



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

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Table 1.1 Number of known secondary metabolites from higher plants

Type of secondary metabolite	Number ^a
<i>Nitrogen-containing</i>	
Alkaloids	21 000
Non-protein amino acids (NPAAs)	700
Amines	100
Cyanogenic glycosides	60
Glucosinolates	100
Alkamides	150
Lectins, peptides, polypeptides	2000
<i>Without nitrogen</i>	
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

^aApproximate number of known structures.

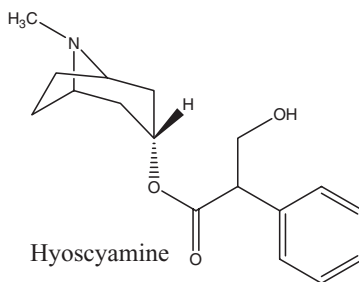
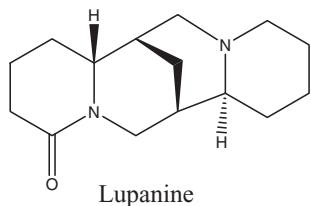
^bTotal of terpenoids number exceeds 22 000 at present.

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 (¹H-NMR, ¹³C-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).

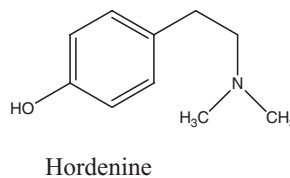
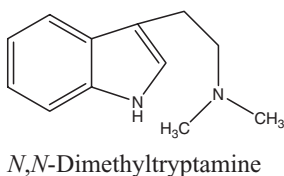
1.2 Biosynthesis

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.*,

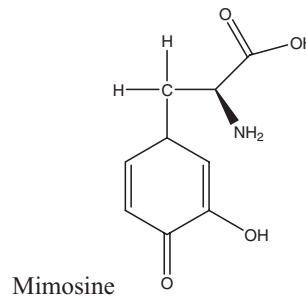
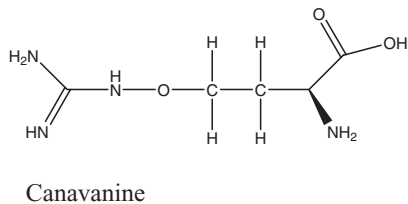
Alkaloids



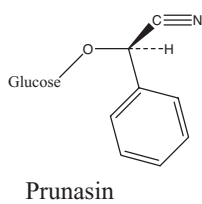
Amines



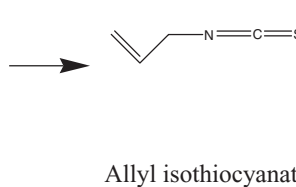
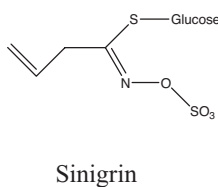
Non-protein amino acids



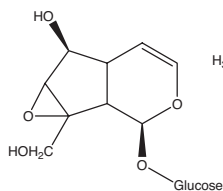
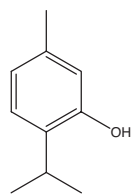
Cyanogenic glucosides



Glucosinolates/isothiocyanates



Monoterpenes



Sesquiterpenes

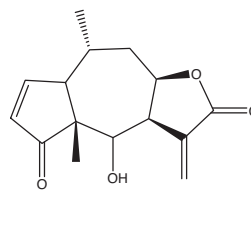
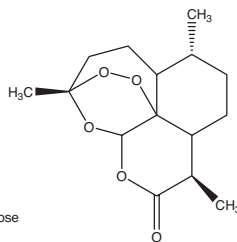


Figure 1.1 Structures of selected secondary metabolites.

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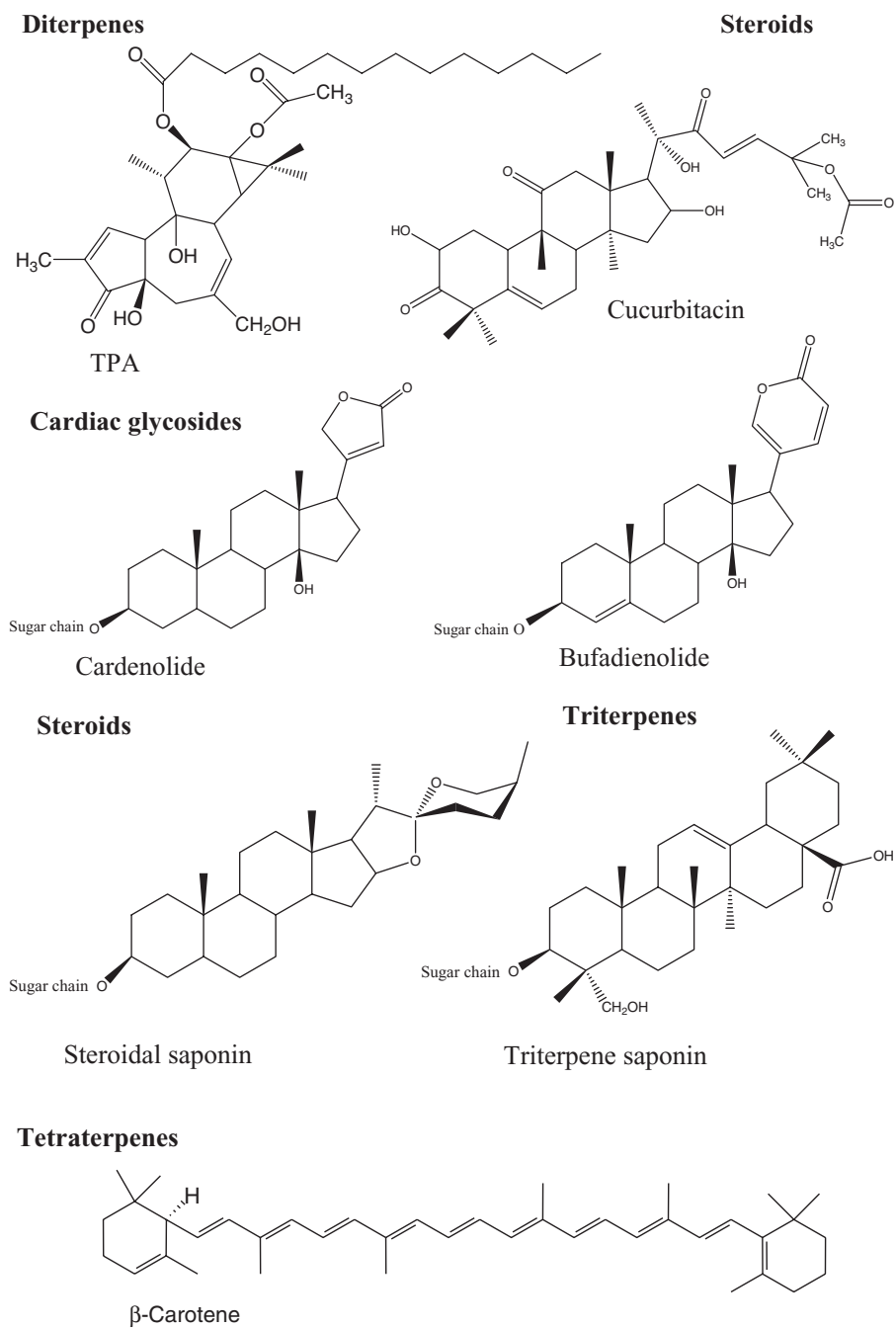
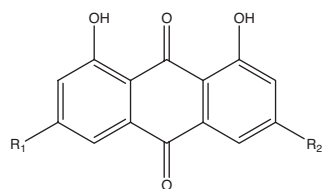
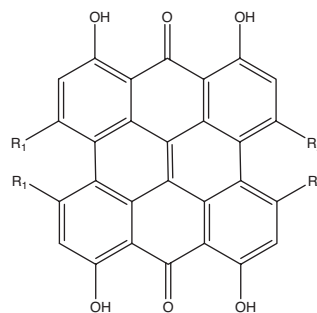


Figure 1.1 (Continued)

Polyketides

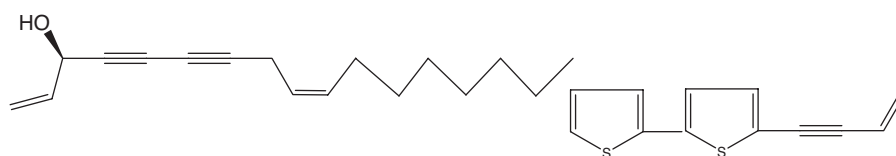


Anthraquinone



Naphthodianthrone

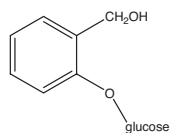
Polyacetylenes



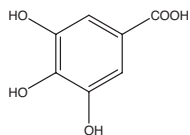
Falcarinol

Thiophene (bbt)

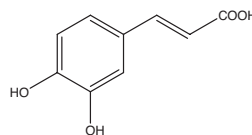
Simple phenolics



Salicin

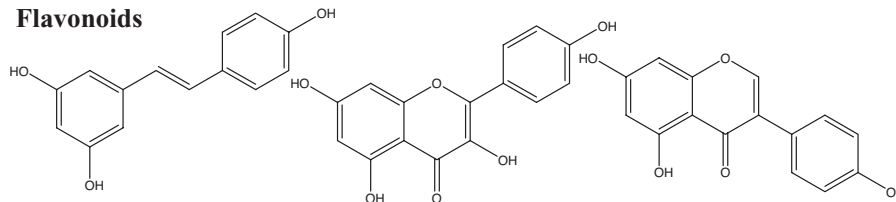


Gallic acid



Caffeic acid

Flavonoids



Stilbene (resveratrol)

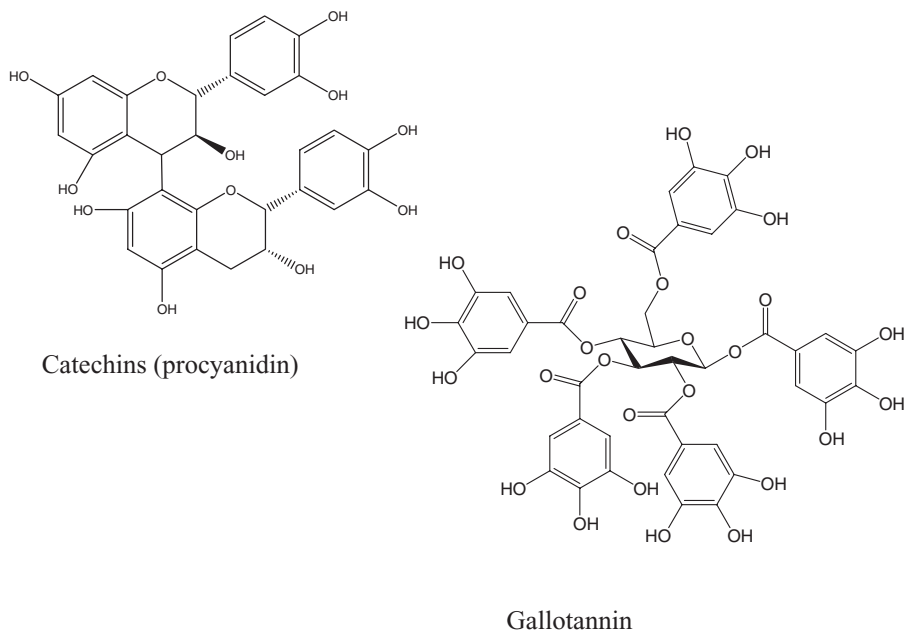
Flavonol (quercetin)

Isoflavone (genistein)

Figure 1.1 (Continued)

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Tannins

**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

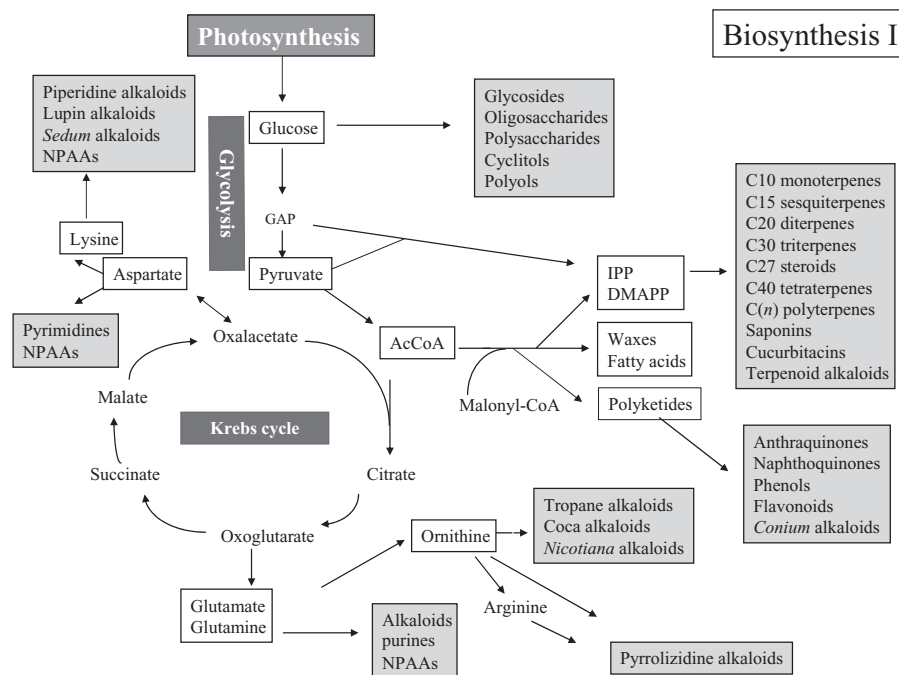


Figure 1.2 Main pathways leading to secondary metabolites. Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethyl allyl diphosphate; GAP, glyceraldehyde-3-phosphate; NPAAs, non-protein amino acids; AcCoA, acetyl coenzyme A. (See Plate 1 in colour plate section.)

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; Jørgensen *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,

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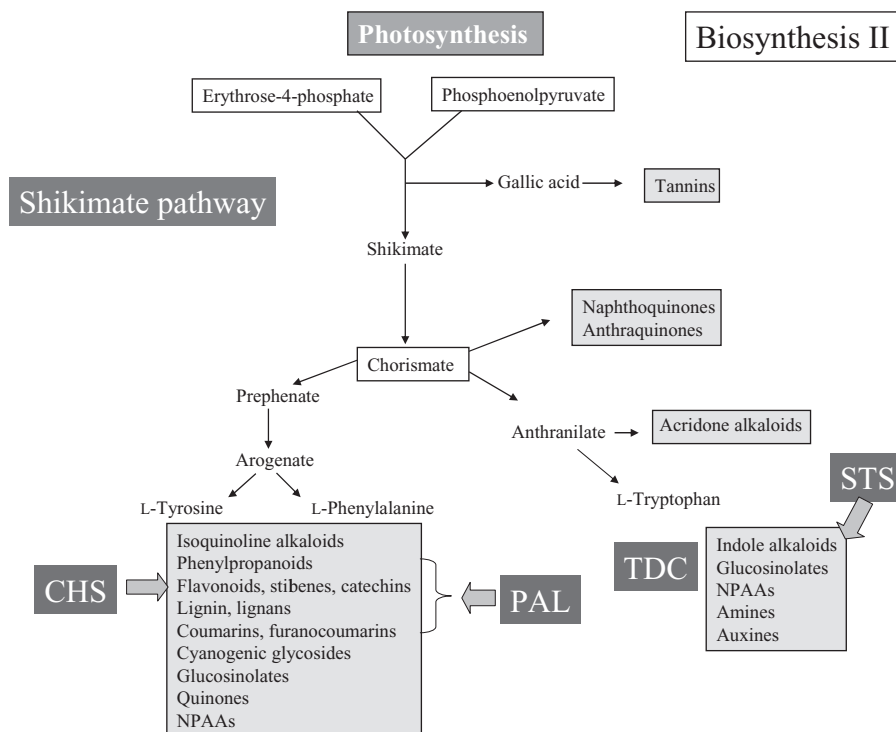


Figure 1.3 Several pathways of secondary metabolites derive from precursors in the shikimate pathway. Abbreviation: NPAAAs, non-protein amino acids; PAL, phenylalanine ammonia lyase; TDC, tryptophan decarboxylase; STS, strictosidine synthase; CHS, chalcone synthase. (See Plate 2 in colour plate section.)

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

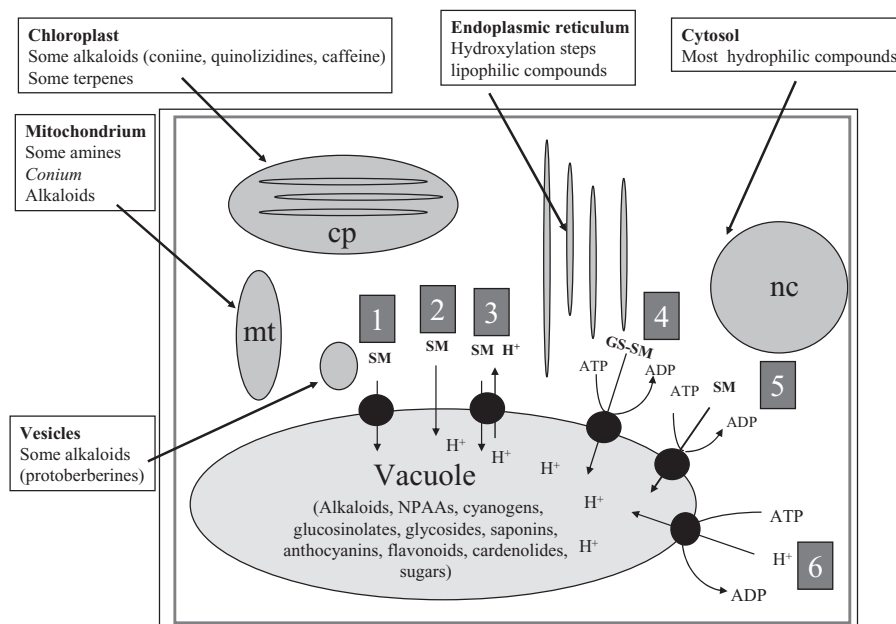


Figure 1.4 Compartmentation of biosynthesis and sequestration. Abbreviations: SM, secondary metabolites; GS-SM, conjugate of SM with glutathione; NPAAAs, non-protein amino acids; ATP, adenosine triphosphate; ADP, adenosine diphosphate; mt, mitochondrion; cp, chloroplast; nc, nucleus; 1, passive transport;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

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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
Kaempferol 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

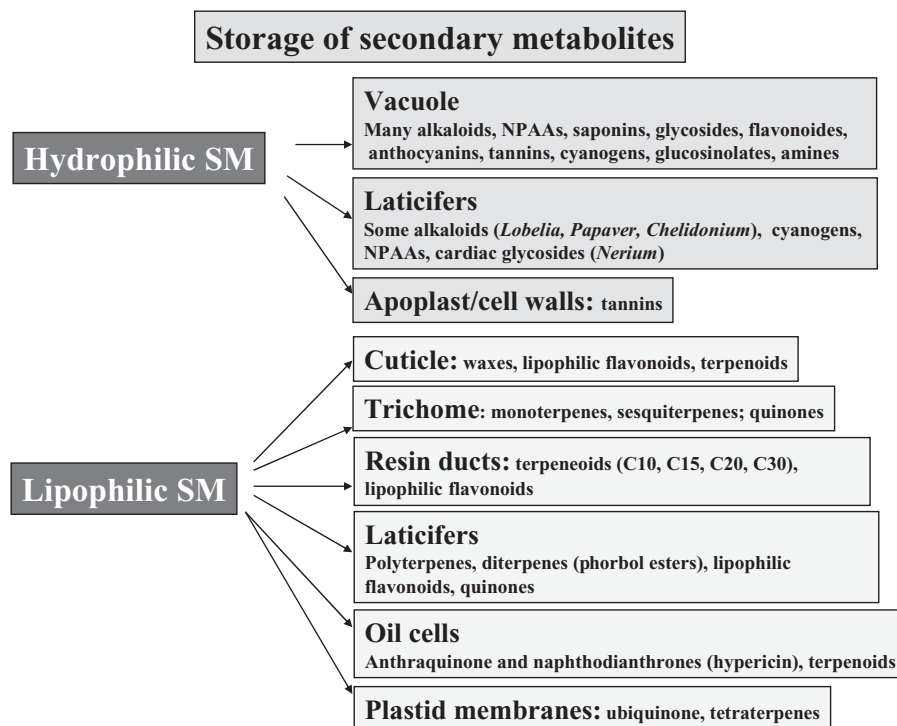


Figure 1.5 Storage compartments for hydrophilic and lipophilic compounds. Abbreviation: NPAAAs, non-protein amino acids. (See Plate 4 in colour plate section.)

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

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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, NPAAAs, 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 form) (NADPH₂). In order to exhibit their function as defence or signal compounds, allelochemicals need to be present in relatively high concentrations at the right

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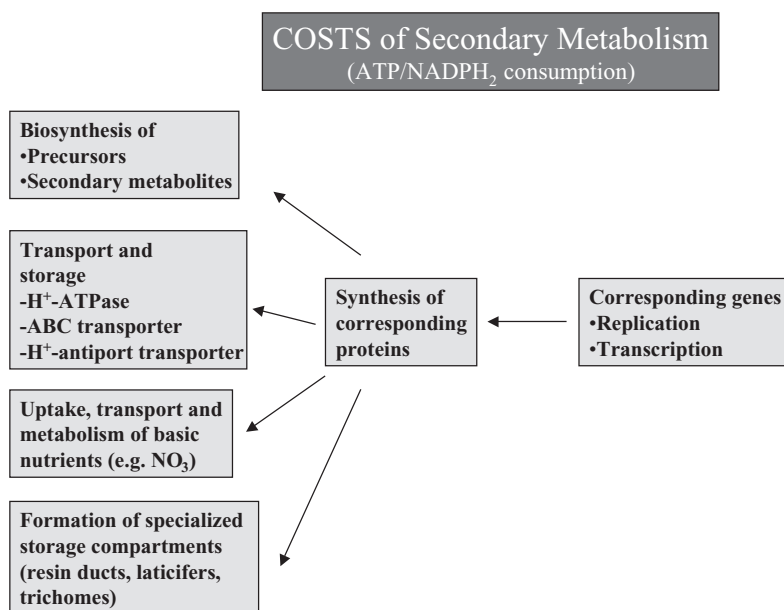


Figure 1.6 Costs of chemical defence and signal compounds. Abbreviations: ATP, adenosine triphosphate; NADPH₂, nicotinamide adenine dinucleotide phosphate (reduced form). (See Plate 5 in colour plate section.)

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.

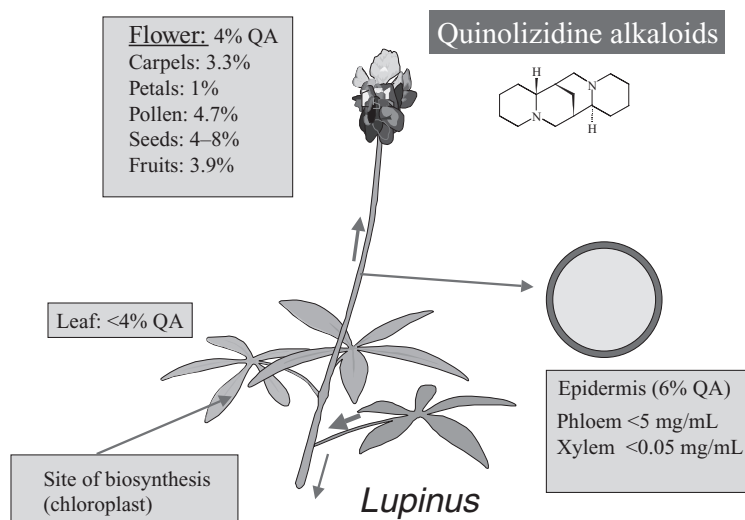


Figure 1.7 Example of the complicated biochemistry and physiology of alkaloid formation: quinolizidine alkaloids (QAs) in lupins (genus *Lupinus*, Fabaceae). QAs are formed in leaf chloroplasts and exported via the phloem all over the plant. QAs predominantly accumulate in vacuoles of epidermal tissue. 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 micro-organisms (N-fixing *Rhizobia* or mycorrhizal fungi)
- (g) Protection against UV light or other physical stress
- (h) Selected physiological functions

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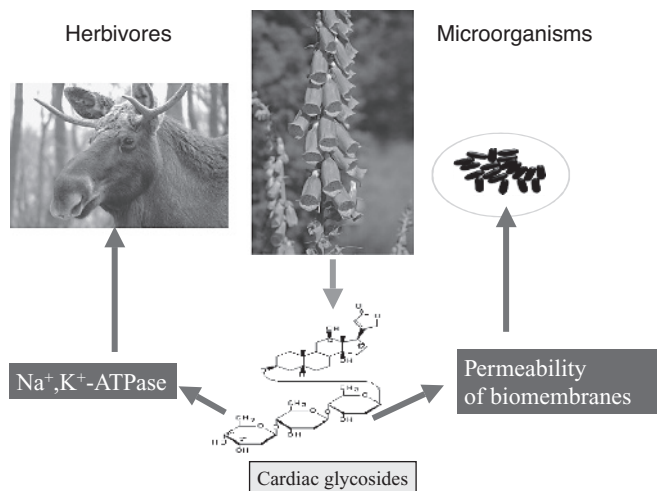


Figure 1.8 Schematic view of the ecological roles of plant SM. Foxglove (*Digitalis purpurea*) produces cardiac glycosides, which are very toxic to animals (vertebrates, insects) because they inhibit Na^+ , $\text{K}^+\text{-ATPase}$, one of the most important transporters in animal cells. Cardiac glycosides 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 livestock.

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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