A....F LI.EQ..EG. .ARL.R.I.N ANWLA.QIKN SDDWKL.A.V VLQTL.I.HE DNYTRSWAEK
Aspergillus_clavatus  .YR..S.SL. .R..A..I.F VM....LNGM K.F..KTI.L .DI.ADLI.R PDL...ITRP A.C.TV..I. NSLT.EVYEL
Neosartorya_fischeri  .YR..S.SL. .R..A..I.F VM....LSGM KAY..KTIGL .NI.ADL..R SDL...ITKP A.C.TV..V. NAIT.EVYEL
Homo_sapiens          .YRH...PL. .R.....M.F VF.M...KG. .AY..KH.QL SHE..SL..Q .P....C.EV ILG..... NK..EA..QR
Papilio_xuthus        .YRH...PL. .R..A....F V..L...E.. .K...KHI.L AHL..KLCTC .E.....EEV TMG..... NDI.EE..RR
Conserved sites             x x   x x    x  x                                                  x
Conserved sites (pl)  x x       x x   x x    x xx   x          x    x       x xxx   xx  x  xxxx
```

Table 7.5 Sequence alignment (amino acids) of phenylalanine ammonia-lyase (PAL) of selected taxa from plants, fungi and bacteria. Conserved sites are marked by 'x'

```
Arabidopsis_thaliana     TIGQVAAIST VKVELSETAR AGVNASSDWV MESMNTDSYG VTTGFGATSH RRTKNGVALQ KELIRFLNAG IFLPHSATRA
Nicotiana_tabacum        .VA.....AV .......E.. ...K...... .D........ .......... ......G... .......... V.........
Solanum_tuberosum        .VA...S.AN F......S.. ...K...... .D..S..... .....C.... ......G... ....K..... V.........
Stellaria_longipes       .VA....VAR .T...CNA.. DR.K...... .D..S..... .......... ...SK.G... .......... V....T.S..
Helianthus_annuus        .VS...G..A .......A.. ...K...... .......... .......... ......G... .......... ..........
Triticum_aestivum        ..AM...VAA TR...D.S.. GR.KE..... .N..M..... .......... ....E.G... R......... A...AA....
Pinus_pinaster           QTSD....AR ...K.D.A.K SR.EE..N.. LTQ.T..T.. .......... ...NQ.AE.. .......... VL.SEDI...
Lycopodium_tristachyum   .VA....V.R ...C.DSA.K HR.DE..N.. LQNVM..T.. .......... ...NQ..E.. Q...G..... VM..A.....
Rhodotorula_mucilaginosa .L.D.VGAAR ...AD.PHI. EKID..VEFL RTQLDNSV.. ......GSAD T..EDAIS.. .A.LEHQLC. VL..LEVV.G
Anabaena_variabilis      ..ND..RVAR .SLTNNTDIL Q.IQ..C.YI NNAVEEPI.. ..S...GMAN VAISQASE.. TN.VW..KT. AG..LADV..
Nostoc_punctiforme       ..DE.VNVAR .RLTDNADVI R..Q..C.YI NNAVEQPI.. ..S...GMAD VVISQAAE.. TN..W..KS. AG.SLADV..
Conserved sites              x                      x              xx xx xx             xx   x      x    x     x
Conserved sites (plants)     xx       x     x     x  xx xx       xx xx xxxxx xxx  xxx  x  xx  xxx xxxxx   x     xx

Arabidopsis_thaliana     AMLVRINTLL QGFSGIRFEI LEAITSFLNN NITPSLLRGT ITDLVPLSYI AGLLTGRPNS KATGPNAFKL AGISSGDLQP
Nicotiana_tabacum        .......... ..Y....... .....KLI.S ....C..... .......... .......... ..V.....RV ..VNG.E...
Solanum_tuberosum        .......... ..Y....... .....KLI.S ....C..... V......... .......... ..V..S..RV .AV.G.E...
Stellaria_longipes       .......... ..Y...W... .....KL..S DV..C..... .......... .........A ..V......A .K.D.PE...
Helianthus_annuus        .......... ..Y....... .....K.... ....C..... .......... .......... ..V..A..AQ ..VEG.E...
Triticum_aestivum        .....V.... ..Y....... ..T.ATL..A .V..C..... .......... ...V...... M..A.D...I ...QH.E...
Pinus_pinaster           S....T.... ..Y....WD. ..TVEKL..A GL..K..... .S........ .......... RVRSRD.L.K V.LEKPE...
Lycopodium_tristachyum   .....T...M ..Y....WQ. ...MAKL..A G...K....S .......... ...V...K.. ..VLAD.M.. V.VENPV...
Rhodotorula_mucilaginosa ..TI.V.S.T R.H.AV.IVV ...L.N...H G...IV.... .S..S..... .ASI..H.D. .VHVDGSAQE .IALK.VVL.
Anabaena_variabilis      ...L.A.SHM R.A....L.L IKRMEI...A GV..YVEF.S .G........ T.S.I.LDP. FKVDF..LRQ LNL.PLT.L.
Nostoc_punctiforme       ...L.A.SH. Y.A....L.L IQR.ET...A GV..HVEF.S .G........ T.A.I.LDP. FTVDFD.LSR L.LPKLQ...
Conserved sites           x  x x      x x  x            x     xx    x  x xx xxxxx       x                         x
Conserved sites (plants)  xxxx xxx   xx xxxx  x xx      x     xx  xxx  x xxxxxxxx xxx xxx x       x            xxx

Arabidopsis_thaliana     KEGLALVNGT AVGSGMASMV LFETNVLSVL AEILSAVFAE VMSGKPEFTD HLTHRLHHPG QIEAAAIMEH ILDGSSYMKL
Nicotiana_tabacum        .......... .......... ..DS.I.A.M S.V...I... ..N....... ....K..... .......... .......V.A
Solanum_tuberosum        .......... ........I. .YDS.I.A.M F.V...I... ..N....... Y...K..... .......... .......V.A
Stellaria_longipes       .....M.... ........I. ...A.I.A.. ..VI...... ..N....... ....K..... .......... .........A
Helianthus_annuus        .......... .......... ...A...AL. S.V...I... ..Q....... ....K..... .........Y .....D.V.A
Triticum_aestivum        .....M.... .....L.... ...A....L. ..V..G..C. ..N....... ....K..... .......... ..E.....M.
Pinus_pinaster           .....I.... S..AAL..I. C.DA...AL. S.VI..M.C. ..N....... P...K..... .M.......Y V........H
Lycopodium_tristachyum   .....M.... S..A.L.AT. CYDA...A.F ..VA..L.C. A.Q.....A. P......... .........F ..Q......A
Rhodotorula_mucilaginosa ....G..... ..SAS..TLA .TDAH...L. .QA.T.LTV. A.V.HAG.HP F.HDVTP..T ...V.RNIRT L.E..K.AVH
Anabaena_variabilis      .....MM... S.MT.I.ANC VYD.QI.TAI .MGVH.LDIQ ALN.TNQSFH PFI.NSP... .LW..DQ.IS L.AN.QLVRD
Nostoc_punctiforme       .....MM... S.MT.I.ANC VYDAK..LA. TMGVH.LAIQ GLY.TNQSFH PFI.QCP... .LWT.DQ.FS L.KD..LVRE
Conserved sites          xxxx   xxx  x    x           x                   x              xx  x   x        x  x
Conserved sites (plants) xxxx  xxxx  xx    x  x      x x        x  x x  x xxxxx x  xxx xxxx  x xxxxxxx   x xx x

Arabidopsis_thaliana     AQKLEMDPLQ KPKQDRYALR TSPQWLGPQI EVIRYATKSI EREINSVNDN PLIDVSRNKA IHGGNFQGTP IGVSMDNTRL
Nicotiana_tabacum        .......... .......... .......... ....A...M. .......... .......... L......... .......A..
Solanum_tuberosum        .......... .......... .......... ....A...M. .......... .......... .......... ..........
Stellaria_longipes       .KA.D..... R.R.A..... A......... G...W..... .......... .......... .......... ..........
Helianthus_annuus        ...V...... .......... .......... ....S...M. .......... .......... L......... ..........
Triticum_aestivum        .K...L...M .......... .......... ....A..... .......... .......G.. .......... ..........
Pinus_pinaster           .A....N... .......... .........V .I..S..HM. .......... .V...A.D.. L......... .......L..
Lycopodium_tristachyum   .A...T...K .......... .......... ....S..Q.. Q......... .......DL. L......... ..........
Rhodotorula_mucilaginosa HETEVK.DEG ILR....P.. C.......LV SDMIH.HAVL SL.AG.TT.. ....LENKMT H...A.MASS V.NT.EK...
Anabaena_variabilis      ELDGHDYRDH ELI....S.. CL..Y...IV DG.SQIA.Q. .I.....T.. .....DNQAS Y.....L.QY V.MG..HL.Y
Nostoc_punctiforme       ELDGHEYRGK DLI....S.. CLA.FI..IV DGVSEI..Q. .V.M...T.. .....ENQVS Y.....L.QY V..T..RL.Y
Conserved sites                        x xx xx     x  xx                x  x  xx x xx          xxx x     x  x   x
Conserved sites (plants) x       x    x x xx xx  xxxxxxxx    xx xx  x  xxxxxxxxx x xxx x  x  xxxxxxxxx xxxxxxx xx

Arabidopsis_thaliana     AIAAIGKLMF AQFSELVNDF YNNGLPSNLT ASNPSLDYGF KGAEIAMASY CSELQYLANP VTSHVQSAEQ HNQDVNLGLI
Nicotiana_tabacum        .L.S...... .........Y .......... .......... .......... .....F.... ..N....... ..........
Solanum_tuberosum        .L.S...... .........Y .......... .G........ .......... .....F.... ..N....... ..........
Stellaria_longipes       .......... .........Y .......... ..E....... .......... .....F.... ..N....... ..........
Helianthus_annuus        .......VTI .......... .......H.S GG.....S.. ..G....... .....F.... ..N....... ..........
Triticum_aestivum        .......... .......... .........S GG........ .......... .....F.G.. ..N....... ..........
Pinus_pinaster           ..S....... .........Y ..G......S GG.......M .......... T...L..... ..T....... .........G
Lycopodium_tristachyum   .L.S...... .......... .........S GG........ .......... T....F.... ..N....... ..........
Rhodotorula_mucilaginosa .V.LM..VS. T.LT.ML.AG M.RA...C.A .ED...S.HC ..LD..A.A. T...GH.... .ST...P..M G..AI..A..
Anabaena_variabilis      Y.GLLA.HLD V.IAL.ASPE FS....PS.L GNERKVNM.L ..LQ.CGN.I MPL.TFYG.S IADRFPT... F..NI.Q.YT
Nostoc_punctiforme       Y.GLLA.HID V.IAL..SPE FS....PS.V GNDRKVNM.L ..LQ.SGN.I MPL.SFYG.S LADRFPT... F..NI.Q.Y.
Conserved sites                x      x             xx  x            xx  x         x    x         xx   xx  x
Conserved sites (plants) x   xxx    xxxxxxxxx  xx xxxx x     xxx  x  xx xxxxxxx  xxx  x xx xx xxxxxx   xxxxxxxxx

Arabidopsis_thaliana     SSRKTSEAVD ILKLMSTTFL VAICQAVDLR HLEERQTVKN TVSQVAKKVL TTGVNVVDRE QVYTYADDPC SATYPLI
Nicotiana_tabacum        .A...A.... ......S.Y. ..L...I... ....KNA... .......RT. .M.A.I.... YLFA...... .CN...M
Solanum_tuberosum        .A...A.... ......S.Y. ..L...I... ....KSV... .......RT. .I.AI..... YLF....... .S....M
Stellaria_longipes       .A...A.... ......SS.. .......... .I..QSA... S....CRR.. I......E.. H.FA...... .S....L
Helianthus_annuus        .A...A.... ......S.Y. ..L..SI... ....KS.... .......... .M........ Y.FA...... LT....M
Triticum_aestivum        .....A..I. ......S... ..L...I... ....KNA..S C.KT..R.T. S.DN.TI... A.FA...... ..N...M
Pinus_pinaster           .A..SA..I. .....LS.Y. T.L....... ....LA...Q I.......T. S..L....N. H.FS.I.... N.S...T
Lycopodium_tristachyum   .A...A..IE .....T..Y. ..L...I... ....QAV..Q A..L....T. ...SDL..HQ PIF..I...T ..A...L
Rhodotorula_mucilaginosa .A.R.A..N. V.S.LLA.H. YCVL...... AM.FTKAFEP M.TELL.QHF GALATRLQQN NS.DLEQRWH DTFSVAT
Anabaena_variabilis      .ATLARRS.. .FQNYVAIA. MFGV...... TYKKHYDARA CL.PATERLY SAVRH..GQK PTSDRPYIWN DNEQG.D
Nostoc_punctiforme       .ANL.RRS.. .FQNYMAIA. MFGV...... TYKMHYDART CL.PNTVQLY .AVCE..GKP LTSVRPYIWN DNEQC.D
Conserved sites          x                   x     x  xxx
Conserved sites (plants) x xxx xx x xxxxx    x  x xx  xxx x xx   xx   x  x    x          x     x xxx     xxx
```

- strictosidine synthase (STS) (combining tryptamine with secologanin to strictosidine, from which other monoterpene indole alkaloids derive)
- berberine bridge enzyme (BBE) (leading to protoberberine alkaloids)
- codeinone reductase (CR) (converting codeinone and morphinone to codeine).

Chalcon synthase occurs in many angiosperms, but also in early landplants, such as the moss *Physcomitrella*. As can be seen from Fig. 7.17a, the plant enzymes form a sister to fungal CHS, which share ancestry with polyketide



(a)

Figure 7.17 Phylogenetic relationships in key enzymes of later steps in SM pathways, based on amino acid sequences. (a) Chalcone synthase (CHS). (b) Strictosidine synthase (STS). (c) Berberine bridge enzyme (BBE). (d) Codeinone reductase (CR).

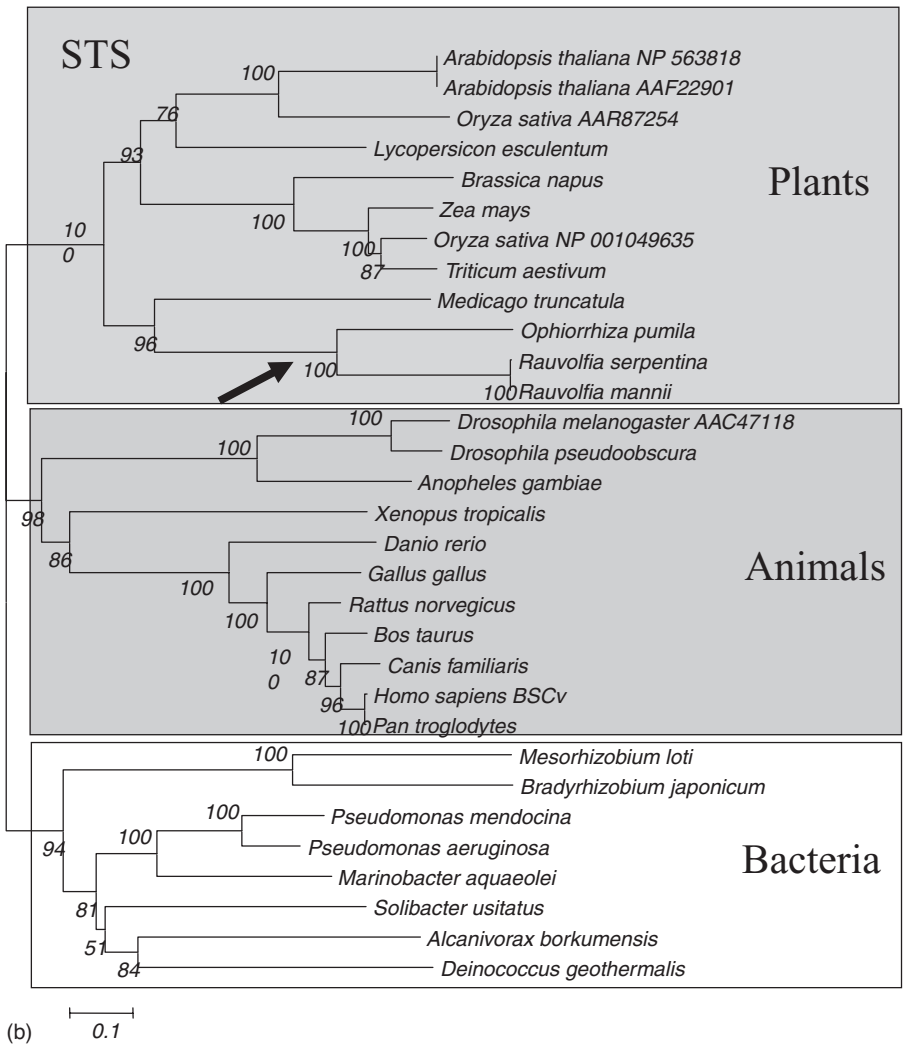


Figure 7.17 (*Continued*)

synthases from cyanobacteria. CHS proteins from plants share many conserved sites in pro- and eukaryotes (Table 7.6), indicating their origin from a common ancestral protein. It is remarkable that CHS is absent in animals. This suggests that CHS in plants might have been imported by cyanobacteria (progenitors of chloroplasts).

Strictosidine synthase has a wide distribution among plants, although it is functionally expressed in a small group of taxa producing monoterpene indole alkaloids (see arrow in Fig. 7.17b). Related genes occur in animals and

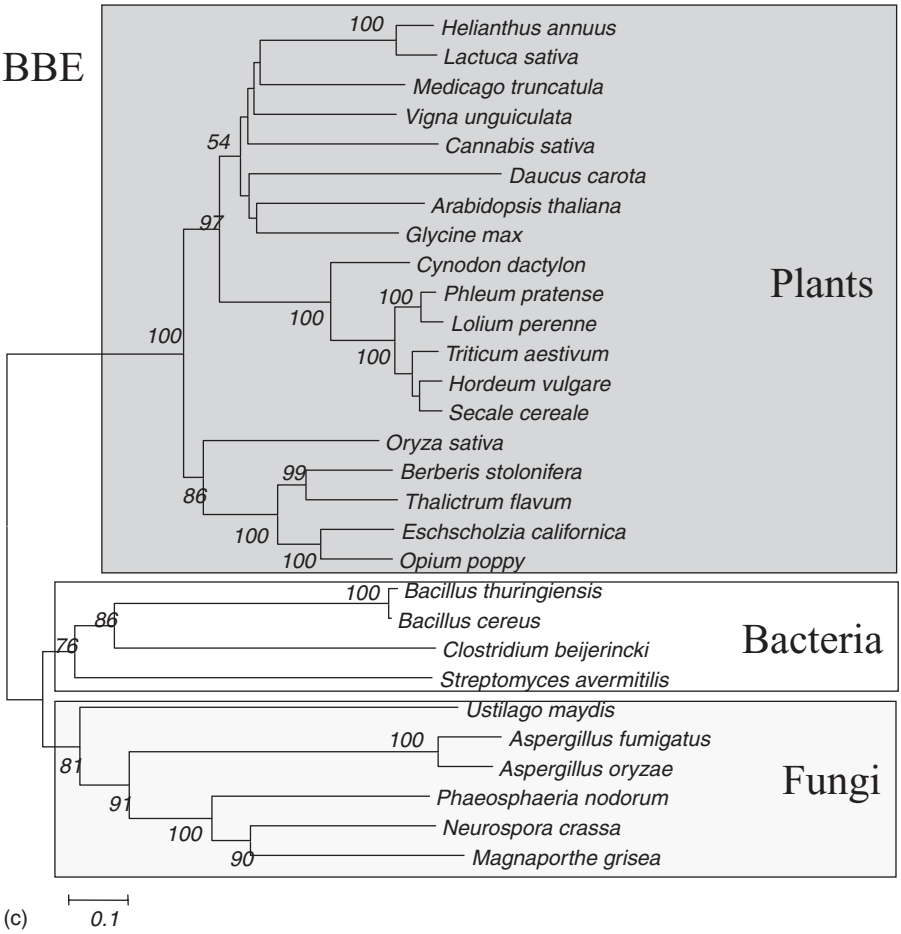


Figure 7.17 (*Continued*)

bacteria. Common conserved amino acid sites (Table 7.7) suggest a common ancestry of STS genes.

Berberine bridge enzyme catalyses are specialized step in protoberberine alkaloid biosynthesis; similar genes and proteins are, however, widely present in higher plants, indicating common ancestry (Fig. 7.17c). BBE or similar proteins, which share a number of common conserved sites (Table 7.8), could also be found in fungi and bacteria. A similar pattern can be seen in the distribution of CR (Fig. 7.17d, Table 7.9).

Whereas we know the function of these proteins in SM biosynthesis, it is not clear whether the corresponding genes (which are present) are expressed in other non-producing organisms. If they are, their functions are mostly unknown. These first analyses support hypothesis II in Fig. 7.15, suggesting

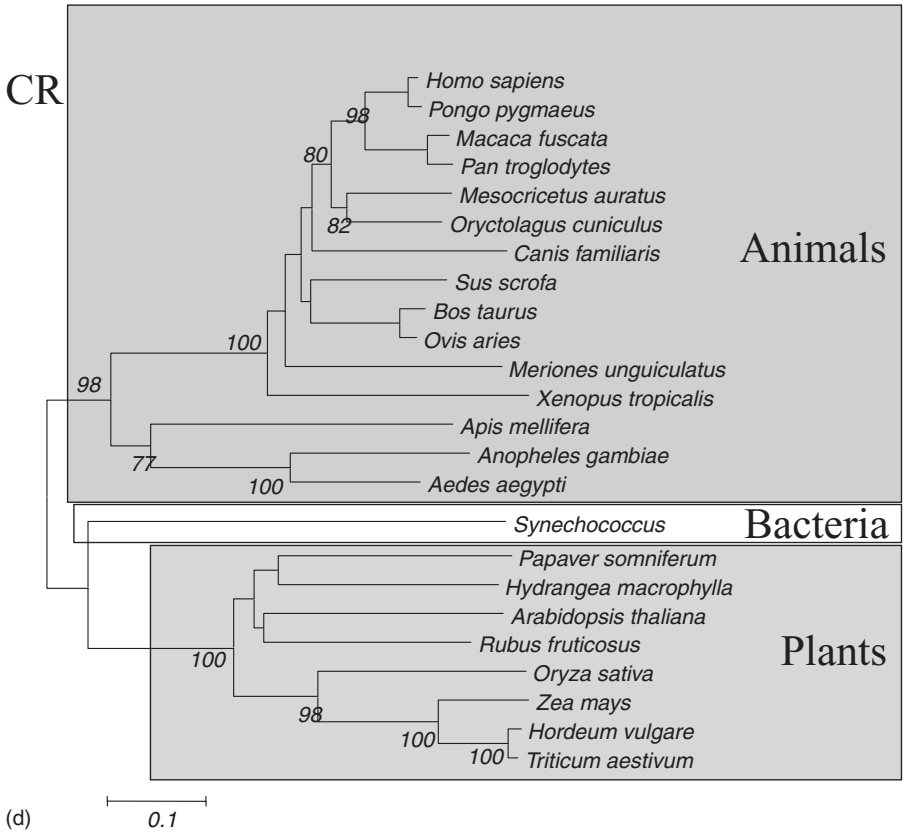


Figure 7.17 (*Continued*)

that the genes of SM have an old common root and are widely distributed in plant kingdom (Fig. 7.18), but silent or functionally inactive in several places. It would be interesting to isolate and express the corresponding genes of non-producing taxa, and to test whether the proteins can carry out the same specific catalytic reactions as seen in SM-producing taxa.

7.6.2 Contribution of horizontal gene transfer to plant secondary metabolism

7.6.2.1 Role of mitochondria and chloroplasts

Summarizing the enzyme data sets, discussed in the last chapter, we can detect a common theme: Specific enzymes of SM pathways, both at the base or more advanced steps, are present in plant species, which produce a corresponding SM. Furthermore, related proteins can be seen in many other members of the plant kingdom and, surprisingly, also in fungi and bacteria.

Table 7.6 Sequence alignment (amino acids) of chalcone synthase (CHS) of selected taxa from plants, fungi and bacteria. Conserved sites are marked by 'x'

```
Petroselinum_hortense  PANILAIGTA TPSNCVYQAD DYYFRITTDL LKFKRMCEKS MIRKRYMHIT EEYYEAPSLD ARQDLVVVEV PRLGKEAASK
Medicago_sativa        ..T....... N.A...E.ST .F..K...E. E..Q...D.. ..KR...YL. ..I.M..... ....M..... ........V.
Arabidopsis_thaliana   ..G....... N.E.H.L..E .......... E......D.. T....H..L. ..F.M..... T...I..... .K......V.
Allium_cepa            ..TV...... ..K.M...DE ........G. E......D.. ........LS .DI.M..... ....I..... .K...S..QS
Solanum_lycopersicum   ..T.M..... ......D.ST ........E. E......D.. ..N.....L. ..I.M..... ....I..... .K......Q.
Nicotiana_tabacum      ..TVM..... ......D.ST .......VE. E.......... ..K.....L. ..I.M..... ....I..... .K......Q.
Physcomitrella_patens  ..CV.G.... V.PAEFL.SE .FF.N..EA. A....I.D.. G....H.FL. ..V.ME...N V.H.I...Q. .K.AA...Q.
Pseudomonas_fluoresc.  MSTLCKPSLL F.HYKIT.QQ MIDHLEQPRM ALA...IQNT QVNE..LVLP IDEAVHTGFT H.SIVYER.A R.MSSI..RQ
Streptomyces_griseus   M.TLCRPAI. V.EHVITMQQ TLDLAREPQR DLVL.LIQNT GVQT.HLVQP I.KLAH.GFE V.NQVYEA.A KTRVP.VVRR
Prochlorococcus_marinus.LT.RGM... V.EQQ.N.S. ALAELVSPER ALVR.IQRRT QVQN.GSVLL GDDAKS.TTA ..MNEFDHHA SA.AIN.CRL
Synechococcus_sp.      ALTLHG.... V.QGSITRDE ALAEHVSGRQ ALLQ.IHQR. GVSR.GSVLI TDHSTN..TG E.MTEFQRHA AE.AF..SAT
Neurospora_crassa      GLS.TGL.VQ Y.PYSLGPDA ILSK.YHSPA K.VLAINRYT G.DQ.SSIGN PDHPNP.TVK ELHEVFMSDG VP.AV..SR.
Aspergillus_oryzae     SVS.VG.ASR CAPHKLGADE AIAR.HY.PS E.MLEINR.T R.DH..SVFS SDHPTI..FS ECDS.FKEYG IP.ASA.SAR
Conserved sites                                                                 x
Conserved sites (plant)xx    xxxx  x x x     x  x xx     xx x x xx  x  x x    x  x  xxx   x x xxx x x x   xx

Petroselinum_hortense  AIKEWGSKIT HLIFCTTSGV DMPGADYQLT KLLGLRPSVK RFMMYQQGCF AGGTVLRLAK DLAENNAGAR VLVVCSEITA
Medicago_sativa        .......... ...V...... .......... .......Y.. .Y........ .......... ......K... .......V..
Arabidopsis_thaliana   .......... .VV....... .......... .......... .L........ .......I.. ......R... ..........
Allium_cepa            ......AH.. .......... .......... .......... .......... .......... ......R... ..........
Solanum_lycopersicum   .......... .VV....... .......... .......... .L........ .....I.... ......K... ..........
Nicotiana_tabacum      .......... ..V....... ....C..... .......... .......... .......M.. ......K... ..........
Physcomitrella_patens  .......D.. .IV.A..... N.....HA.A .....K.T.. .V....T... G.AS...V.. ......K... ..A.A..V..
Pseudomonas_fluoresc.  ..ENA.DD.R MVAVTSCT.F M..SLTAH.I ND....T.TV QLPIA.L..V ..AAAINR.N .F.SLSPDNH A.I.SL.FSS
Streptomyces_griseus   .LANAE.E.D LIVYVSCT.F M..SLTAWII NSM.F..ETR QLPIA.L..A ...AAINR.H .FCVAYPDSN ..I.SC.FCS
Prochlorococcus_marinus.LE.AEDV.. ..VTVCCT.F KA..V.LA.I DQ.E.D.G.Q .THVGFM..H GALNG..V.R AF..AD.N.V ..LCSV.LCS
Synechococcus_sp.      .FS.S.EA.. ...TVCCT.F EA..V.LA.I ER....AD.Q .THVGFM..H .ALNG..V.R AF..AD.D.V ..IC.V.LCS
Neurospora_crassa      .MA.ARAQ.. .MVST.CTDS AN..Y.HYVA .E...SDRLE KVLLHGI..S G.LAA..T.A N.CLGHKP.. I..LAL.VST
Aspergillus_oryzae     ..QD..DE.. ..VAV.CTNT AH..F.SV.C RK...KCN.R .VLLHGI..G G.ISAM.V.H E.LLGSVP.. A.I.AC.VPT
Conserved sites        x       x             x                                  xx          x             x   x
Conserved sites (plant)xxxxxx  xx x    xxxxx  xxx x  x  xxxxx x xx x xxxx xxx  x  xx  xx xxxxxx xxx xx x xx xx

Petroselinum_hortense  VTFRPSDDSL VGQALFGDGA AAVILGSDLS VRPLFILPDS DGAIDGHLRE VGLTFHLLKD VPGLISKNIE KSLKEFGISD
Medicago_sativa        .......... .......... ..L.V...PE IK.I..A... E......... A......... ...IV....T .A.V..E...
Arabidopsis_thaliana   .......... ......S... ..L.V...T. .E.I...... .......... .......... .........V ...D..K...
Allium_cepa            .........M .......... ..LVV....E ...I..... E......... .......... .......... .....L..E.
Solanum_lycopersicum   .........M ........R. ..M.I...PE .....L.... E......... .......... .......... ...I..Q...
Nicotiana_tabacum      .....N.... .......... ....I...PE .....L.... E......... .......... .......... ...V..Q...
Physcomitrella_patens  ..Y...E.G. ..S....... GVYVV...PE .K......E. ........T. A..I...M.. .......... .F.N.RKSPA
Pseudomonas_fluoresc.  LCYQ.Q.HAF ISA.....AV S.CVMRA.AP GKAKTF.... EHY.KYDVKD S.FH.T.D.A .MNS.KDVAP MMEEL.EQHC
Streptomyces_griseus   LCYQ.T.G.. LSNG....AL S.AVVRGQGT G.ERNLV..T EDW.SYAV.D T.FH.Q.D.R ...TMEMLAP VL.DLVDW.V
Prochlorococcus_marinusLHLHGW.EKV .AN...A.... ..LVASQA.A EKQSLVI.NT SSLMHWQVGD H.FAMG.SPQ ..EA.ADALQ PW.SSLRSDL
Synechococcus_sp.      LHLQGG.EQV .AN...A.... ...VASAQRP IF.ALVI.G. A.LMHWRIAD H.FSMG.SPQ ..QTVAQALR PW.DDL.LTP
Neurospora_crassa      TMV.ELEETR I.I...S.C. S....SNGEA PG.AIVI... EHDLGFDVDP M.WKVV.SPR ..V.AKASLQ PTYADLSYQK
Aspergillus_oryzae     .FA.EL..VN .AMC....C. ..LV.SNGHK AERPIQFDGT EDIAHFNVHD K.YHAIID.R I.Q.TG.CVP AGFQSISNYV
Conserved sites                  xx x
Conserved sites (plant)xx xx  x   xx xxx x x      xxx     x x  x x  xxxxxxx x  xx xxx xx xxx  xxxx  x x x  x x

Petroselinum_hortense  WNSLFWIAHP GGPAILDQVE LKLGLKEEKM RATRQVLSDY GNMSSACVLF ILDEMRKKSE EGVLFGFGPG LTVETVVLHS
Medicago_sativa        Y..I...... .......... Q..A..P... N...E...E. .......... .........Q N......... ..I.....R.
Arabidopsis_thaliana   .......... .......... I......... ....H...E. .......... .....R..K D......... .........
Allium_cepa            Y.EV...... .......... A.V...P.R. ....E..KE. .......... .M.....N.K .......... ........R.
Solanum_lycopersicum   ...I...... .......... ....S..P..L .......... .......... .......A.K .......... ..........
Nicotiana_tabacum      .......... .......... ......Q..L K...K...N. .......... .......A.K .......... ..........
Physcomitrella_patens  ..EM..AV.. .......... A..K.TKD.. QGS.DI..EF ......S.... V..QI.HR.K M.FFI..... ..L.VL..RA
Pseudomonas_fluoresc.  AQND.F.F.T ..RK...ELV .Q.D.EPGRV AQS.DS..EA ..IA.VV.FD V.KRQFDSGP A.M.AA.... F.A.MA.GKW
Streptomyces_griseus   P.MD.F.V.A ...R...DLC HF.D.PP.MF .YS.AT.TER ..IA.SV.FD A.ARLFDDGA A.LIA..... I.A.VA.GSW
Prochlorococcus_marinusTEIRS.AL.. ...R..QACA DA.A..H.HL AES.SI.QAH ......T... ..ER..H.AS GCLALA.... .CA.VALFDL
Synechococcus_sp.      ASISS.AM.. ...RV.SACG EV.E.SP.QL QTS.A..H.H .....T.... ..QRL.RSAP GCLALA.... .CA.VALFRL
Neurospora_crassa      PADFD.AM.. ..AT..SGA. SAM..TP.H. ..SYDRYINH ..S...TIFS V.NRL.E.DD AV.GCA.... IN..MCM.KR
Aspergillus_oryzae     PSNYG.AV.. ..Y.V.VAAQ DA...TADDL ..SYDAYR.G ..TI.TTIIR ..EKL.DEHH GL...AAI.H. I.L..AI.TR
Conserved sites              x  xx   x        x                    xx  x                          x x    x
Conserved sites (plant) x  xx  xx xxxxxxxxxx  x  x  x      x  x    xxxxxx xxx   x  x  x   x   xxxxx xx x  xx
```

This would favour hypothesis II in Fig. 7.15. Even animals share some of the proteins (TDC, ODC, TyrDC, STS and CR). These observations indicate that the proteins very likely evolved in prokaryotes and were transferred into eucaryotes via either protobacteria (Fig. 7.18) or cyanobacteria, the progenitors of mitochondria or chloroplasts, respectively. A number of SM (e.g. many terpenoids, QAs, the piperidine alkaloid coniine) are produced completely or partly in chloroplasts and/or mitochondria (see Chapter 1). The corresponding genes are mostly nuclear today. It is tempting to speculate that these localizations are indirect indicators of a former bacterial origin of the corresponding pathways. The introduction of bacterial genomes into eukaryotes was

Table 7.7 Sequence alignment (amino acids) of strictosidine synthase (STS) of selected taxa from plants, animals and bacteria. Conserved sites are marked by 'x'; species with a functional STR are in bold

```
Ophiorrhiza_pumila      AMVVSILCAL FLSSSLSFFE FIEAPSYGPN AAFDSDGLYA SVEDGRIIKY DPSNLKPLCG RVYDFGFHYE TRLYIADCYF
Rauvolfia_serpentina    T.ALFTVFL. ....LA.KEI L......A.. ST...TNF.T ..Q...V... E.NSKR.... .T..ISYNLQ NQ...V...Y
Rauvolfia_mannii        T.ALFTVFL. ....LA.KEI L......A.. ST...TNF.T ..Q...V... E.NSKR.... .T..ISYNLQ NQ...V...Y
Medicago_truncatula     MV.SV..L.I ..LCPSVNKL QLPP.LT..E S...RN.P.V TSS....F.. VSNEVQAI.. .PLGL..NHQ .D..V..A..
Arabidopsis_thaliana    LAKIFLVF.I YCAIPFHSEI RFLNEVQ..E S...PQ.P.T G.A....LFW NGTRKEDI.. .PLGLR.DKK ND.....A.L
Lycop._esculentum       ILLLI.VVQ. VSVNAFKSKI IHLNG.I..E S...PN.P.I G.A....L.L QG..KEHI.. .PLGLR.DTK .E.....A.L
Oryza_sativa            HLFFAA.ALA L.LTPFLGRL EFVGEVF..E SE..RH.P.. GLA...VVRW MEDAEERR.. .PLGLR..G. .E..V..A.Y
Oryza_sativa_AAR87254   VA.AVAAL.A .CGTP.TAEI RFRGEVQ..E S...PL.P.T G.A...VVRW .GARKEHI.. .ALGLR.DRR .D.....A..
Brassica_napus          VLCIIA.SVV .IAIPFMGKL EFVDRVF..E SE..GL.P.T GLA...VVRW MEAVKEK... .PLGLR.VK. .N.....A.Y
Triticum_aestivum       HLFIAA.ALA LVLMPFLGRL EFVNEVF..E SE..RQ.P.. GLA...VVRW MDKAGEQW.. .PLGLR..R. .E.F...A.Y
Zea_mays                HLFFAA.ALA L.VAPFLGRL EFVGEVF..E SE..LQ.P.. GLA...VVRW MEEAEEEF.. .PLGLR..G. .E..V..A.Y
Danio_rerio             SGK.FRVTL. TMVAL.LAER LF.ERLV..E SLANIGDF.T GTA..K.V.I ERNIEEHT.. .PLGIRVGPN GT.FV..A.L
Homo_sapiens_BSCv       SGR.FRVTF. M.AVTVLAER LF.NQLV..E SIAHIGDMFT GTA...VV.L EGEIDE.V.. .PLGIRAGPN GT.FV..A.K
Marinobacter_aquaeolei  WLL..L.VVF L.LGF.LADL LARGEV...E DTTIGPD..S GTQ..W.VRV HDGTHLETG. .PLGLV.DSN GN.IV..AWK
Pseudomonas_aeruginosa  KLSGI.VLL. AGAAY.LAEL LGQGQLH..E DTAVDSQV.. GLA...VVRL .SGKTVDTG. .PLGMD.DAA GN.IL..AWK
Conserved sites                                    x                xx              x x            x   x
Conserved sites (plants)                           x    xx     x    xxx             xx x            xx  xx

Ophiorrhiza_pumila      GLGFVGPDGG HAIQLATSGE FKWLYALAID QQFVYVTDVS TKYDDRGVQD RINDTTGRLI KYDPSTEEVT VLMKGLNIPG
Rauvolfia_serpentina    H.SV..SE.. ..T.....VP ......VTV. .RI..F.... .L.......Q DTS.K..... ......K.T. L.L.E.HV..
Rauvolfia_mannii        H.SV..SE.. ..T.....VP ......VTV. .RI..F.... .L.......Q DTS.K..... ......K.T. L.L.E.HV..
Medicago_truncatula     ..VK...N.. N.T..VGPTS TMFADG.DV. PDI..F..A. .N.KLKDF.T ASG.NS...L R.....NQT. ..LRN.T..S
Arabidopsis_thaliana    .IMK...E.. L.TS.TNEAP LRFTND.D.. DEN..F..S. SFFQR.KFML VSGEDS..VL ..N.K.K.T. T.VRN.QF.N
Lycop._esculentum       ..QV...K.. L.TP.VQKFP LVFTNDVD.. DDVI.F..T. ...QRWQFLT SSG......M ...K..KK.. ..LGD.AFAN
Oryza_sativa            ..MS...N.. V.TS..REVP VNFAND.D.H RNS.FF..T. .R.NRKDHLN LEGEG....L R...E.KAAH .VLS..VF.N
Oryza_sativa_AAR87254   ..LK...... L.TP...EAR .NFTND.DL. DDN..F..S. IH.QR.HFMQ FSG.PS...L ....N.KKA. ..HRNIQF.N
Brassica_napus          ..LV...E.. V.TP...HVP ILFAND.D.H RNSIFF..T. KR..RANHFF LEGES....L R...P.KTTH IVQE..AF.N
Triticum_aestivum       ..MA..ES.. V.TS..REAP VHFAND.D.H MNSIFF..T. .R.SRKDHLN LEGEG....L R..RE.GA.H .VLN..VF.N
Zea_mays                ..MV..QS.. V.SSV.REAP IRFAND.DVH RNS.FF..T. MR.SRKDHLN LEGEG....L R...E.SG.H .VL...VF.N
Danio_rerio             ..FE.N.VT. EVKS.VSTER LGFVND.DVT .DK..F..S. SRWQR.DFMH L.MTAD..VL E..TE.K..N .M.EN.RF.N
Homo_sapiens_BSCv       ..FE.N.WKR EVKL.LS.EN MSFVND.TVT .DKI.F..S. S.WQR.DYLL LVMTDD...L E..TV.R..K ..LDQ.RF.N
Marinobacter_aquaeolei  ..LSIT.Q.D ITVLTREAEP .RFTDDVV.A PDRI.F..A. SRFQQPDYVL DLLRPH...L R.N.K.RKTE ..LGN.HFAN
Pseudomonas_aeruginosa  ..LRID.Q.K VETLATEADP .AFTDD.D.A SDRI.F..A. S.FHQPDYIL DLLRPH...L R.....GKTE ..L.D.YFAN
Conserved sites          x                                    xx x                xx    x   x
Conserved sites (plants)x  xx  xx  x  x                       xx x                xx    x   x

Ophiorrhiza_pumila      GTEVSKDGSF VLVGEFASHR ILKYWLKGPK ANTSEFLLKV RGPGNIKRTK DGDFWVASSD NGITVTRGIR FDEPGNILEV
Rauvolfia_serpentina    .A...A.S.. ...A..L..Q .V....E... KG.A.V.V.I PN......NA ..H...S..E .HGR.DK..K ..........
Rauvolfia_mannii        .A...A.S.. ...A..L..Q .V....E... KG.A.V.V.I PN......NA ..H...S..E .HGR.DK..K ..........
Medicago_truncatula     .VA...E.... ...S.YLAN. .QRV.....R ..S..LFMLL A..D....NS G.Q..ISV.S S.CSTLS.V. VN.N.LV.QI
Arabidopsis_thaliana    .LSLG..... FIFC.GSIG. LR......E. .G...VVALL H..D..RTN. .......VHC Q.GWPHVAVK YS.E.KV.K.
Lycop._esculentum       .VAL..NK.. ...T.TTNF. ..R......L VG.HDVFVEL P..D..RINP K......LQA GDGELHTALK LS.D.RV...
Oryza_sativa            .VQI.D.QQ. L.FS.TTNC. .MR...E..R .GQV.VFADL P..D.VRLSS G.R....IDC MSMRMHLVAL L.GE.DVV..
Oryza_sativa_AAR87254   .VSM....L. FVFC.GSRG. LSR.....E. .G.VDLFAIL P..D.VRTND K.E....IHC Q.GKLHLI.K YNPE.EV.DI
Brassica_napus          .IQL...Q.. L.FT.TTNC. LV....E.A. TGEV.VVVDL P..D.VRMN. K.E....IDC A.MKMYVIS. ..AD.EV...
Triticum_aestivum       .VQI.Q.QQ. L.FS.TTNC. .MR...E..R .GQV.VFANL P..D.VRLNS K.Q....IDC MSMKMYLLAL L.GE..VV..
Zea_mays                .VQI.E.HQ. L.FS.TTNC. .MR...E..R .GEV.VFANL P..D.VRSNG R.Q....IDC KARRMHVLAL L.GE.RVV..
Danio_rerio             .IQLFP.EES ...A.TTMA. .KRVHVS.LN KGGMDTFIEL P..D..R.SS S.GY...M.A KVPRYSLVVE LQSD.TCVRS
Homo_sapiens_BSCv       .VQL.PAED. ...A.TTMA. .RRVYVS.LM KGGADLFVEM P..D..RPSS S.GY...GM.T KVPRYSLVLE LSDS.AFRRS
Marinobacter_aquaeolei  .VA..PQ.DY ...N.TWKY. ..R..IS... .GRA.VFADL P..D.LAVDG G.RY...FPT KPQNYGLVVA ..RK.RM.TS
Pseudomonas_aeruginosa  .VAL.ANED. ...N.TYRY. .TR.....E. .GQH.VFIDL P..D.LQGDR K.T....LPT KPTAYGLV.A I..Q.K.VRS
Conserved sites         x             x                                x x       x  xx                    x
Conserved sites (plant)x             x          xx x                  x x       x  xx                    x
```

an early and major event of horizontal gene transfer (HGT). The introduced genomes were apparently further modified in the host organisms by duplication and mutation. The novel metabolites formed from these new pathway genes were probably further transformed by endogenous plant enzymes.

7.6.2.2 Contribution from endophytes and ectomycorrhizal fungi

The distribution of particular SM in unrelated plant families can also be due to endophytes and ectomycorrhizal fungi, which may produce the SM themselves or have introduced the pathway genes to their host plants (this would be another example of HGT (Fig. 7.18)).

Mycorrhizal associations with vascular plants apparently evolved about 400 million years ago and are present in about 80% of our plants (Simon *et al.*, 1993; Remy *et al.*, 1994). Ecto- and endomycorrhiza are common associations with roots; they enlarge the root surface area and thus help to catch more

Table 7.8 Sequence alignment (amino acids) of berberine bridge enzyme (BBE) of selected taxa from plants, fungi and bacteria. Conserved sites are marked by ‘x’

```
Berberis_stolonifera     SNGVSNFTAL SSSDSYHRLL YVSMQNQIFT RPPRPSMIIL PQSKEELAAS VVCSNRGLWT IRLRSGGHSY EGLSYVFVVI
Eschscholzia_californica F...R.H.VF .D...FN.F. HL.I..PL.Q NSSK..A... .G.....SNT IR.IRK.S.. .......... .....T.IL.
Opium_poppy              .H..H...T. .DTN..FK.. HA....PL.A K.SK..F.VM .G.....SST .H.CT.ES.. .......... .....T..IV
Thalictrum_flavum        IH..H.Y.TH QT.N...... .......... .S.Q.RV... .E.MDQ..NV IS.CT..S.. .......... ....HI..I.
Arabidopsis_thaliana     VYFPLEK.FF ATKNVFSQV. ESTA..LR.L KK.K.GF.FS .IHESHVQ.. II..KKLRMH L.V.....D. .......ILM
Glycine_max              G.SEYIEKIT FT.S..PQVW DSLA..PRWV NIRK.L..LT .FHES.IQ.A IL..KELKLQ L.V.....D. .....L..MV
Vigna_unguiculata        NTNSISSVLY TTNS..FSV. DAT...LR.S DSRK.LV.VT ..VVSHIQ.T IK..Q.HGLQ ..T.....D. ........IL
Hordeum_vulgare          CLVKEIPAR. LAKS.FPAV. EQTIR.SRWS S.VK.LY..T .TNTSHIQSA ...GR.HGVR L.V.....D. .....R.A.V
Helianthus_annuus        PSFPITGEVY TGNS.FPTV. QNYIR.LR.N ET.K.FL..T AEHVSHIQ.A ...GKQNRLL LKT.....D. .....L.FIV
Daucus_carota            NSESISQVVF TANA..NPI. QLNL..LR.N TSRK.LA.VT .IEETQIQTV IY.ARKNSMN V.T.G...DF ..V..T..LL
Lactuca_sativa           PSFPLSGQLY TDNS.FPSV. QAYIR.LR.N ES.K.IL..T ALHPSHIQ.A ...AKTHRLL MKT.....D. .......F.V
Streptomyces_avermitilis VAANWTAL.R DDGALPGDRS WPAAHQLYN. .FLK.AAVAY VAHADD.RTT MAYARAHGVK VAI.N..... A.W.SGRLIL
Bacillus_thuringiensis   MERSKKLKQT KTGRIPTDPD .NVARMNLNL SIKL.CI.VF C.NNKDICNA LKWARERHIP F.....R... .NF.LLGLI.
Ustilago_maydis          NSTSLDQCLS TGGELSTSSN .TALSSSYNP LFYK.LV.VE .GTSDQV..I .K.VSAQNQK LTPK...... TAY.LGS...
Aspergillus_oryzae       RDCLVSALGG NGLVANQP.Y QTTAVHEYNL NIVT.AA.TY .ETA.QI..V .K.ASQYDYK VQA......F GNYGLGA..V
Conserved sites                                                x                                 x x
Conserved sites (plant)                             x         x  x                x           x xxx   xx x  x

Berberis_stolonifera     DLMNLNSIDL ESKTAWVESG ATLGEIYCAI SEDTLGFSGG YCPTVGSGGH ISGGGFGMMS RKYGLAADNV IDALIVDANG
Eschscholzia_californica .......... ..E....... S....L.Y.. T.SK...TA. W.....T... .......... .......... V..ILI....
Opium_poppy              .M.......V L.E....... .....L.Y.. AQ.....TA. W......... .......... .......... V..ILI.S..
Thalictrum_flavum        .......... DTQ....... .......H.. GKG.MA..A. ....G..... .AP....... .......... V...L.....
Arabidopsis_thaliana     ..SKMRN.NI QDNS...Q.. ..V..L.YR. A.KVH..PA. L.SSL.I... .T..AY.S.M .....G.... L..K......
Glycine_max              ..I.IRE.N. ADE....QA. .SI..L.YK. .KKVH..PA. T..S..I... .....Q.L.L ..H....... V..YLI....
Vigna_unguiculata        ..L.FRKV.V .NR....QV. .....L.YT. .QK....PA. V.YS..A... .....Y.FLM .......... ...H.I.V..
Hordeum_vulgare          ..NKMRLVNE KAR....D.. .Q...L.Y.. AKPV.A.PA. V..SI.V..N FA......LL ....I..E.. ..VKL.....
Helianthus_annuus        .MF..RNV.I .QE....QA. .....V.YR. A.NKH..PA. V.....V... F....Y.NLM .....SV..I V..Q.I.V..
Daucus_carota            .MI.F.N... KTS....Q.. IS...F.YR. .Q.V.A.PA. LLSS..LT.L LG...Y..LK ...A.....T L..R...Y..
Lactuca_sativa           .MF..RNVSI .DE....QA. .....V.YR. A.NSHA.PA. V.....V... F....Y.NLM G....SV..I V..QLI.V..
Streptomyces_avermitilis .VSK..KTRA SGG..V.GA. SK.IDV.R.L AAKGVTIPA. S.....VS.L TL...H.VV. .A...TC.SL TQ.TLIT.D.
Bacillus_thuringiensis   .VSEMHTVNT .KL..TI.A. .N..TV.KEL WNYGVTIPA. TSAS..IV.L AL...I..L. .LF..KC.QL MEVEM.Q.C.
Ustilago_maydis          ..RQ.D.V.R DA...S.GA. VR..SLAQQ. WDGNFALPH. T..Y..VS.. AL.....YAT .AW.FLL.RI VEMQF..I..
Aspergillus_oryzae       .MKYF..M.D QTYE.VIGP. T...DVDVEL YNGKRAMAH. V...IST... FTM..L.PTA .QW...L.H. EEVEV.L..S
Conserved sites          x              x    x                     x         x     x
Conserved sites (plant)  x             xxxx  x    xx x  x       x  x      x  x     x       xx      x   x    x xx

Berberis_stolonifera     AVLDRSMGED VFWAIRGGGG VWGAIYAWKL QLLVKQVTVF KLMKFDNMLH KWQVVAPALE DDFTLSVLAG ADSFLGLYLG
Eschscholzia_californica .I...Q.... .......... .........I K...EK.... RVT.NVAL.. ...F..EE.. E.......G. ..TM..FHF.
Opium_poppy              .I...E..D. .......... .........I K...EKL... RVT.NVGL.. ...Y..DE.D E...V...G. VNM....H..
Thalictrum_flavum        V....E.... .......... ....V..... ..V..N..I. R...HSEL.. ...L...K.. ...S.A.... TNT.......
Arabidopsis_thaliana     KL...A.... T.......A. SF.I.L...I K.V..T.... TVT.TLQIIS ...R..DK.V EELFIR..FN VA.YNA.F..
Glycine_max              KI...K.... ........DA SF.V.L...I K.V.PI..G. NVPRTPELI. R..YI.HD.H E.LVIR.I.Q IST.NSIF..
Vigna_unguiculata        NL...K.... L......... SF.V.VS..I K.V.ST.... NVERILEIIE ...L..NK.D ERIF.RMDLA RAN.VAMFQ.
Hordeum_vulgare          KL..K..SP. H...V..... SF.IVVS.QV K...PT.... QIP.TVQLIN ...L.....P G.IMIRII.M G.T.EAM...
Helianthus_annuus        KL...K.... L....T.... SF.VVL.Y.I K.V.EV.... TIERREEIAE R.VQ..DK.D R.LF.RMTFS VII.PT....
Daucus_carota            KI...K.... L.......DP SFCVVLEL.. ..V..S..Y. AVQRTLELFQ ...AT.APRD L.VRVV.DTI TSV.QC....
Lactuca_sativa           KL.N.K.... L....T.... SF.VVV.Y.I K.V.TT.... NVQRTSEIA. R.IQ..DK.D N.LF.RMTFN VIL.PT....
Streptomyces_avermitilis L.ANATENK. L...L..A.N NF.VVTELHF KTHAP.GVSA YMTWWSKAAA VVKAWQEDQP .EIWS.CHLA NA.VAAFS..
Bacillus_thuringiensis   IRANEQENSN L...C..... NF.I.TSLTF RVHP.N.SI. SITWWEDFIA AF.AWQNYID ERL.S.IELF .KEAQ.EFV.
Ustilago_maydis          RSVTHNSEH. LW..L..A.S NF.IVTQFTF S.QAT.IQNY AYSYTNEIVA LQEMTLS.SG FEPNFGGELL VVQLS.QH.L
Aspergillus_oryzae       VRASNTQNQE ..F.VK.AAA SF.IVTEF.V RTQAGIAVQY SYTFLGSKAQ FIKDWQS.KN LTRQFYTNMV IFILE..FF.
Conserved sites                           x      x
Conserved sites (plants)   x   x  x  xxx  xx     x         x x   x x             x   x  x                      x

Berberis_stolonifera     PKEISSVDQN FPELFAHLAG LNSVEMNNDD RAFKTKVDFV KEPIPLEGIK GALTLTRGMA FNGQGGLMSR ISSDSTPFPH
Eschscholzia_californica L.TK.TF.LL ......Y... .ET.SL...E ........LT ...L.SKAFY .L.E.SN.I. L..F..Q..K ....F.....
Opium_poppy              R.DKTII.EK ....M.F.S. .DTISL...E .........T .VSV..NVFR H..E.SG.I. L..F..K..E ..T.F.....
Thalictrum_flavum        ...S..MHKK ....T.E... .K..SLKD.. .........P ..A......Q ...E.K...V M.....M.D. ..T.AS....
Arabidopsis_thaliana     G.GMNVMKKS ....I.YIS. FPTHTLLQPK VS..A.S... .T...ES.LQ .IFK.LIP.I W.PY..M.AK .PESQI....
Glycine_max              GVD.PLMNES ....VMFI.. Y.IEDLL.FK .S..A.S..F ...V.KS.LE ..WK.LIALI MEPY..R.NE ..ESEI....
Vigna_unguiculata        GV..PLMQK. ....AVFTN. ALIGSLL.RS GKY.G.S.Y. RK...VD.LR .LWR.NVQLQ .APY..K.DN ..ESEI..A.
Hordeum_vulgare          TCKTPLMSSK ....IPFIHL GKQATLL.FK PFAEY.S.Y. YQ.V.KPVWE QLFGWLGA.V MDPY.ATI.A TPEAA.....
Helianthus_annuus        NSRVTLLNKD ....VLYYT. FP.GTLLSRL NP..I.S.Y. QN..SKRQFE FIFEMKLEL. ..PY..R..E ..EFAK....
Daucus_carota            KIDLPIMQKY ....APMFS. FPVGTLL.PR NSV.I.SS.T TQ..S...LN .IWDWLVQIQ YTPF..I.NE FAESAL....
Lactuca_sativa           NSTVALLNKD ....VLFYTN FPIGTLLSRL NP..I.S.Y. .NT.SKQ.FE SIFEMKLEL. ..PY..R..E ..EFAK....
Streptomyces_avermitilis TYGQNA..RL ADKIAGCSSF PTDAQGRSGR ETYAARS..F DRS.SSA.TL LNQMAVA.I. LTAL..AVN. V.PTA.A.V.
Bacillus_thuringiensis   SPSH.LLSPL LETGSPS.FI EEVPYGG.IP EN..RSGSY. YK....K.LM QYFLHADAIW HQSLI.AVEN ..PNE.AYF.
Ustilago_maydis          ASRHDALMHS .HSKSVKEFT SWIESDVSHE QFYAKSLVQP STCTYDYELD AYAGQGNSDF LGPLSYQSGT A.FNAHNASF
Aspergillus_oryzae       S..QYEALRL EERFLVLTDW .GM.GILRPT WFYAKSLG.T PDTLIPSSEF FKYINNTSVT LSLE..AIND VPA.A.AYG.
Conserved sites (plants)            xxxx                                                       x             xx x

Berberis_stolonifera     RKGMMEYIVA DRDDAKSIGW LHGFYNYMGQ FLPSAYVNDL DLGRLDWGEK YFLSNYERLV RAKTLIDPKN VFHHPQSI
Eschscholzia_californica .S..V..... NQSQK.KLD. .EKV.EF.KP .VSKG..... ...GI....S .........I ........N. ..N.....
Opium_poppy              ....F...I. NQ.ES.ISE. .AK..D.LEP .VSKG..... .I.GI....R ..S....... K.......N. ..N.....
Thalictrum_flavum        .S..V..... .KH.LH..H. ..QLFD...K .VSNG..... ....I..... ..M...D... ....M..... ..N.....
Arabidopsis_thaliana     ...KVQ.VTS LDS.KRP.N. IRDL.S..TP YVS....... ....NT..AN ..KN.FN..M MI.AKV..E. F.R.E...
Glycine_max              ...NLQ.L.N EVN.EA.LQ. AKMV.K..TP YVSK..F... ...KNK..K. ..KG.FR..A QI..KF..L. F.RNE...
Vigna_unguiculata        .S.HIH.V.V QEE.EATVN. IRRL.K..EP YVSN...... .I.VNN..L. ..SN.FK..A TV..KV..H. F.RNE...
Hordeum_vulgare          ...NIQ.VNY FAEAGAALQ. SKDI.KF.EP .VSK..A..I ....NE.... ..KG.FQ..A IT.GKV..QD Y.RNE...
Helianthus_annuus        .S.KIQ.E.N EDL.EAELNF TRLM.D..TP .VSK.FL... .I.INSY.H. ..KE..K... SV..KV..D. F.RNE...
Daucus_carota            .P..INMA.T AQNEEATLQ. INDLFK.YAP YVTKS....A ...IGS..K. .YKN.FD... KI.SIV..L. F.N.K...
Lactuca_sativa           .S.KIQ.E.N .ELVEAALNF TRVM.D..TP .VSK.FL... .I.VNSY.H. ..KE..K..T MV..RV..S. F.RNE...
Streptomyces_avermitilis .RSR.LAQYI SWRGTSGQS. .T.AHAA.QR HASG..Q.YT .PTLTN.RKA .YGDAAT..T .L.HQY..NG F.TY..GL
Bacillus_thuringiensis   ..AIIAQEYI SWK.DEE.R. VKDLRESLDP YTLGD...WP .IDIKN.QTS .YG..FH..R KV..MY..CD ...FQ...
Ustilago_maydis          VNQFYS.GFP SNHPDAQYNA FDDLVQTAKN SS.D....YV .ARLH..P.Q .YGNALA..K NL..KW..ND ..WF..GL
Aspergillus_oryzae       .DVLFWVQIF VSPGPV.YDF AD.L..VLTK AV.E..LGCP .PKMANAQQ. .WRQ.LP..E EL.ETL...D T..N..G.
Conserved sites                                                      x          x      xx        xx        x
Conserved sites (plants) x                                       xx  x x    x   x   x  xx    x   xx        xxx
```

Table 7.9 Sequence alignment (amino acids) of codeinone (CR) of selected taxa from plants, fungi and bacteria. Conserved sites are marked by 'x'

```
Papaver_somniferum    SNGVPMITLS MPALGMGTAT EREKLAFLKA IEVGYRHFDT AAAYQSEECL GEAIAEALQL GLIKRDELFI TSKLWCADAH
Hydrangea_macrophylla AFTI.EVP.. ..V..L...P .TVRK.VTE. LKL....... ..L.N..QP. .D......GE .......... ......S...
Arabidopsis_thaliana  LTT..TLAIR ..V..F...P TML.ETVIE. .KL....... SPR..T..PI ...L...VS. ..VR.S.F.V .T........
strawberry            ATQI.EVV.E ..V..F...P .VLIE.V.E. .KL....... .SI.G..QT. .V...Q..K. ..VA..... .....PN.G.
Hordeum_vulgare       MGAGDRTVAG ..RI.....P DPIRR.V.R. ..I....... ..H.ET.API ...A...VRS .AVA..D.. ......S...
Triticum_aestivum     MGAGDRTAAG ..RI.....P DPIRR.V.R. .......... ..H.ET.API ...A...VRS .AVA..D.. ......S...
Zea_mays              MSATGRAPCG L.RV.L...P DPVRA.V.R. .QL....... ..H.AT.API ...A...VRT ..VA.ED..V ...V......
Oryza_sativa          MMPEAALSSG ..RV.....P STV.DVV.R. ..A....... ..V..T.AI. .D.V...VRA ..VA....Y. .....V.H..
Synechococcus         KDVI.TMQYA ..L..L..GS RQVYKSVRE. .KI....I.C .SI.GN..EV .D..RD.I.N HE.T.S..W. .....SNCHG
Aedes_aegypti         AAKI.NAIF. I.MI.L..WP GVVTQ.VKD. ..I....I.C .HV..N.HEV .DG..AKI.D .T...EDI.V .....NTFHR
Pan_troglodytes       DPKYQRVE.N ..V..F..YR N.AVEVTKL. ..A.F..I.S .YL.NN..QV .L..RSKIAD .SV..EDI.Y ......TFFQ
Conserved sites                  x  x xx            x x  x xx x     x  x    x             x      x x x
Conserved sites (plants)         x  x xxx           x x  xxxxxxx    x  x    x x x x    x   x      x x x    x

Papaver_somniferum    ADLVLPALQN SLRNLKLEYL DLYLIHHPVS LKPGKVNEIP KDLPMDYKSV WAAMEECQTL GFTRAIGVSN FSCKKLQELM
Hydrangea_macrophylla REN.E....K T.K......I .M....W... S...NEYP.K .E.Q...... .E......K. .L.K..... ......SDVL
Arabidopsis_thaliana  GG..V..IKR ..K....D.. ...I..W... S....KFP.D E.M...FEV. .SE.....R. .LAKC..... .......HIL
strawberry            PN..I...KK ..Q..E.... ......W.I. A....SHALE EKM...F.G. ..D...A.R. .L.KS..I.. ..T..T.N.L
Hordeum_vulgare       G.R.V...RH T....QM..V ....V.W... M...RKAPFT AEV.F.MRA. .E.....HR. .LAK....A. ......DT.L
Triticum_aestivum     R.R.V...RQ T....QM..V ....V.W... M...RKAPFT A.V.F.MRA. .E.....HR. .LAK....A. ......DT.L
Zea_mays              R.R.....RR T.S..QM..V ...MV.W..T M.A.RTAPFT PEE.F.MRA. .E.....HR. .LAK....C. ......ET.L
Oryza_sativa          PGH...S.RR A..KMQM... ......F... MRLAESMTYS ..VM..MEG. .KE.....R. .L.K..... ......ET.L
Synechococcus         KNH.EA..SQ ..Q..GVD.. N.....W..G IR.E.TFAES V..TPEISET .E...STRDK .L.KH..... .TV....Q.V
Aedes_aegypti         P...EG..KV T.K....A.. ......W..A Y.E.DPMGPD GKFIFS.VDT .KE..KLVDA .LVKN..L.. .NS.QI.RVL
Pan_troglodytes       PQM.Q...ES ..KK.Q.D.V ....L.F.MA .Q..EPKDEN GKVIF.LCAI .EV..K.KDA .LAKS..... .NRRQ.EMIL
Conserved sites                  x      x    x  x x                          x  xx      x    xx  x x
Conserved sites (plants)  x      x      x  x x  x x                 x      x  xxx   x x    xx  x xx xx

Papaver_somniferum    AAAKIPPVVN QVEMSPTLHQ KNLREYCKAN NIMITAHSVL GAIGPWGSAV MDSKVLHQIA VARGKSVAQV SMRWVYQQGA
Hydrangea_macrophylla .N..V..A.. ...VN.CWQ. .Q.T.F..S. G.LVV.YAA. ..V.FY.TR. .G.E..NE.. RI..NT.... CL..A.E..I
Arabidopsis_thaliana  SI.T...S.. ......IWQ. RK...L.RS. D.VV..Y... .SR.F..TKI .E.D..KE.. E.KE.T.... ....A.E..V
strawberry            SF.T...S.. ......FWQ. .K..DF...S G.VV..F.P. ....S..TH. LE....NE.. E.H..T.... CI.....V..
Hordeum_vulgare       SF.T...T.. ...VN.VWQ. RK...F.RGK G.QLC.Y.P. ..K.H..... ..AG..QD.. AS........ CL....E..D
Triticum_aestivum     SF.T...T.. ...VN.VWQ. RK...F.RGK G.QLC.Y.P. ..K.HR.... ..AG..QE.. AS........ CL....E..D
Zea_mays              SF.T..... ...IN.VWQ. RK...F.R.K G.QLC.Y.P. ..K.H...S. ...G...E.. KSK..T.... CL....E..D
Oryza_sativa          SF.T.S.AA. ...VH.YCR. NK...F..EK G.QLC.Y.P. .GK...SN.. ..CPL.K... ME...TI.... CL....E..D
Synechococcus         SHC.QK.E.. ...HH.L.Q. PT.I...ASE E.L...Y.P. .SMD.QDA.. L.HP.IRA.. ET..C.P.... VLA.DV.R.I
Aedes_aegypti         DV.R.K..C. .I.NHAY... SK.TAF.REK G.IV..Y.P. .SPARPDIVL LHDPI.KT.. DKH..EP..I LI.YQI.L.H
Pan_troglodytes       NKPGLK..C. ...CH.Y.N. SK.LDF..SK D.VLV...A. .TQRELSPVL LEDP..CAL. KKHKRTP.LI AL.YQL.R.V
Conserved sites             x  x x x      x   x   x     x  x x                  x        x          x
Conserved sites (plants)x x x  x xxx  x   x   x   x     x   x   x x               x  xx        xxx   xx x xx

Papaver_somniferum    SLVVKSFNEG RMKENLKIFD WELTAEDMEK ISEIPQSRTS SAFLLSPTGK EEEFW
Hydrangea_macrophylla GVL.....KE ..EQ..Q..N .T.SDDESK. ......G.AC LGDYT.VH.. I..L.
Arabidopsis_thaliana  .M.....TKE .LE....... .S..EDETQR ......F.NV HGVYT.KK.. VA.M.
strawberry            T.A...Y.KE .L.Q.VQV.. ....E..L.. .NQ...RKMM PRE.VTA... LDDL.
Hordeum_vulgare       C.I....D.A ..R...DVDG ....E.ERRR .A....RKIN LGRYV.DH.. L..L.
Triticum_aestivum     C.I....D.A ..R...DVDG ....E.ERRR .A....RKIN LGRYV.EH.. L..L.
Zea_mays              C.I....D.. ......D.V. ...SE.ERQR ..K...RKIN QGRYV.EH.. F..L.
Oryza_sativa          CVI.....KS .LR...G... ....ND.RH. ..TL.EW.GT LDIFVHK... VD...
Synechococcus         .AIP..VKPS .LL...QAA. IQ.SNTELQ. .ELDQNF.LV NG.WVMEG.T LQSL.
Aedes_aegypti         VVIP..VTKS .IAS.FDV.N F..D.D..KQ LALERNE.IC PEGAFGHPHH F.KEE
Pan_troglodytes       VVLA..Y..Q .IR..VQV.E FQ..S...KV LDLNRNY.YV VM..MDHPDY FSDEY
Conserved sites          xx     x   x        x
Conserved sites (plants) xxx    x   x      x x        x   x             xx     x
```

water and nutrients. Some of these fungi apparently produce toxic SM and support their host plants in the chemical defence against herbivores and pathogens. An interesting example of a symbiotic fungus–plant interaction can be seen in ergot alkaloids, which have been discussed in Section 7.2.11. Ergot alkaloid formation in the Convolvulaceae is apparently due to an endophytic clavicipateceous fungus (Ahimsa-Müller *et al*., 2007; Markert *et al*., 2008). This observation explains the isolated occurrence of ergot alkaloids in Convolvulaceae.

Certain simple PAs, such as loline, were detected in a grass (*Lolium pratense*, ex. *Festuca pratensis*) and in a root hemiparasitic plant (*Rhinanthus serotinus*). The alkaloids derived from a symbiotic endophytic fungus (*Neotyphodium uncinatum*; Clavicipitaceae), which lives on the grass (Lehtonen *et al*., 2005). Similar to the situation in *Claviceps*, the fungus provides defence compounds, which help the grass and its hemiparasite to ward off herbivores. It is well

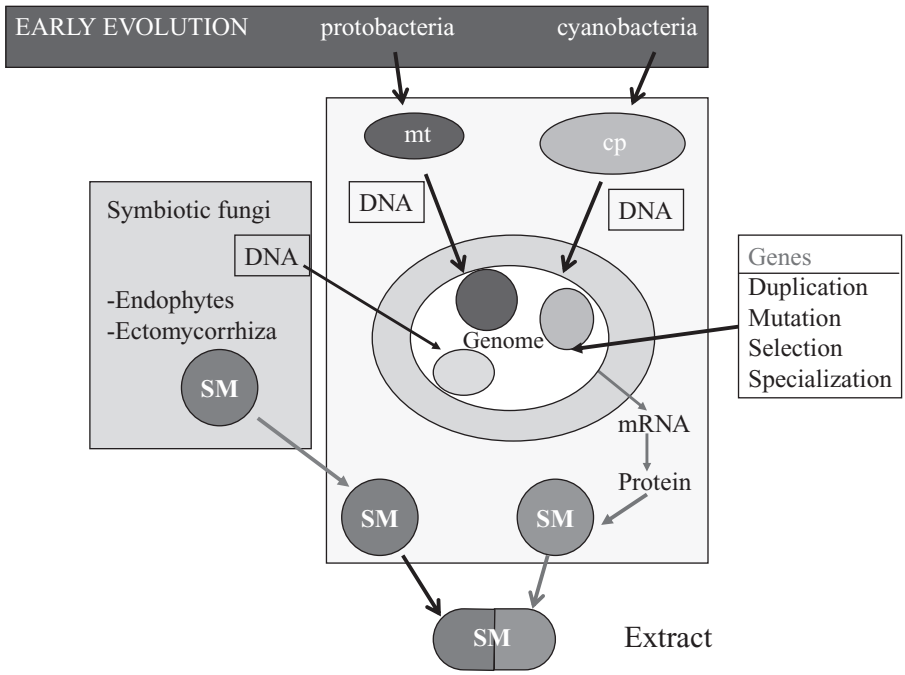


Figure 7.18 Schematic illustration of a possible origin of SM in plants. (See Plate 21 in colour plate section.)

known that parasitic and semi-parasitic plants can tap the secondary metabolites of their host plants (Stermitz, 1998). Therefore, the occurrence of a particular SM in such a plant has nothing to do with a common genetic base or with HGT or common phylogeny. Such phenomena are abundant in families with parasitic and semi-parasitic species, such as Scrophulariaceae, Santalaceae (including the former Viscaceae) and Loranthaceae.

Naphthodianthrones, such as hypericin, are well-known constituents of St John's wort (*Hypericum perforatum*; Clusiaceae). Kusari *et al*. (2008) have isolated an endophytic fungus from *H. perforatum* that produces hypericin in culture. Also emodin, a precursor of hypericin, is produced by both endophyte and the plant. Fungi are known producers of anthraquinones; in lichens, which are symbioses between an alga and a fungus, anthraquinones represent common defence metabolites, which are probably produced by the fungal partner. Since anthraquinone-producing plants are isolated over the plant kingdom (Asphodelaceae, Fabaceae, Rhamnaceae, Polygonaceae), it would be interesting to search for a former or extant endophyte association.

The maytansinoid ansa antibiotics are produced by *Actinosynnema pretiosum* (Actinomycetes), but also in a number of angiosperms, including Celastraceae, Rhamnaceae and Euphorbiaceae. It has been suggested that the

occurrence of these SM depends on infection by this actinomycete (Cassady *et al*., 2004).

The anticancer drug taxol is produced by endophytes from *Taxus brevifolia* (Stierle *et al*., 1993). An isolated occurrence of the taxan alkaloids has been reported from *Corylus avellana* (Betulaceae), which is not associated with an endophyte (although the pathway genes might have been imported by an endophyte at an earlier stage) (Ottaggio *et al*., 2008). Podophyllotoxin is produced by endophytes of *Podophyllum peltatum* (Eyberger *et al*., 2006).

Within Fabaceae, *Astragalus* and *Oxytropis* are famous for the production of toxic indolizidine alkaloids, such as swainsonine, which inhibit glucosidases. These alkaloids are apparently produced by an endophyte (*Embellisia* spp.; Pleosporaceae) (Ralphs *et al*., 2008).

Camptothecin (formally a quinoline alkaloid, but derived from the tryptamine/secologanin pathway) occurs in unrelated families such as *Camptotheca acuminata* (Cornaceae), *Nothapodytes foetida, Pyrenacantha klaineana, Merrilliodendron megacrapum* (Icacinaceae), *Ophiorrhiza pumila, O. mungos* (Rubiaceae), *Ervatamia heyneana* (Apocynaceae) and *Mostuea brunonis* (Gelsemiaceae). It has been shown recently that camptothecin can be produced by endophytes from *Nothapodytes foetida* (Puri *et al*., 2005). It is tempting to speculate that the patchy distribution was originally caused by endophytes, which have infected the respective plants or which have transferred their genes.

Although the endophytes, which were isolated from these plants, are capable of biosynthesizing hypericin, taxol, camptothecin and podophyllotoxin in vitro, it is less likely that they alone perform the productions in the plant. It has been speculated that an HGT has taken place at some stage, thus importing the respective pathways from the fungi into the host plant (Kusari *et al*., 2008). It is a challenging question to determine the degree and contribution of endophytic and ectophytic SM pathways to the SM profiles of plants. If it was a more common phenomenon than usually assumed, it would offer an additional explanation for the patchy distribution of certain SM in the plant kingdom. It has been speculated that HGT could also be taking place when viruses or insects invade plants.

7.6.3 Conclusions and outlook

One of the main questions discussed in this chapter is about the origin and evolution of plant secondary metabolism. We have started with the observation that some SM (such as phenolics and terpenoids) are produced by nearly all vascular plants, whereas others, especially those with alkaloids, cardiac glycosides, anthraquinones, etc., show a more restricted but usually patchy distribution.

The patchy distribution could be due to simple convergence, which is certainly the case in several instances. It could also be due to a wider distribution of SM pathway genes in the plant kingdom, which are silent or

inactivated in most places but become activated under certain conditions or in particular clades. Evidence is provided from the distribution of a few key genes/proteins that SM pathways might have been introduced into plants from SM- producing bacteria via an early HGT; it is established that protobacteria became mitochondria and cyanobacteria plastids. Another external source for plant SM could be ectomycorrhizal fungi and endophytes, which either directly produce a particular SM or indirectly by transferring the pathway genes from fungi to plants. Some parasitic and hemiparasitic plants sequester SM from their host plants. Because fungal infections and parasitic sequestration do not necessarily follow plant phylogeny, they could cause (at least partly) the patchy distribution seen in some SM groups.

It is likely that HGT only introduced a limited number of pathway genes and that the host plants developed and contributed their own set of genes/enzymes, leading to the various structural variations seen in nature as a sort of biotransformation reaction.

Acknowledgements

We would like to thank various co-workers and collaborators who have contributed to the results in the past (E. Käss, M. Kaufmann, B. Gemeinholzer, G. Mohamed, T. Morazova, F. Sporer, H. Sauer-Gürth, H. Staudter, B.-E. van Wyk and J. Boatwright). Theodor C. H. Cole kindly allowed me to use an Indesign file of an APG-III tree.

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 ISBN: 978-1-405-18397-0