ANNUAL PLANT REVIEWS VOLUME 40

Biochemistry of Plant Secondary Metabolism

Second Edition

Edited by

Michael Wink
Professor of Pharmaceutical Biology
Institute of Pharmacy and Molecular Biotechnology
Heidelberg University
Germany

WILEY-BLACKWELL
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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

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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

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.*,

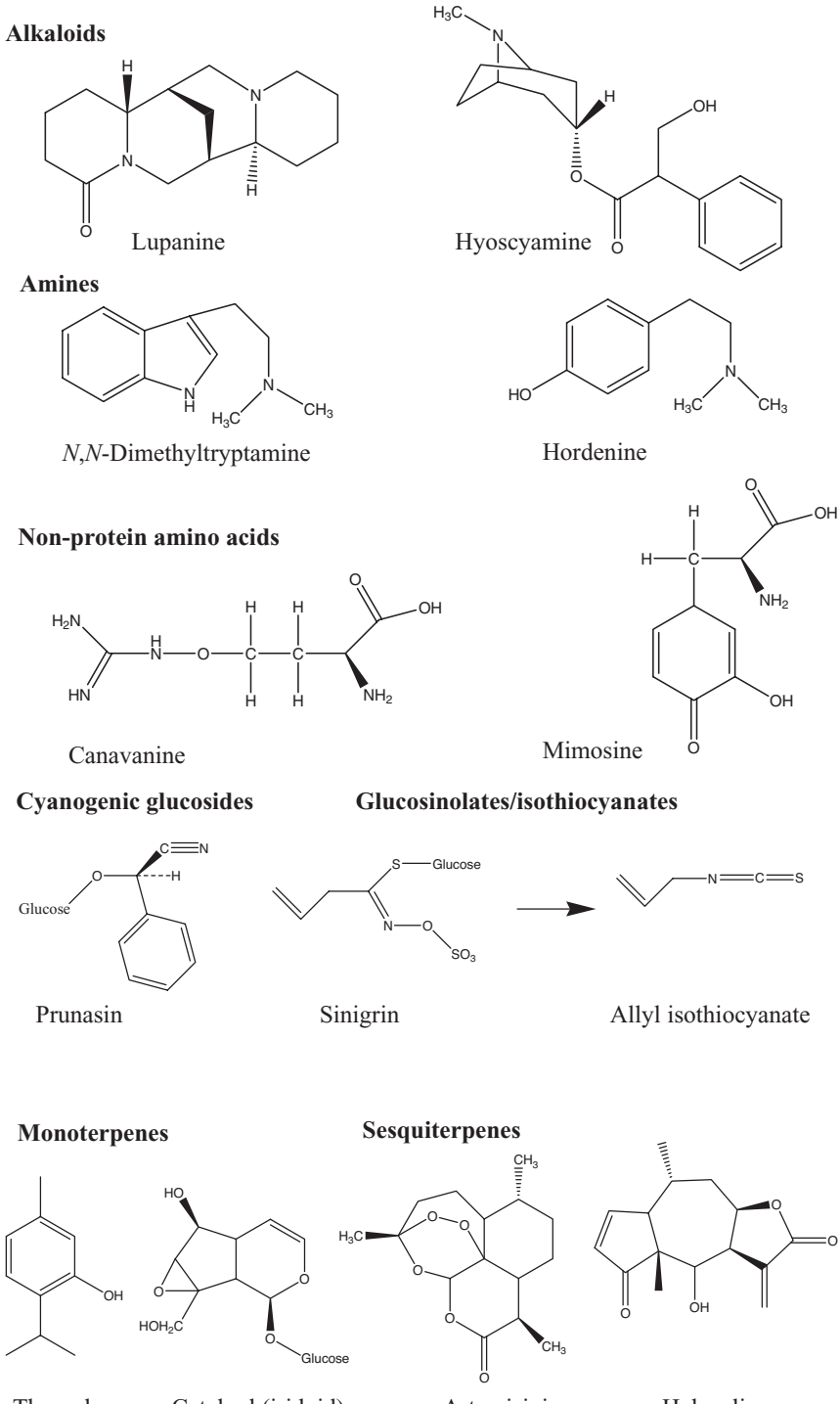


Figure 1.1 Structures of selected secondary metabolites.

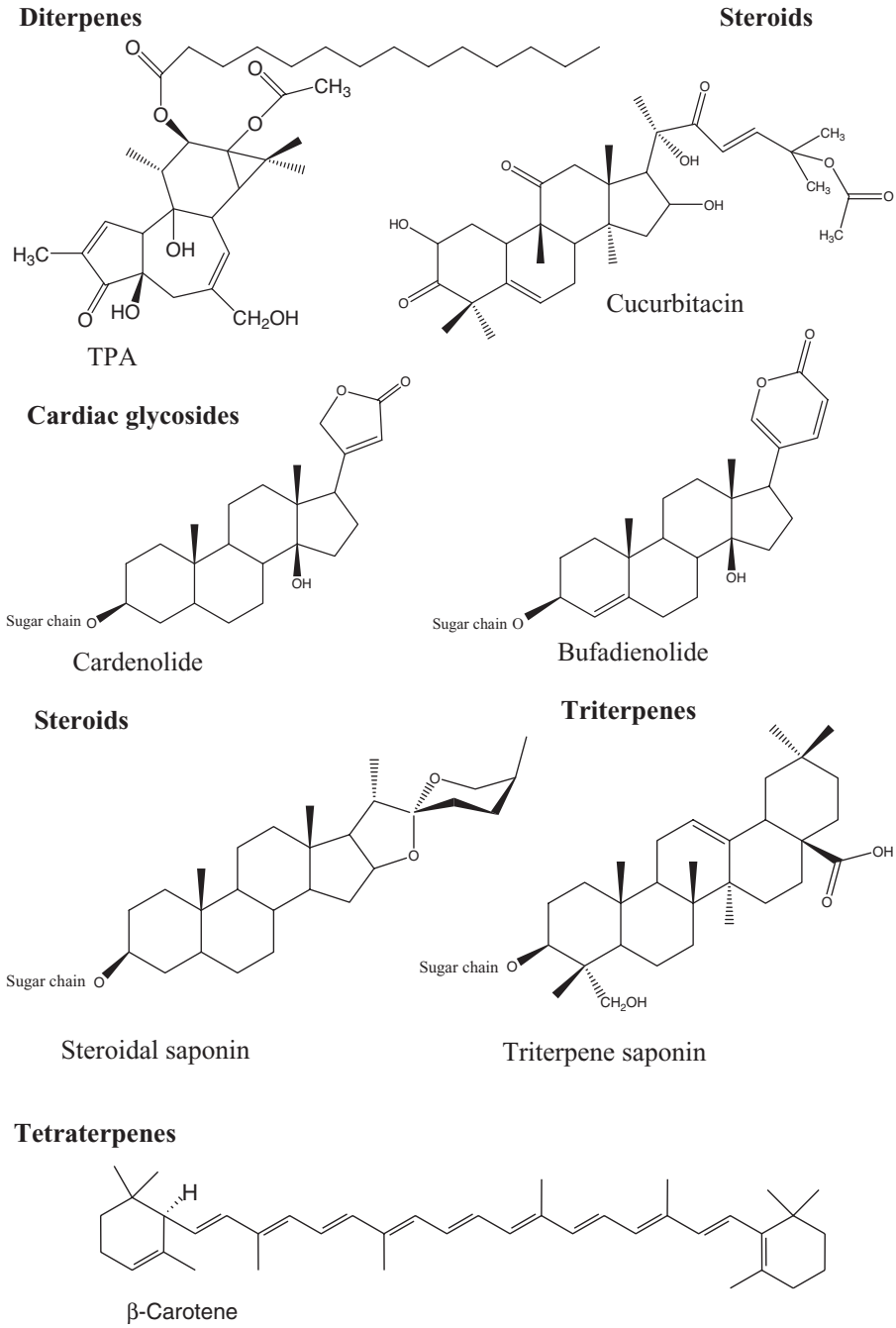
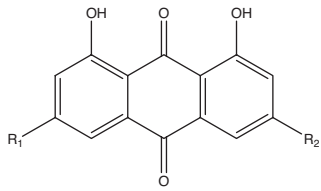
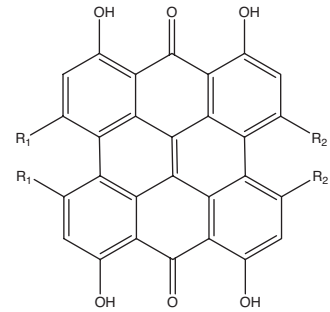


Figure 1.1 (*Continued*)

Polyketides

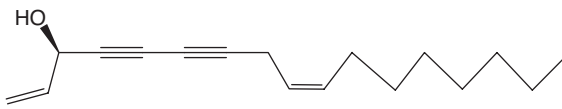


Anthraquinone

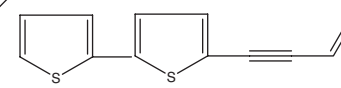


Naphthodianthrone

Polyacetylenes

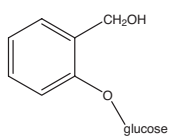


Falcarinol

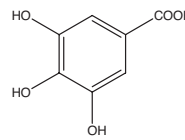


Thiophene (bbt)

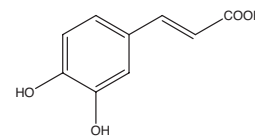
Simple phenolics



Salicin

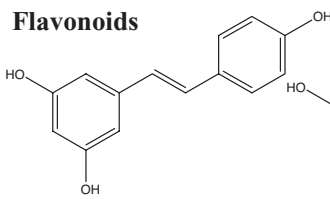


Gallic acid

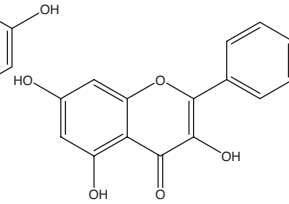


Caffeic acid

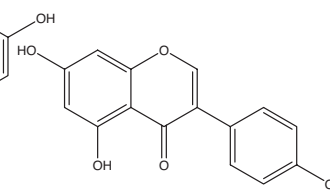
Flavonoids



Stilbene (resveratrol)



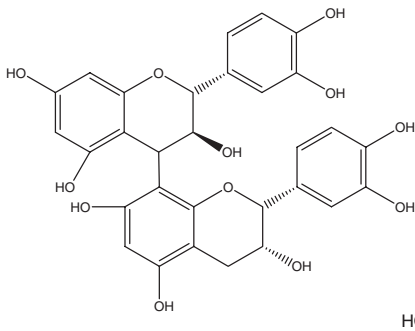
Flavonol (quercetin)



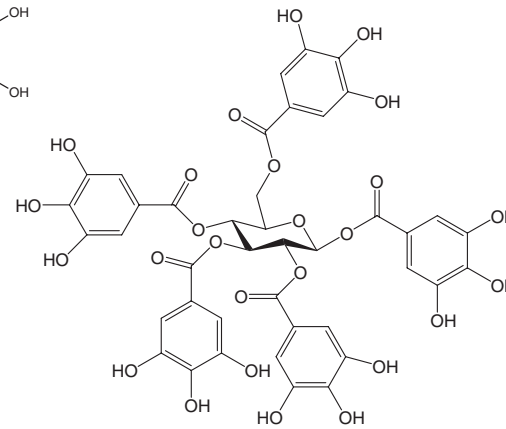
Isoflavone (genistein)

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

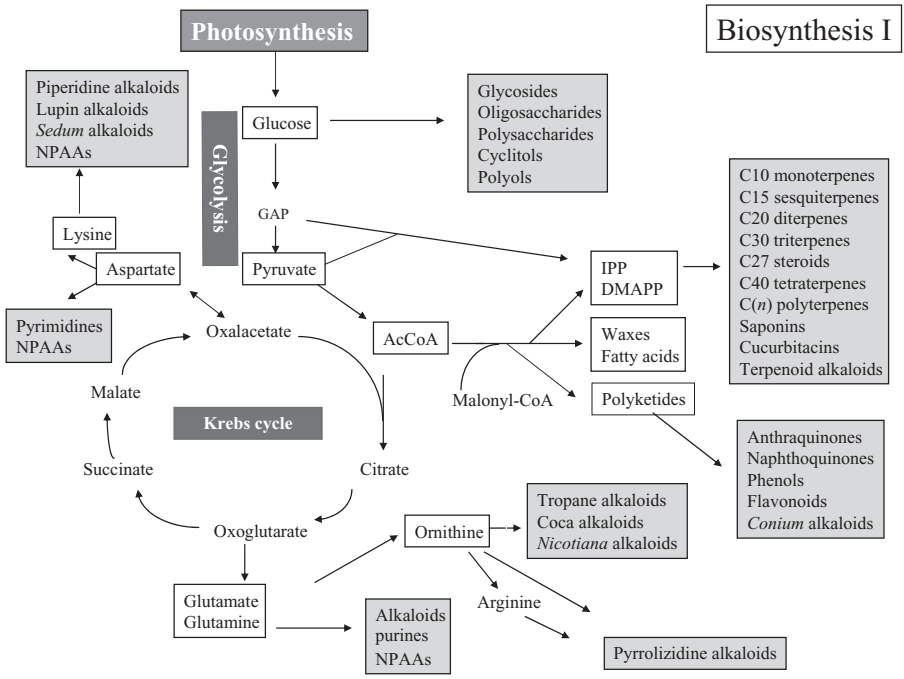


Figure 1.2 Main pathways leading to secondary metabolites. Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethyl allyl diphosphate; GAP, glyceraldehyde-3-phosphate; NPAAs, non-protein amino acids; AcCoA, acetyl coenzyme A. (See Plate 1 in colour plate section.)

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,

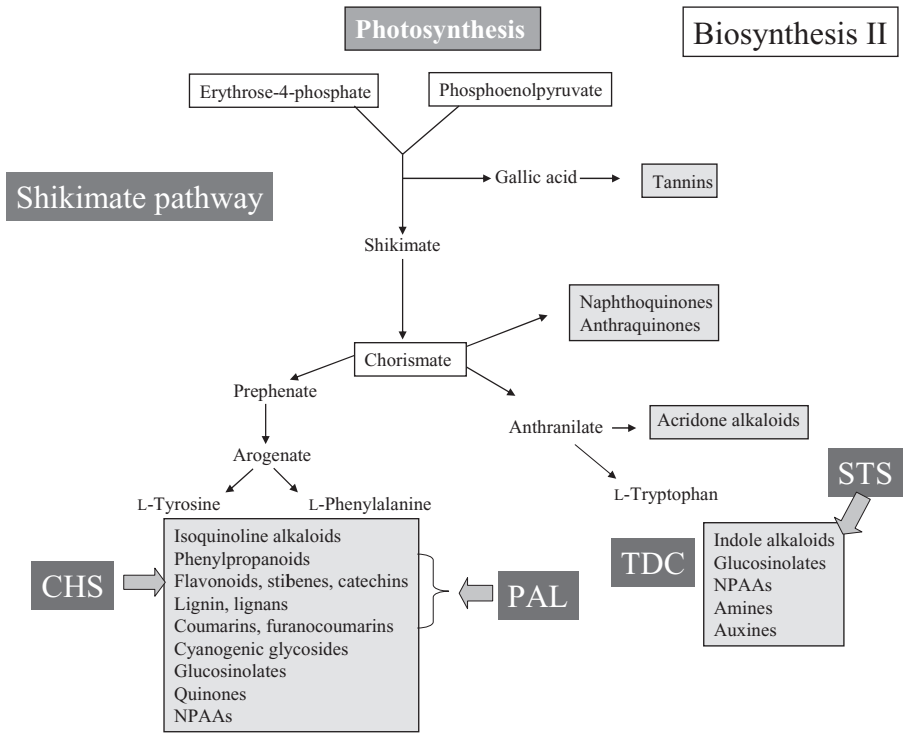


Figure 1.3 Several pathways of secondary metabolites derive from precursors in the shikimate pathway. Abbreviation: NPAAs, non-protein amino acids; PAL, phenylalanine ammonia lyase; TDC, tryptophan decarboxylase; STS, strictosidine synthase; CHS, chalcone synthase. (See Plate 2 in colour plate section.)

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

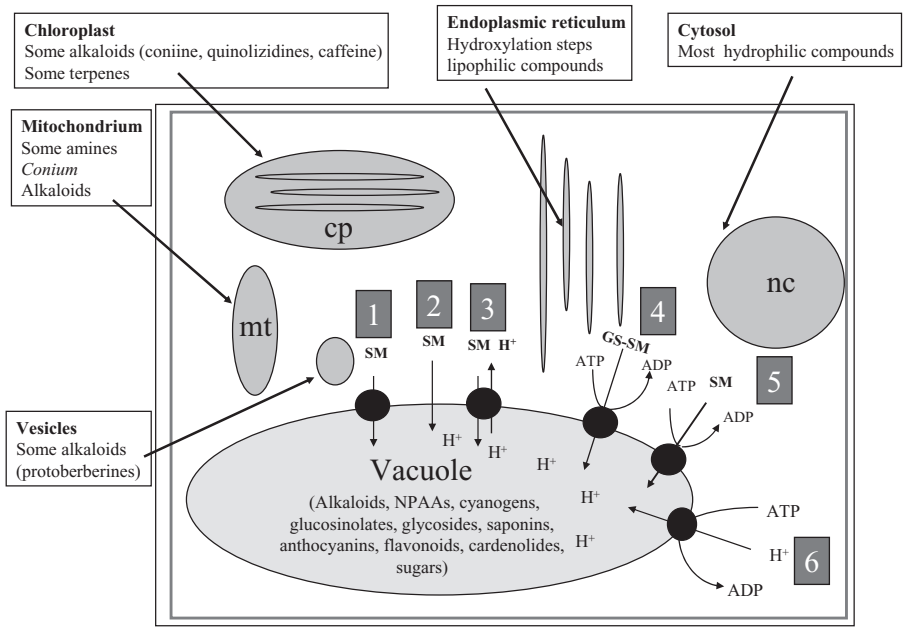


Figure 1.4 Compartmentation of biosynthesis and sequestration. Abbreviations: SM, secondary metabolites; GS-SM, conjugate of SM with glutathione; NPAAs, non-protein amino acids; ATP, adenosine triphosphate; ADP, adenosine diphosphate; mt, mitochondrion; cp, chloroplast; nc, nucleus; 1, passive transport; 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

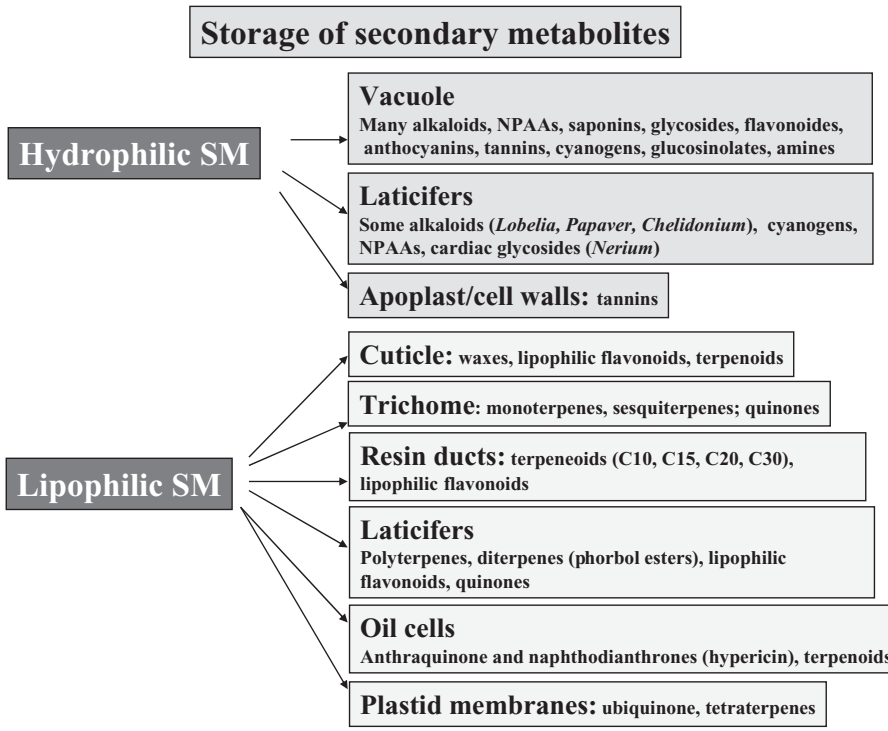


Figure 1.5 Storage compartments for hydrophilic and lipophilic compounds. Abbreviation: NPAAs, non-protein amino acids. (See Plate 4 in colour plate section.)

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,