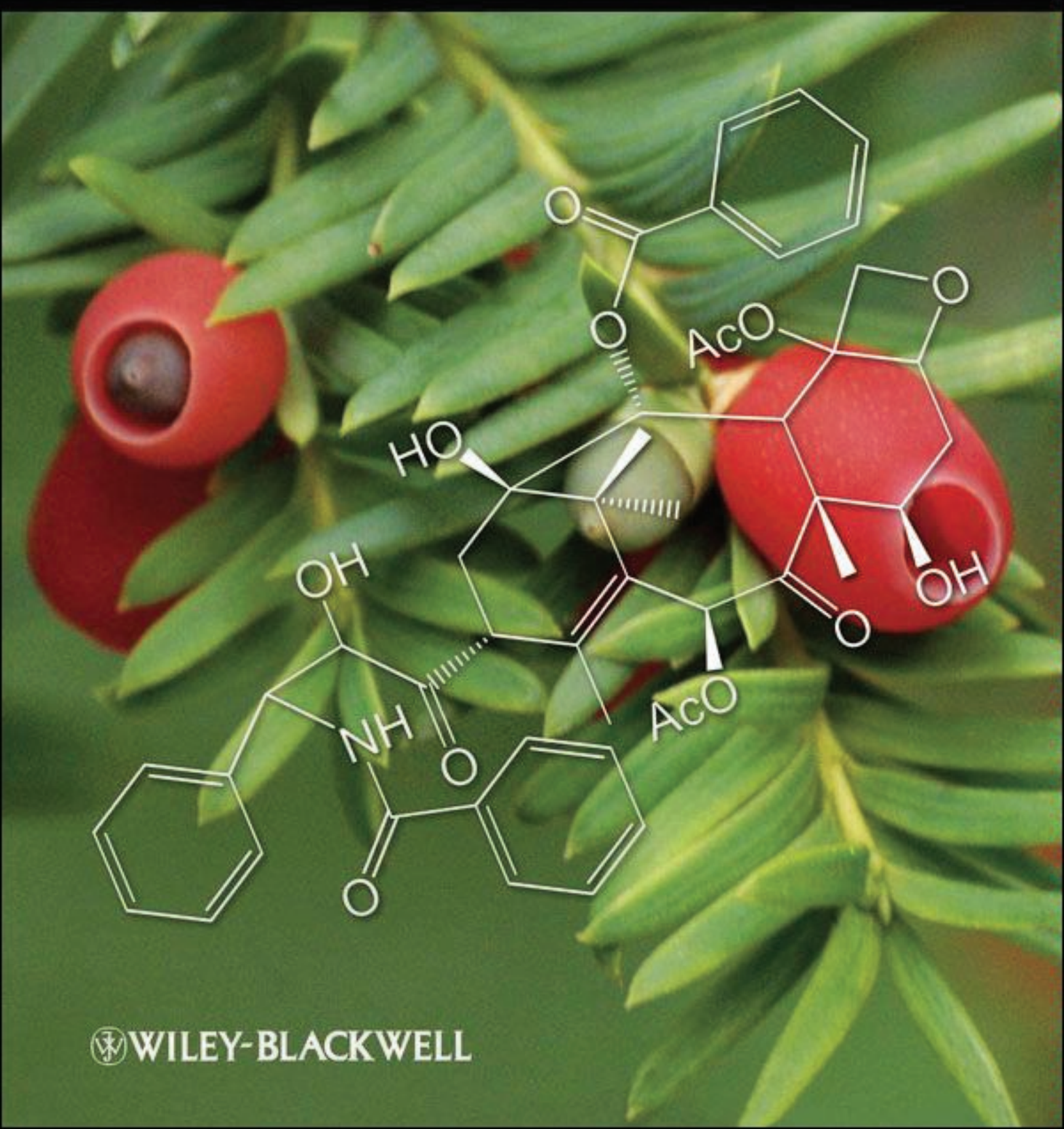
Annual Plant Reviews, Volume 39

Functions and Biotechnology of Plant Secondary Metabolites

Second Edition

Edited by **Michael Wink**



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Functions and Biotechnology of Plant Secondary Metabolites

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Edited by

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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 (SM)** 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 SM usually occur in a limited number of plants, indicating that they are not essential for primary metabolism, i.e. anabolism or catabolism.

Whereas SM had been considered to be waste products or otherwise useless compounds for many years, it has become evident over the last three decades that SM 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 SM serve as defence chemicals against herbivorous animals (insects, molluscs, mammals), microbes (bacteria, fungi), viruses or plants competing for light, water and nutrients. Therefore, SM are ultimately important for the fitness of the plant producing them. Plants usually produce complex mixtures of SM, 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 SM have been shaped and optimised 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 function of plant SM, their modes of action and their use in pharmacology as molecular probes, in medicine as therapeutic agents, and in agriculture as biorational pesticides. A companion volume – *Biochemistry of Plant Secondary Metabolism* edited by M. Wink – published simultaneously provides overviews of the biosynthetic pathways (enzymes, genes) leading to the formation of alkaloids, glucosinolates, cyanogenic glucosides, non-protein amino acids, flavonoids and other phenolics and terpenoids. The mechanisms of transport and storage were also discussed as well as a general outline of the evolution of secondary metabolism.

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. The present volume draws together results from a broad area of biochemistry, pharmacology and pharmacy 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 function and utilization of SM (M. Wink), followed by a more detailed overview over the various modes of action of SM (M. Wink and O. Schimmer), including interactions with the major molecular targets, such as biomembranes, proteins and DNA. Some emphasis is placed on DNA modifying metabolites, on mechanisms involved in cytotoxicity and on SM interfering with elements of neuronal signal transduction (neuroreceptors, ion channels). The production of SM for defence is not restricted to plants, but can also be seen in other sessile organisms. SM are especially abundant in marine organisms. A. Putz and P. Proksch explore chemical defence in marine ecosystems. Because plants have to defend themselves against bacteria, fungi and viruses, it is not surprising that many SM exert antibacterial, antifungal and antiviral properties. The antimicrobial properties are reviewed with a special emphasis on medical application (J. Reichling). Because many pathogens have become resistant against antibiotics (e.g. MRSA), antibiotic substances from plants with different modes of actions become more important in the future. Mankind has used medicinal plants for thousands of years to treat health disorders and diseases. Although many of the traditional applications have been replaced by synthetic drugs these days, phytomedicine and phytotherapy is still in use and receiving much attention. J. Heilmann reviews new findings of plant-derived drug in the context of anticancer and chemopreventive properties, and drugs with anti-inflammatory, antidepressant, anti-ischaemic, antimalarial and immunostimulatory activities. The final chapter addresses the problem of the production of SM as some of them are difficult to obtain and thus very costly. An alternative to the plantation of medicinal plants in the field is the production of SM in plant cell and organ cultures or by recombinant microorganisms. The recent results and developments are reviewed by W. Alfermann.

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 thank all those who have taken part in writing and preparation of this book. I thank Theodor C. H. Cole for help 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.

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Chapter 1
INTRODUCTION

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Abstract: Secondary metabolites (SM) occur in plants in a high structural diversity. A typical feature of SM is their storage in relatively high concentrations, sometimes in organs which do not produce them or as inactive 'prodrugs' that are enzymatically activated in case of danger. Biochemical and physiological features of secondary metabolism are strongly correlated with the function of SM: SM are not useless waste products (as assumed earlier) but important tools of plants needed against herbivores, microbes (bacteria, fungi) and viruses. Some of the SM also function as signal molecules to attract pollinating arthropods or seed-dispersing animals. During more than 500 million years of evolution, plants have evolved SM with a wide variety of biochemical and pharmacological properties. Many SM interact with proteins (receptors, ion channels, enzymes, cytoskeleton, transcription factors), DNA/RNA and/or biomembranes. Some of the interactions with molecular targets are highly specific, others have pleiotropic properties. Potential modes of action are summarized. As a consequence of the pharmacological properties of SM, several of them are used in medicine to treat disorders and infections. Others are interesting in biotechnology as rational pesticides. Phytomedicine normally employs complex mixtures, as they are present in the producing plant, which may exert additive or even synergistic properties.

Keywords: secondary metabolites; ecological functions; herbivores; microbes; signal compounds; molecular modes of action; targets; phytomedicine

1.1 Ecological function of secondary metabolites

A typical trait of plants is the production of a high diversity of secondary metabolites (SM) (the number of identified substances exceeds 100 000 at present), including many nitrogen-free (such as terpenes, polyketides, phenolics, saponins and polyacetylenes) and nitrogen-containing compounds (such as alkaloids, amines, cyanogenic glycosides, non-protein amino acids, glucosinolates, alkamides and peptides). In plants, several major SM, usually from different classes and biochemical pathways, are commonly accompanied by dozens of minor components. Complex mixtures, which

differ from organ to organ, sometimes between individual plants and regularly between species, are the result.

These compounds are synthesized in plants in a tissue-, organ- and developmental-specific way by specific biosynthetic enzymes (Facchini and De Luca, 2008; Murata *et al.*, 2008). The corresponding genes are regulated accordingly and gene regulation shows all the complexity known for genes encoding enzymes of primary metabolism. It is a particular feature of SM that they are accumulated and stored in high concentrations in the plant organs important for survival and reproduction; SM levels of 1–3% dry weight are regularly seen. In general, hydrophilic compounds are stored in the vacuole, whereas lipophilic substances are deposited in resin ducts, laticifers, trichomes, oil cells, or in the cuticle. As sites of synthesis are not necessary, the sites of storage, long-distance transport by xylem, phloem or via the apoplast have been discovered in some instances (see *Biochemistry of plant secondary metabolism*, for a more detailed discussion).

Although SM were known to mankind for thousands of years (Mann, 1992; Roberts and Wink, 1998) and have been used as dyes (e.g. indigo, shikonin), flavours (e.g. vanillin, capsaicin, mustard oils), fragrances (e.g. rose oil, lavender oil and other essential oils), stimulants (e.g. caffeine, nicotine, ephedrine), hallucinogens (e.g. morphine, cocaine, scopolamine, tetrahydrocannabinol), insecticides (e.g. nicotine, piperine, pyrethrin, rotenone), vertebrate and human poisons (e.g. coniine, strychnine, aconitine, colchicine, cardiac glycosides) and even as therapeutic agents (e.g. atropine, quinine, cardenolides, codeine), their putative biological functions have been a matter of controversy.

Whereas most animals can run or fly away when attacked by a predator (Edmunds, 1974), or possess an immune system to protect them against invading microbes or parasites, these means are apparently not available for plants when molested by herbivores, microbes (bacteria, fungi) and even other plants competing for light, space and nutrients. In contrast to most animals, plants can replace the parts, which have been diseased, wounded or browsed. This capacity for open growth and regeneration, which is most prominent in perennials, allows a certain tolerance towards herbivores and microbes. A number of plants employ mechanical and morphological protection, such as thorns, spikes, glandular and stinging hairs (often filled with noxious chemicals), or develop an almost impenetrable bark (especially woody perennials); these features can be interpreted as antipredatory means (in analogy to weapons and shells in animals).

Sessile or slow-moving animals, such as sponges, nudibranch molluscs, corals (see Chapter 3 in this book) and amphibia (e.g. salamanders, poisonous frogs, toads) are infamous for their ability to produce a wide range of chemicals that are usually toxic (for reviews, see Braekman *et al.*, 1998; Proksch and Ebel, 1998). Some insects either produce SM themselves or sequester them from their host plants (for overviews, see Duffey, 1980; Blum, 1981; Bernays and Chapman, 1994; Eisner *et al.*, 2005). Zoologists have never

doubted that these compounds serve for chemical defence against predators. Surprisingly, the defence function of SM in plants has been and sometimes is still controversial.

It had often been argued that SM are waste products or have no function at all (Hartmann, 2007). This hypothesis fails to explain several observations: (1) waste products are characteristic and necessary for heterotrophic animals that cannot degrade their food completely for energy production. These organisms excrete waste products that are often rich in nitrogen (i.e. urea, uric acid). However, plants are essential autotrophs and, therefore, do not need elaborate excretory mechanisms. Furthermore, nitrogen is a limiting nutrient for plants. Consequently, the production of nitrogen-containing excretions, such as alkaloids, would be difficult to explain. In addition, alkaloids are often found in young or metabolically active tissues but not in dying or senescing cells, as would be expected according to the waste product hypothesis. (2) SM are often not inert end products of metabolism (an expected trait of waste products), but many of them can be metabolized by plant cells. For example, nitrogenous SM, such as alkaloids, non-protein amino acids, cyanogenic glucosides or lectins, are often stored in considerable quantities in leguminous seeds. During germination, a degradation of these compounds can be seen, indicating that their nitrogen is reused by the seedling. (3) Secondary metabolism is often highly complex and regulated in a tissue- and developmentally specific manner, which would be surprising for a waste product without function.

Alternatively, it was argued as long as 100 years ago by E. Stahl in Jena (Germany), that SM serve as defence compounds against herbivores (Hartmann, 2007). This hypothesis has been elaborated during recent decades (Fraenkel, 1959; Ehrlich and Raven, 1964; Levin, 1976; Swain, 1977) and a large body of experimental evidence supports the concept that follows (for reviews, see Wink, 1988, 1992, 1993a; 2003b; Harborne, 1993; Bernays and Chapman, 1994). Several SM have evolved for protection against viruses, bacteria, fungi, competing plants and, importantly, against herbivores (e.g. slugs and snails, arthropods and vertebrates). In addition, SM can serve as signal compounds to attract animals for pollination (fragrant monoterpenes, coloured anthocyanins or carotenoids) and for seed dispersal (reviewed by Cipollini and Levey, 1997) (Fig. 1.1). In several instances, both activities are exhibited by the same compounds: anthocyanins or monoterpenes can be insect attractants in flowers but are insecticidal and antimicrobial at the same time. This makes sense, since insects need to be attracted as pollinators, but should not eat the flowers. The pollinators are rewarded by nectar instead. In addition, some SM concomitantly exhibit physiological functions, for example they can serve as mobile and toxic nitrogen transport and storage compounds or ultraviolet-protectants. These multiple functions are typical and do not contradict their main role as chemical defence and signal compounds. If a trait can serve multiple functions, it is more likely to be maintained by natural selection. In this book, Chapters 2 and 4 review some of these aspects.

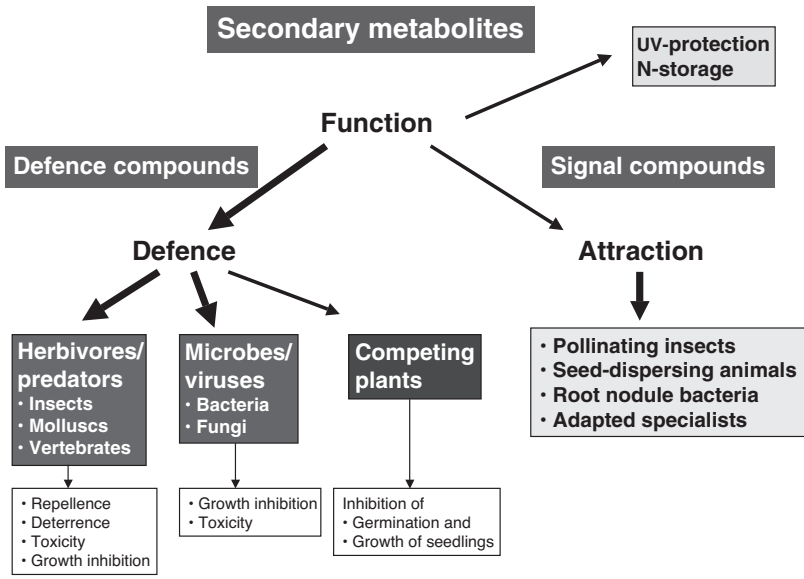

Figure 1.1 Ecological and physiological functions of plant secondary metabolites. (See Plate 1 in colour plate section.)

1.2 Presence of defence and signal compounds at the right time and place

In most plants, synthesis and accumulation of SM is regulated in space and time. As a rule, vulnerable tissues are defended more than old, senescing tissues. For example, it is usually observed that seeds, seedlings, buds and young tissues either sequester large amounts of a compound or actively synthesize them – 'optimal defence theory'. Organs that are important for survival and multiplication, such as flowers, fruits and seeds, are nearly always a rich source of defence chemicals.

The specific localizations of SM make sense if their role as defence and/or signal compounds is accepted. Trichomes and glandular hairs are always on the surface of the plant; a herbivore cannot avoid direct contact with them if it tries to feed on the plant. If membrane-active terpenes reach their lips, tongue or mandibles, many herbivores can be deterred before they actually start feeding on the plant. Another example is the sequestration of high concentrations of SM in vacuoles, which are often positioned in a favourable site for defence, as many of them are stored in epidermal and subepidermal cells (Saunders and Conn, 1978; Kojima *et al.*, 1979; Matile, 1984; Wink *et al.*, 1984; Werner and Matile, 1985; Wink, 1992, 1997; Gruhnert *et al.*, 1994). If a small herbivore or microbe attacks such a plant, it will encounter a high SM concentration immediately at the periphery when wounding or entering the

tissue, which might deter further feeding. Compounds that are sequestered in resin ducts or laticifers are often under high pressure and readily squirt out when these elements become wounded. For a small herbivorous insect, this will be a dangerous situation, since these effluents will make their mandibles sticky. A few 'clever' beetles and caterpillars cut the veins of leaves upstream to the area on which they want to feed. The fluids emerge from the cuts but can no longer reach the parts downstream, which are eaten later (Dussourd and Eisner, 1987; Becerra, 1994).

Several defence compounds are transported via the phloem from the site of synthesis to other plant organs. Since the phloem is a target for many sucking insects, such as aphids, these insects encounter a high load of alkaloids in the plants producing them. For lupins, in which alkaloid-rich and almost alkaloid-free varieties (sweet lupins) are known, it could be shown that aphid generalists (e.g. *Myzus persicae*) sucked only on 'sweet' lupins but never on alkaloid-rich varieties, with high alkaloid contents (Table 1.1) in the phloem (Wink, 1992). Moreover, many other animals, from leaf miners (Agromyzidae) to rabbits (*Oryctolagus cuniculus*) showed a similar discrimination, in that alkaloid-rich plants were left alone, while 'alkaloid-free' cultivars were highly susceptible. The only exception is a specialized aphid, *Macrosiphum albifrons*, which lives on lupins and sequesters the dietary alkaloids, using them for defence against predators (Wink and Römer, 1986).

In general, a series of related compounds is found in each plant: often a few major metabolites and several minor components, which differ in the

Table 1.1 Relationship between alkaloid content and percentage herbivory by aphids (generalists and specialists) and other herbivores

Species	Alkaloid content mg/g FW	Herbivory (%) by			
		Myzus spp.	Leaf miners	Rabbits	*Macrosiphum albifrons*
Lupinus albus					
var. *lucky*	<0.01	20	100	100	< 5
var. *lublanc*	<0.01	15	100	100	< 5
var. *multolupa*	0.03	15	100	80	<10
Wild-type from Syria	**2.0**	**0**	**<1**	**<10**	**100**
Wild-type from Crete	**2.2**	**0**	**<1**	**n.d.**	**100**
L. luteus	0.01	100	n.d.	n.d.	n.d.
	0.25	50	n.d.	n.d.	n.d.
	0.7	< 1	n.d.	<5	0
L. polyphyllus	**1.0**	**0**	**<1**	**<5**	**80**
L. angustifolius	**1.5**	**0**	**<1**	**<10**	**100**

n.d., not determined; FW, fresh weight.
Source: After Wink and Römer (1986), Wink (1987a, 1988, 1992), Wink and Witte (1991).

position of their functional groups. The profile usually varies between plant organs, within developmental periods and sometimes even diurnally, for example as shown for lupin alkaloids (Wink and Witte, 1984). Furthermore, marked differences can usually be seen between individual plants of a single population and even more so between members of different populations. This variation, which is part of the apparent evolutionary 'arms race' between plants and herbivores, makes adaptation by herbivores more difficult, since even small changes in chemistry can be the basis for a new pharmacological activity (for more details, see Chapter 2 in this book). Furthermore, mixtures can address a multitude of targets and might act additive or even synergistic. There is evidence, for example, that some SM with membrane activities can facilitate the uptake of polar substances and thus increase the bioavailability of allelochemicals (Wink, 2008a).

Defence against herbivores and pathogens is not necessarily constitutive. Research in recent decades has shown that wounding and infection triggers several events in plants. For example, wounding can lead to a decompartmentalization, thus releasing prefabricated defence chemicals and mixing them with hydrolyzing enzymes, such as β-glycosidase, myrosinase, nitrilase or alliinase (Matile, 1980) (Fig. 1.2; Table 1.2). Active allelochemicals are the result.

In other instances, it has been shown that the level of existing defence chemicals is increased substantially within hours or days after wounding or infection, for example nicotine in *Nicotiana tabacum* (Baldwin, 1994) or lupin alkaloids in *Lupinus polyphyllus* (Wink, 1983). After infection, in particular, new compounds with antifungal, antibacterial or herbivore-deterring activities are made and sequestered; phytopathologists have termed these compounds 'phytoalexins' (Fig. 1.2). These compounds include, among others, several isoflavones, pterocarpans, furancoumarins, chalcones and stilbenes.

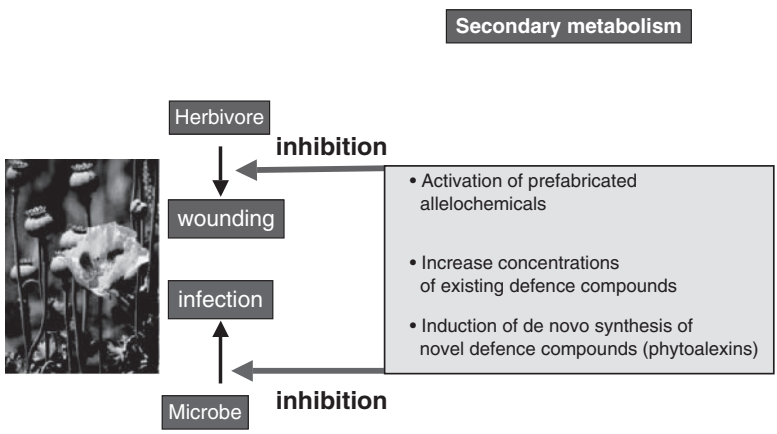

Figure 1.2 Examples of induced defence in plants. (See Plate 2 in colour plate section.)

Table 1.2 Typical 'prodrugs' present in plants that are activated by wounding, infection or in the human body

Secondary metabolites of undamaged tissue	Active metabolite
Cyanogenic glucoside	Hydrogen cyanide
Glucosinolate	Isothiocyanate
Alliin	Allicin
Coumaroylglucoside	Coumarin
Arbutin	Benzoquinone
Salicin, methylsalicylate	Saligenin, salicylic acid
Gein	Eugenol
Bi-desmosidic saponins	Mono-desmosidic saponins
Cardiac glycosides with terminal glucose residues	Cardiac glycosides without terminal glucose residues
Cycasin	Methylazoxymethanol
Ranunculin	Protoanemonine
Tuliposide	Tulipalin
Crocetin	Safranal
Cucurbitacin glycosides	Free cucurbitacins

Many of these metabolites have antifungal properties, so that they are sometimes considered to be part of the specific antimicrobial defence system of plants. However, since most of these compounds also affect herbivores, the plant defence induced appears to be a more general phenomenon (see also Chapter 4 in this book).

Recent research has shown that elicitors, receptors, ion channels, salicylic acid and the pathway leading to jasmonic acid and methyljasmonate are important elements in converting the external signal into a cellular response (Creelman and Mullet, 1997).

The SM defence system works in general but a number of herbivores and microorganisms have evolved that have overcome the defence barrier (analogous to the situation in which some viruses, bacteria or parasites overwhelm the human immune system). In these organisms, a series of adaptations can be observed, allowing them to tolerate or even use the dietary defence chemicals (a schematic overview is presented in Fig. 1.3) (for reviews, see Ahmad, 1983; Brattsten and Ahmad, 1986; Rosenthal and Berenbaum, 1991/1992; Wink, 1993a; Bernays and Chapman, 1994; Brown and Trigo, 1995; Hartmann and Witte, 1995).

Several volatiles are produced by plants when wounded, including aldehydes, esters, amines or ethylene. It has been proposed that some of these volatiles can alert the defence system of neighbouring plants. In addition, they can attract predatory arthropods. A well-studied example is that of spider mites (*Tetranychus urticae*) on *Phaseolus lunatus* leaves. Volatiles from infested plants attract predatory mites (*Phytoseiulus persimilis*), which prey on the mites that induced the reaction in the first place (Dicke *et al.*, 1990; De

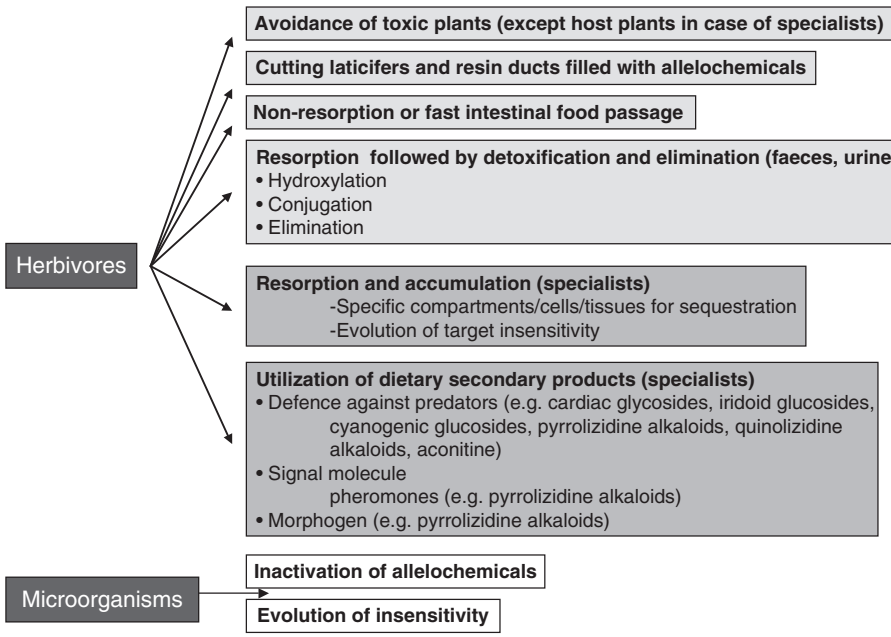

Figure 1.3 Adaptations of specialist herbivores and pathogens. (See Plate 3 in colour plate section.)

Moraes *et al.*, 1998). It is likely that more tritrophic systems work in this way; many of them still await discovery.

Chemical defence is not only obvious in terrestrial ecosystems but also of major importance in the survival of marine organisms. In this book, Chapter 3 provides an overview of this exciting and rapidly growing research field.

1.3 Molecular modes of action of SM

If defence compounds inhibit the growth of microbes or herbivores or are otherwise toxic to them, they must interfere with the physiology and biochemistry of these organisms. A large body of pharmacological and toxicological literature clearly documents that these activities do exist (Wink, 1993a,b, 1999, 2000, 2007, 2008a; Teuscher and Lindequist, 1994). Typical organ systems that are often affected by SM in animals are schematically illustrated in Fig. 1.4. Typical for animals are nerves and muscles; it is not surprising that many SM, especially alkaloids, immediately modulate elements of neuronal signal transduction, neuromuscular signalling and muscle contractions. If these defence compounds are animal specific, plants have the advantage that these compounds are not toxic for them (as nerves and muscles are absent in plants). In case of defence compounds with a broader specificity or which also affects

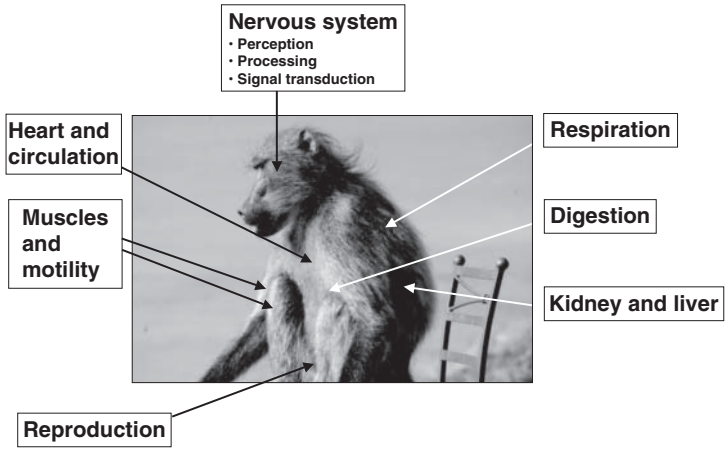

Figure 1.4 Targets for allelochemicals in animals. (See Plate 4 in colour plate section.)

targets in plants, special mechanisms are required in order to avoid autotoxicity, such as sequestration in specialized cells and tissues (see Vol. 2, Chapter 1).

In many instances, the mechanisms, which underlie these effects, have been elucidated; often specific interactions with one or several of the molecular targets shown in Fig. 1.5 can be observed. It has been argued that some defence compounds have been shaped during evolution to specifically interact with particular targets in a process termed 'evolutionary molecular modelling' (Wink, 1997). In this book, Chapters 2 and 4 explore this topic in more detail.

SM or toxins negatively interfere with molecular targets in cells of herbivores or pathogens. Major cellular targets (Fig. 1.5; Table 1.3) include:

- The biomembrane
- Proteins (including receptors, ion channels, enzymes, transporters, regulatory proteins, structure proteins, cytoskeleton, mitotic spindle [microtubules], transcription factors, hormones)
- Nucleic acids (DNA, RNA)

Allelochemicals can act as agonists or antagonists at a given molecular target. If this happens to a crucial cellular target, severe negative consequences result. For example, if a compound is cytotoxic to individual kidney cells, the effect will usually damage the function of the kidney. Organ damage can be so serious that it leads to coma and death. Acute cytotoxins are those, which inhibit or kill cells with high rates of protein synthesis, such as liver cells. Therefore, such cells are a prime target for many cytotoxic substances. In contrast, chronic exposure to mutagenic substances does not lead to immediate death but can cause cancer or teratogenic effects (for details, see Chapter 2 in this book).

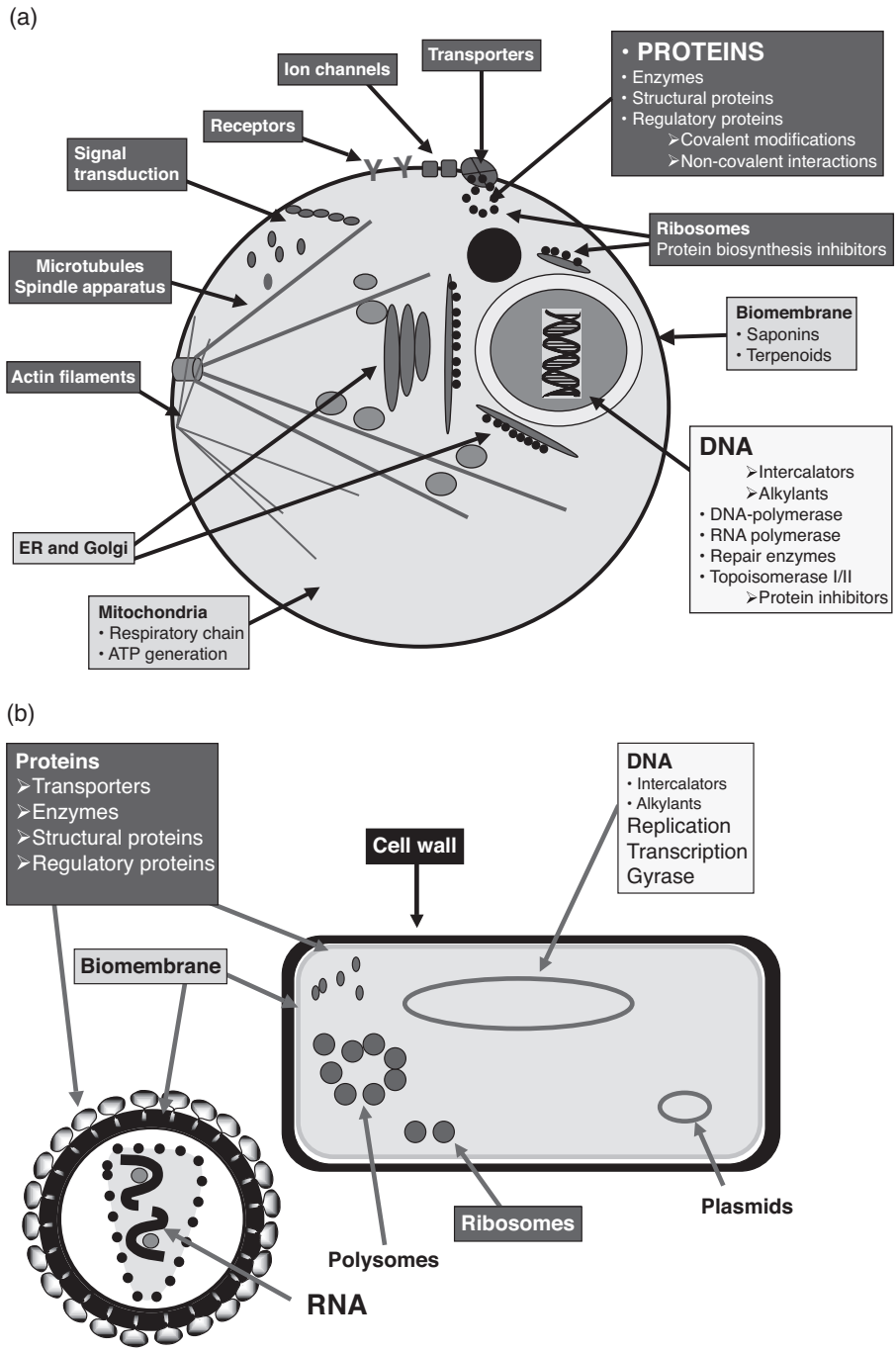

Figure 1.5 Molecular targets of defence chemicals in animal cells (a, b). (See Plate 5 in colour plate section.)

Table 1.3 Interaction of representative secondary metabolites with molecular targets

Target	Activity	Secondary metabolites (examples)
Biomembrane	Membrane disruption	Saponins
	Disturbance of membrane fluidity	Small lipophilic SM
	Inhibition of membrane proteins	Small lipophilic SM
Proteins Changing protein conformations		
	Non-covalent bonding	Phenolic SM (phenylpropanoids, flavonoids, catechins, tannins, lignans, quinones, anthraquinones, some isoquinoline alkaloids)
	Covalent bonding	Isothiocyanates Sesquiterpene lactones, allicin, protoanemonine, furanocoumarins, iridoids (aldehydes), SM with aldehyde groups, SM with exocyclic CH_2 groups SM with epoxide groups SM with cyclopropane rings
Specific interactions		
	Inhibition of enzymes	Hydrogen cyanide from cyanogens, many structural mimics
	Modulation of regulatory proteins	Phorbol esters, caffeine
	Inhibition of ion pumps	Cardiac glycosides;
	Inhibition of microtubule formation	Vinblastine, colchicine, taxol, podophyllotoxin,
	Inhibition of protein biosynthesis	Emetine, lectins
	Inhibition of transporters	Non-protein amino acids

(*Continued*)

Table 1.3 (*Continued*)

Target	activity	SM (examples)
	Modulation of hormone receptors	Genistein, many other isoflavonoids
	Modulation of neuroreceptors	Many alkaloids, some non-protein amino acids
	Modulation of ion channels	Aconitine, many alkaloids; conotoxins, tetrodotoxin, saxitoxin, gonyautoxin,
	Modulation of transcription factors	Cyclopamine, hormone mimics
DNA/RNA	Covalent modifications (alkylation)	Pyrrolizidine alkaloids, cycasine Aristolochic acids, Furanocoumarins, SM with epoxy groups
	Intercalation	Planar, aromatic and lipophilic SM Sanguinarine, berberine, emetine, quinine, furanocoumarins; anthraquinones
	Inhibition of DNA topoisomerase I	Camptothecin, berberine
	Inhibition of transcription	Amanitine

The **biomembrane** is affected by lipophilic or amphiphilic compounds (Table 1.3), which are widely present among terpenoids. These compounds will interact with the lipophilic inner core of biomembranes represented by phospholipids and cholesterol. They can form transient pores or may even solubilize biomembranes. These compounds can additionally interact with membrane proteins, such as ion channels, transporters and receptors, thereby influencing signal transduction and transport processes in cells and tissues. These interactions are widely non-specific. Therefore, membrane-active SM can promote cytotoxic effects in animal, bacterial and fungal cells, even in some enveloped viruses. Some saponins may facilitate the absorption of polar compounds (see discussion in Wink, 2008a).

Proteins are the main players in cells, important for metabolism, structures, motility, cell division, gene regulation and communication. Proteins need to have the correct three-dimensional shape (conformation) in order to recognize their substrates, ligands and other protein partners. SM very often are capable of interfering with proteins (Table 1.3), especially by inducing conformational changes. These conformational changes can either activate (agonists) or inactivate (antagonist) a protein. We can distinguish between covalent and non-covalent interactions. Some SM have highly reactive functional groups, such as aldehydes, epoxides, sulfhydryls, exocyclic methylenes or cyclopropanes, which can make covalent bonds with functional groups of proteins (Table 1.3). Phenolic compounds carry phenolic hydroxyl groups that can dissociate under physiological conditions into negatively charged O^- groups. These O^- groups can form hydrogen bonds or more stable ionic bonds with positively charged amino acid residues (as present in lysine, arginine or histidine). A single of such non-covalent bonds is weak and hardly influences protein conformation. In polyphenols, we usually see several phenolic OH groups; together, they can react cooperatively and effectively induce conformational changes. Such interactions are likely to be the cause for many of the adverse effects of SM on herbivores, microbes and viruses (Wink and Van Wyk, 2008) (for details, see Chapter 2 in this book).

Nucleic acids, such as DNA, rRNA, mRNA and the corresponding enzymes for replication, transcription and repair, are another major target for allelochemicals. Nucleic acids can be modified by alkylation and intercalation (Table 1.3). These interactions with DNA can lead to point mutations, which can cause amino acid substitutions or frame shift mutations, that usually results in detrimental effects if the mutations are not repaired by repair enzymes (for details, see Chapter 2 in this book).

1.4 Biotechnology and utilization of SM

Since SM have evolved as compounds that are important for the fitness of the organisms producing them, many of them interfere with the pharmacological targets, which make them interesting for several biotechnological

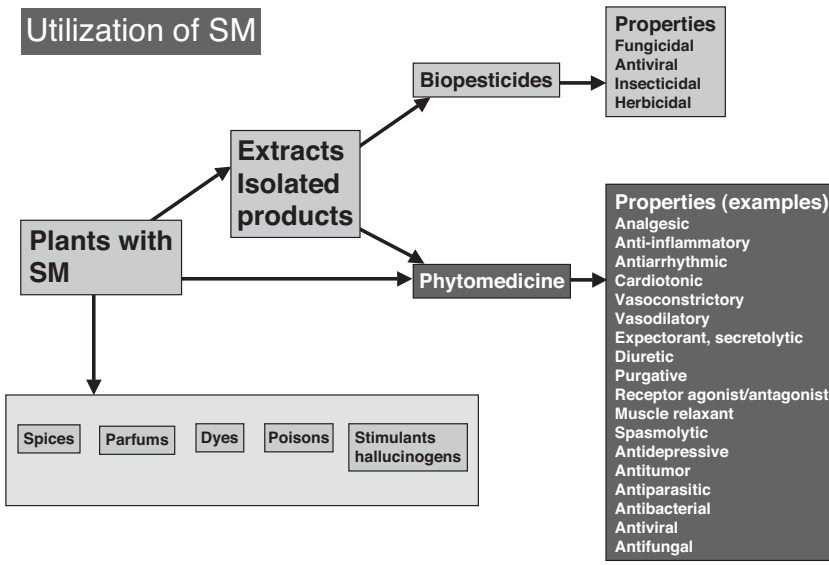

Figure 1.6 Utilization of secondary metabolites (SM) in biotechnology. (See Plate 6 in colour plate section.)

applications (an overview is presented in Fig. 1.6). A main area is phytomedicine, and several thousand plants are in use worldwide to treat human ailments and diseases (Fig. 1.6). In addition to isolated substances with established pharmacological profiles (including potent antineoplasmic drugs, such as the alkaloids vinblastine, vincristine or taxol) (Wink, 2007), complex extracts or even crude plant drugs are often used (Wink, 2008a). Controlled clinical studies have shown the efficacy of several, for example extracts from *Ginkgo biloba*, *Hypericum perforatum*, *Piper methysticum*, *Chamomilla recutita*, *Crataegus monogyna*, *Silibum marianum*, *Melissa officinalis*, *Mentha x piperita*, *Valeriana officinalis* (Wagner and Wiesenauer, 1995; van Wyk and Wink, 2004). The use of stimulants (such as caffeine, nicotine, ephedrine), fragrances (several essential oils), flavours (essential oils, capsaicin, piperine, etc.), natural dyes, poisons (strychnine) and hallucinogens (morphine, heroin, cocaine, tetrahydrocannabinol) is based on SM (Fig. 1.6). In this book, Chapters 4 and 5 explore this wide field in more detail.

Since many SM are insecticidal, fungicidal and phytotoxic, they may be used in agriculture as natural plant protectants. Before the advent of synthetic pesticides about 60 years ago, plant-derived insecticides (including nicotine, rotenone, quassin, ryanodine, pyrethrins and azadirachtins) were a common theme (Jacobson and Crosby, 1971; Wink, 1993b). Applications unequivocally showed that these natural insecticides worked. One ecological advantage, that is that SM are readily degraded in plants and in soil, is also their disadvantage and synthetic pesticides are more resistant and persistent.

Moreover, modern pesticides are usually more potent than biopesticides. On the other hand, plants are easy to grow and biopesticides could be a sustainable source of plant protectants for farmers in countries that do not have access to Western synthetic pesticides. Unfortunately, legislation does not favour mixtures of compounds to be used as pesticides; therefore, the development of biorational pesticides has to face many obstacles. Nevertheless, natural compounds do provide an underexplored alternative (for review, see Wink, 1993b).

As a consequence of these various applications, a world market for plant extracts and isolated SM exists, which exceeds 10 billion US dollars annually (Balandrin *et al.*, 1985). Therefore, it is a challenge for biotechnologists to find ways to produce these compounds in sufficient quantity and quality (Wildi and Wink, 2002; see Chapter 6 of this volume). The main and traditional way is to grow the respective plants in the field or in greenhouses and to extract the products from them (Fig. 1.7). For several species, new varieties have been selected with improved yields and quality. In this context, cell and organ culture are important techniques for in vitro propagation. In a few instances, genetic engineering of secondary metabolism has already had a direct influence; for example, when *Atropa belladonna* plants were transformed with the gene that encodes the enzymes converting L-hyoscyamine into L-scopolamine, new plants were generated which produced scopolamine as the major product (Hashimoto and Yamada, 1992). More often, flavonoid metabolism has been

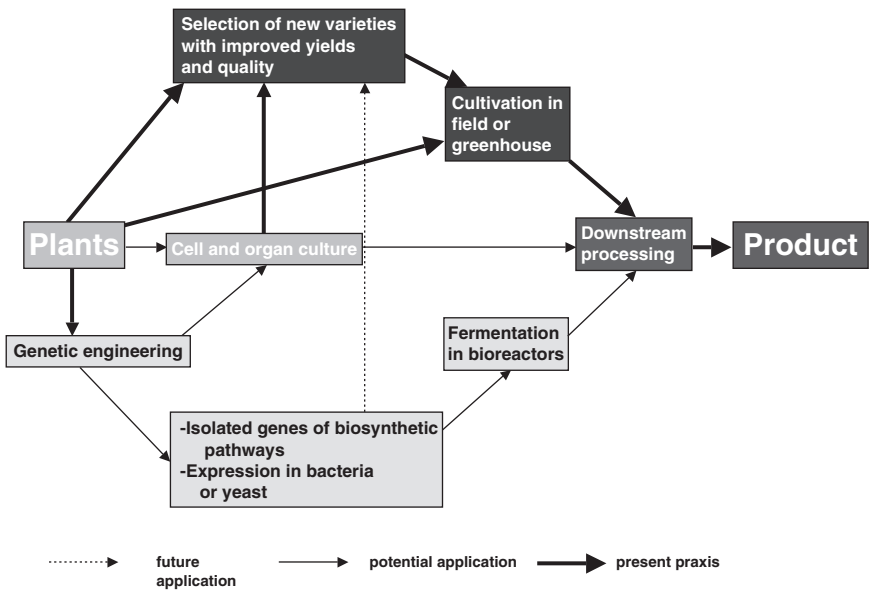

Figure 1.7 Strategies for the production of secondary metabolites. (See Plate 7 in colour plate section.)

altered genetically, producing plants with different flower colours (Mol *et al.*, 1998).

It is a challenge for future research to isolate the genes of biosynthetic pathways and to express them either in transgenic plants or in microbes. If successful, recombinant bacteria or yeasts might be grown some day, which will produce valuable plant SM (Wink, 1989; Kutchan, 1995; Facchini, 2001) (Fig. 1.7); combinatorial biosynthesis might then be an open field. Using genes encoding enzymes for the biosynthesis of antibiotics, this strategy has already been successful (Katz and Hutchinson, 1992; Katz and Donadio, 1993; McDaniels *et al.*, 1993). Interest in growing and manipulating microorganisms and plants in cell culture for commercial purposes (Verpoorte *et al.*, 2007; Oksman-Caldentey *et al.*, 2007) has given impetus to the study of the biosynthesis of alkaloids and other SM and, in particular, to the elucidation of the enzymes involved. It has also brought about renewed interest in the regulation of SM 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; Schäfer and Wink, 2009). Ultimately, it might be possible to produce valuable alkaloids from recombinant bacteria or yeast.

If the corresponding SM (both from plant or microbial origin) confer resistance to insects or pathogens, genetic transformation of susceptible crop plants could be another valuable avenue for exploitation.

For more than two decades, scientists around the world have tried to produce valuable SM in cell or organ cultures (for overviews, see Neumann *et al.*, 1985; Constabel and Vasil, 1987; Kurz, 1989; Charlwood and Rhodes, 1990). Whereas undifferentiated cell cultures have often failed to produce such a compound in reasonable yields, differentiated organ cultures (e.g. transformed root cultures) are often as active as the intact plant (Rhodes *et al.*, 1990; Wink *et al.*, 2005). Cell- and tissue-specific gene expression appears to control these processes. In this book, Chapter 6 addresses the production of SM in vitro. It is possible that genetic engineering may help to improve plant cell cultures as biotechnological production systems in the future.

1.5 Conclusions

Plant secondary metabolism is still an interesting and challenging field of scientific endeavour ranging from botany, plant physiology and biochemistry, chemistry, pharmacology and medicine to evolution, ecology and biotechnology. It is the aim of the two volumes (*Biochemistry of Plant Secondary Metabolism and Functions* and *Biotechnology of Plant Secondary Metabolites*) to highlight recent results and to stimulate young researchers to enter a field that looks back on a long history but nevertheless provides an interesting present-day research topic and will hopefully experience an exciting future.

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Chapter 2

MOLECULAR MODES OF ACTION OF DEFENSIVE SECONDARY METABOLITES

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Abstract: Secondary metabolites (SM) have been shaped by evolution for more than 500 million years. As a result, many of them have distinctive biochemical and pharmacological properties. The molecular modes of action of the main groups of SM are reviewed in this chapter. Details are given on interactions of SM with proteins that can induce conformational changes and thus a modification of their bioactivity. The fluidity and permeability of biomembranes constitute another important target, which is influenced by many lipophilic and amphiphilic SM. A number of SM can either alkylate or intercalate DNA, which can cause mutations and in consequence cancer or malformations. Many SM are cytotoxic because they interfere with biomembranes, proteins of the cytoskeleton or DNA; they often induce programmed cell death (apoptosis). A large number of SM, especially alkaloids modulate neuronal signal transduction by interfering with ion channels, ion pumps, neuroreceptors, choline esterase, monoamine oxidase and other enzymes related to signal transduction pathways. A typical feature of SM is their ability to modulate more than one molecular target; thus, additive and even synergistic activities can be expected.

Keywords: defence; attraction; herbivores; microbes; molecular modes of action; DNA alkylation; DNA intercalation; mutations; cytotoxicity; apoptosis; molecular targets; neuronal signalling; alkaloids; terpenoids; cyanogenic glucosides; phenolics

2.1 Introduction

Since only autotrophic plants can convert light energy via photosynthesis into organic compounds, heterotrophic animals and most microorganisms

depend on plant material as an energy source or for vital precursors and vitamins. In order to survive, plants have had to develop defence strategies against herbivores, microbes (bacteria, fungi), viruses and even against other plants competing for light, space and nutrients. Efficient defence strategies must exist because the world is still green, despite a multitude of herbivores and microorganisms (Hartley and Jones, 1997). Many plants are avoided by herbivores (an obvious exception being crop plants, in which chemical defence compounds have been selected away by plant breeders).

Plants are always a rich source of compounds that do not appear essential for primary metabolism, including thousands of secondary metabolites (SM) and several macromolecules, such as peptides, proteins, enzymes, lignin, callose, cellulose or cuticular waxes. In addition to their function in physiology or in structural maintenance, many serve for defence against microbes or herbivorous animals (allelochemicals). In addition, some SM (e.g. flavonoids, anthocyanins, tetraterpenes and monoterpenes) function as signal compounds to attract pollinating and seed-dispersing animals. Some of these compounds exhibit both defence and signal functions at the same time (for reviews, see Wink, 1992, 1993a,b,c, 1997, 2003, 2007a,b,c 2008c; Harborne, 1993; Bernays and Chapman, 1994).

A large body of experimental, toxicological data and circumstantial evidence clearly shows that many alkaloids, cyanogenic glucosides, glucosinolates, terpenes, saponins, tannins, anthraquinones, polyacetylenes and other allelochemicals are toxic or deterrent to animals (insects, vertebrates), and several display antibiotic or even allelopathic activities (for overviews, see Levin, 1976; Swain, 1977; Rosenthal and Janzen, 1979; Waller, 1987; Wink, 1988, 1993a,b,c; Rosenthal and Berenbaum, 1991, 1992; Harborne, 1993; Bernays and Chapman, 1994; Roberts and Wink, 1998; Wink and Van Wyk, 2008).

Allelochemicals can only function as chemical defence compounds if they are able to influence herbivores or microbes in a negative way. A closer analysis shows clearly that most allelochemicals interfere with one or several molecular targets in animals and microbes; i.e. they are usually multi-target compounds, which exhibit pleiotropic effects (Efferth *et al.*, 2007; Wink, 1992, 1993a, 1998, 2000, 2007a,b,c 2008b,c; Wink *et al.*, 1998a,b). Some structures of allelochemicals appear to have been shaped during evolution in such a way that they can mimic the structures of endogenous substrates, hormones, neurotransmitters or other ligands; this process can be termed 'evolutionary molecular modelling'. Other metabolites are less specific and intercalate or alkylate DNA, inhibit DNA- and RNA-related enzymes, protein biosynthesis, various proteins or disturb membrane stability and permeability.

2.2 Molecular modes of action – an overview

This chapter provides a short overview of the modes of action of some important groups of SM (Wink, 1993a, 1999a, 2007, 2008a,b; Teuscher and

Lindequist, 1994; Roberts and Wink, 1998; van Wyk and Wink, 2004; Wink and Van Wyk, 2008), followed by a more detailed analysis of allelochemicals (especially alkaloids) with cytotoxicity, their influence on neuronal signal transduction and DNA/RNA.

In general, **alkaloids** are infamous as animal toxins and certainly serve mainly as defence chemicals against predators (herbivores, carnivores) and to a lesser degree against bacteria, fungi and viruses (Levin, 1976; Swain, 1977; Wink, 1988, 1992, 1993a, 2008b; Hartmann, 1991; Harborne, 1993; Bernays and Chapman, 1994; Roberts and Wink, 1998). Alkaloids and amines often affect neuroreceptors as agonists or antagonists, or they modulate other steps in neuronal signal transduction, such as ion channels or enzymes, which take up or degrade neurotransmitters or second messengers. Since alkaloids often derive from the same amino acid precursor as the neurotransmitters, acetylcholine (ACh), serotonin, noradrenaline (NA), dopamine, gamma-aminobutyric acid (GABA), glutamic acid or histamine, their structures can frequently be superimposed on those of neurotransmitters. Other alkaloids intercalate DNA, alkylate DNA (see Section 2.2.3), induce apoptosis or inhibit carbohydrate-processing enzymes (Goss *et al.*, 1995). It is apparent that the toxicity of most alkaloids is correlated with their interactions with particular molecular targets (Wink, 1993a, 1999b, 2000, 2007a; Wink *et al.*, 1998a,b; Roberts and Strack, 1999; Wink and Van Wyk, 2008).

Non-protein amino acids (NPAAs) can be considered as structural analogues to 1 of the 20 protein amino acids. NPAAs frequently block the uptake and transport of amino acids or disturb their biosynthetic feedback regulations. Some NPAAs are even incorporated into proteins, since transfer ribonucleic acid (tRNA) transferases cannot usually discriminate between a protein amino acid and its analogue; resulting in defective or malfunctioning proteins (Rosenthal, 1982). Other NPAAs interfere with neuronal signal transduction or enzymatic processes (Teuscher and Lindequist, 1994; Selmar, 1999, 2010; Wink and Van Wyk, 2008).

Cyanogenic glucosides are stored in the vacuole as prefabricated allelochemicals ('prodrug' principle). If tissue decomposition occurs due to wounding by a herbivore or a pathogen, then a β-glucosidase comes into contact with the cyanogenic glucosides, which are split into a sugar and a nitrile moiety that is further hydrolyzed to hydrocyanic acid (HCN) and an aldehyde. HCN binds to cytochrome oxidase and therefore effectively blocks mitochondrial respiration; in consequence, adenosine triphosphate (ATP) production is blocked. Therefore, HCN functions as a strong poison in most animals (Conn, 1980; Selmar, 1999, 2010; van Wyk and Wink, 2004; Wink and Van Wyk, 2008).

Glucosinolates also function as prefabricated vacuolar defence compounds. The resulting mustard oil, which is released after cleavage by myrosinase, is highly lipophilic and can disturb the fluidity of biomembranes (thereby exhibiting a substantial antimicrobial effect) and bind to various enzymes, receptors or other macromolecules, such as DNA (see Sections 2.2.2.1 and 2.2.1.3) (Selmar, 1999, 2010; Wink and Van Wyk, 2008).

Terpenes (mono-, sesqui-, di- and triterpenes) are usually highly hydrophobic substances and are stored in resin ducts, oil cells or glandular trichomes. Most of them readily interact with biomembranes. They can increase the fluidity of the membranes, which can lead to uncontrolled efflux of ions and metabolites, modulation of membrane proteins and receptors or even to cell leakage, resulting in cell death. This membrane activity is rather non-specific; therefore, terpenes show activities against a wide range of organisms, ranging from bacteria and fungi to insects and vertebrates. Many terpenes are even effective against membrane-enclosed viruses. Even if the concentrations were not critical for a large vertebrate herbivore, terpene-rich food is usually avoided, since these terpenes would inhibit the growth of rumen microorganisms, which are important for the breakdown of cellulose. A number of terpenes have special additional activities because their structures figure as analogues to natural substrates, hormones (e.g. steroidal hormones, sex hormones, ecdysone, juvenile hormone) or neurotransmitters (Kreis and Müller-Uri, 2009). Several diterpenes are quite toxic; phorbol esters (present in Euphorbiaceae and Thymelaeaceae) activate protein kinase C and therefore cause severe inflammation. Grayanotoxin I (or andromedotoxin), which are common in Ericaceae, are potent inhibitors of sodium channels and thus strong neurotoxins (Wink and Van Wyk, 2008).

Saponins are the glycosides of triterpenes or steroids and include the group of cardiac glycosides and steroidal alkaloids. Some of them are stored as bidesmosidic compounds in the vacuole, which are cleaved to the active monodesmosidic compounds by β-glucosidase upon wounding-induced decompartmentation (Wink and Van Wyk, 2008). Monodesmosidic saponins are amphiphilic compounds, which can complex cholesterol in biomembranes with their lipophilic terpenoid moiety and bind to surface glycoproteins and glycolipids with their sugar side chain. This leads to a severe tension of the biomembrane and leakage. This activity can easily be demonstrated with erythrocytes, which lose their haemoglobin when in contact with monodesmosidic saponins. This membrane activity is rather unspecific and effects a wide set of organisms from microbes to animals. Some saponins have additional functional groups, such as **cardiac glycosides** (carrying a five- or six-membered cardenolide or bufadienolide ring), which enable them to inhibit one of the most important molecular targets of animal cells, the Na^+-, K^+-ATPase (Ashour *et al.*, 2010; Kreis and Müller-Uri, 2010; Wink and Van Wyk, 2008). Among steroidal glycosides, the cucurbitacins (occurring in members of the Cucurbitaceae) express substantial cytotoxic activities (Wink and Van Wyk, 2008).

Polyketides include anthraquinones, which produce severe diarrhoea in vertebrates by interfering with intestinal Na^+-, K^+-ATPase and adenylyl cyclase. These compounds can also interact with DNA (see Section 2.2.2.3).

Flavonoids and phenylpropanoids (including coumarins, furanocoumarins, catechins and tannins) are widespread in plants (Petersen *et al.*, 1999, 2010). They exhibit a wide range of biological activities. In several instances,

they act as analogues of cellular signal compounds or substrates. Afflicted mechanisms range from prostaglandin and leukotriene formation, enzyme inhibition, estrogenic properties (coumarins, isoflavones, stilbenes) to DNA alkylation (e.g. by furanocoumarins) (see Section 2.2). These molecules usually have several phenolic hydroxyl groups in common, which can dissociate in negatively charged phenolate ions. Phenolic hydroxyl groups form hydrogen and ionic bonds with proteins and peptides. The higher the number of hydroxyl groups, the stronger the astringent and denaturing effect (Wink, 2008b). Tannins inhibit enzymatic activities very effectively; however, most digestive enzymes of herbivores have apparently adapted to tannins during evolution and are less sensitive than other enzymes.

2.2.1 Physicochemical mechanisms

A short overview of the localization of potential targets in animal, bacterial cells and viruses has been given in Figs 1.5a–1.5c. The modulation of a molecular target will negatively influence its communication with other components of the cellular network, especially proteins (crosstalk between proteins), elements of signal transduction (including gene expression) or membrane functions. As a consequence, the metabolism and function of cells, tissues, organs and eventually the whole organism will be affected. Although we know the structures of many SM, our knowledge concerning the molecular modes of action is largely fragmentary and incomplete. Such knowledge is, however, important in order to understand the functions of SM for the producing organism, and for the rational utilization of SM in medicine or plant protection.

Major cellular targets for allelochemicals include:

- proteins and three-dimensional structure of proteins, i.e. their conformation (including receptors, enzymes, ion channels, transporters, regulatory proteins, structural proteins, cytoskeletal proteins, microtubules forming the mitotic spindle, transcription factors, hormones),
- the biomembrane (fluidity, permeability),
- nucleic acids (DNA, RNA).

2.2.1.1 Proteins and protein conformation

Proteins are probably the most important molecular target in cells and viruses. Proteins have multiple functions, ranging from catalytic enzymes, transporters, ion channels, receptors, microtubules, histones to regulatory proteins (signal molecules, transcription factors controlling gene expression, etc.) and structural proteins (as present in the viral coat or capside). Proteins can only work properly if they have the correct three-dimensional structure, called conformation. Conformational changes alter their properties and can prevent effective crosstalk between proteins and between proteins and other targets (DNA, RNA). Protein activities are often regulated by

phosphorylation or dephosphorylation. The addition or subtraction of such a bulky group induces a conformational change. Probably most SM that have been found in nature interact with proteins in one or another way (binding, complexing, denaturing) by changing protein conformation.

Most SM interfere with proteins in an **unselective** way, i.e. they affect any suitable protein that they encounter (Figs 2.1 and 2.2). An important strategy is to form covalent bonds with a protein, often by binding to free amino-, SH- or OH- groups (Table 1.3; Fig. 2.1). SM with aldehyde, epoxide and cyclopropane groups (found in several terpenes, iridoids, phenylpropanoids)

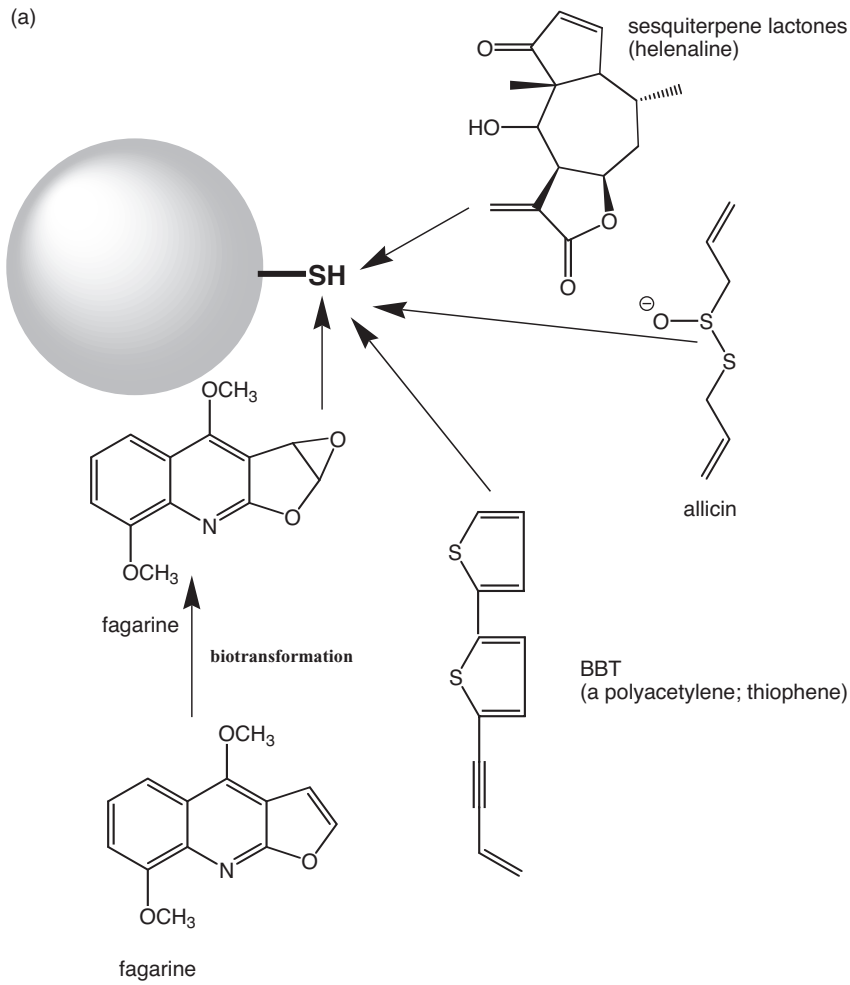

Figure 2.1 Examples of secondary metabolites that can modify proteins by forming covalent bonds. (a) Reactions with SH-groups of proteins;

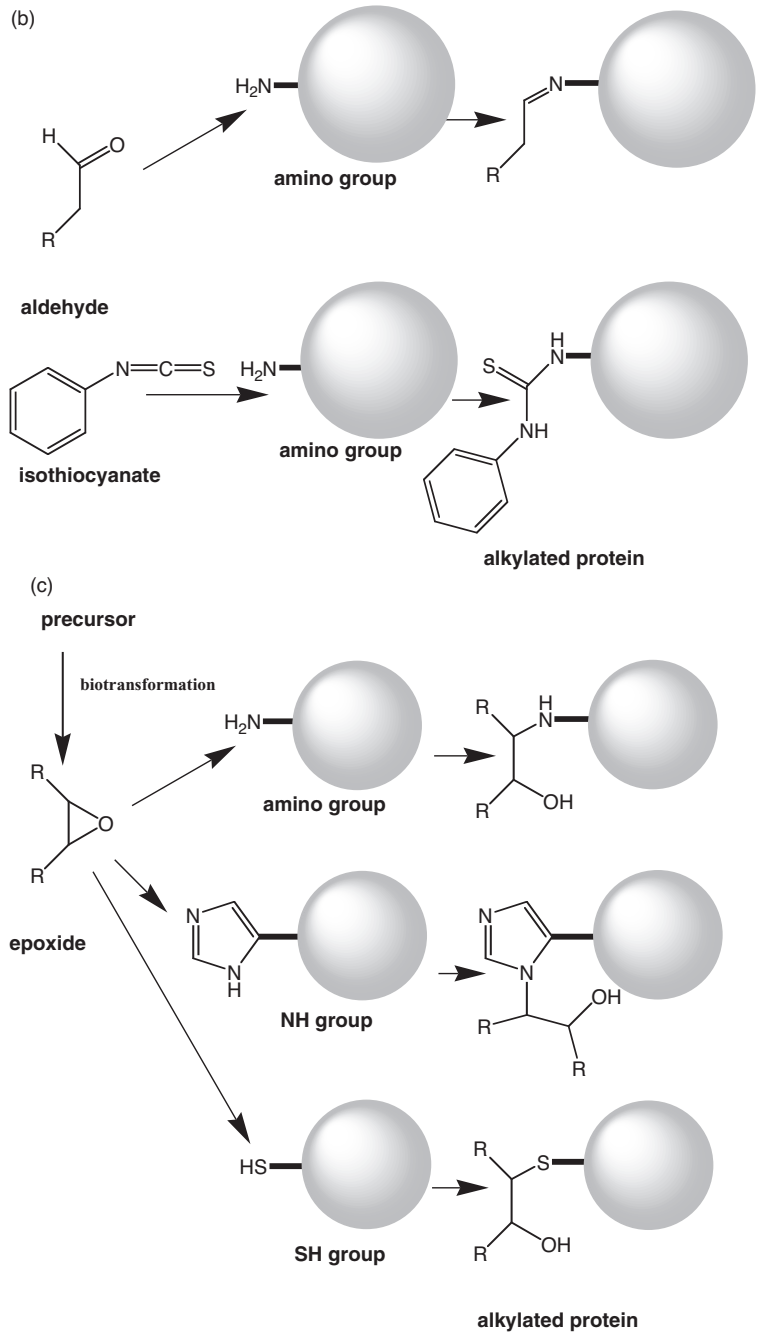

Figure 2.1 (*Continued*) (b) reactions of aldehydes with amino acids of proteins; (c) reactions of epoxides wuth amino groups of proteins. (See Plate 8 in colour plate section.) (© Bentham Science Publishers Ltd.)

bind to amino groups, SH reagents (compounds with activated double bonds or exocyclic methylene groups; such as sesquiterpene lactones, furanocoumarins, phenylpropenes) and epoxides couple to free SH groups or quinones attack metal proteins or oxidize/reduce target molecules. The covalent modification can lead to a conformational change and thus loss of activity; or protein turnover is altered because proteases can no longer break down the alkylated protein. SM with reactive functional groups that are able to undergo electrophilic or nucleophilic substitutions are represented by isothiocyanates, allicin, protoanemonin, tulipalin, iridoid aldehydes, furanocoumarins, valepotriates, sesquiterpene lactones and SM with active aldehydes, epoxide or terminal and/or exocyclic methylene groups (Table 1.3; Fig. 2.1). In several instances, the reactive metabolites are not natively present in plants. They can be converted to active metabolites by the wounding process (by released metabolizing enzymes) either inside the producing organism or in the body of a herbivore/predator (after biotransformation in intestine or liver).

A major class of plant metabolites are phenolic substances. Polyphenols, include structures, such as phenylpropanoids, flavonoids, catechins, tannins, lignans, quinones, anthraquinones, and several alkaloids with one or several with phenolic hydroxyl groups (Fig. 2.2a and 2.2b). The phenolic hydroxylgroups can partly dissociate under physiological conditions resulting in -O^- ions. The polyphenols have in common that they can interact with proteins by forming hydrogen bonds and the much stronger ionic bonds with electronegative atoms of the peptide bond or the positively charged side chains of basic amino acids (lysine, histidine, arginine), respectively. A single of these non-covalent bonds is quite weak. But because several of them are formed concomitantly when a polyphenol encounters a protein, a change in protein conformation or a loss in protein flexibility is likely to occur that commonly leads to protein inactivation. Since most polyphenols are quite polar and therefore hardly absorbed after oral intake, they are usually not regarded as serious toxins (Wink and Van Wyk, 2008).

If an SM covalently binds to a protein of the human body, new epitopes are generated that can be recognized and attacked by the immune system. As a consequence, antibodies are formed against these modified proteins. If an animal or person comes into contact again with such a toxin and if identical protein derivates are formed, an acute immune response can occur in form of allergies or sometimes with life-threatening anaphylactic shock.

Other allelochemicals are more selective. They can recognize and bind to the active binding site of an enzyme or receptor; they often mimic endogenous ligands (Table 1.3). Well-studied examples of specific interactions include alkaloids that are structural analogues of neurotransmitters, e.g. nicotine and hyoscyamine are mimics of ACh; nicotine binds to nicotinic acetylcholine receptors (nAChR), whereas hyoscyamine is specific for muscarinic acetylcholine receptors (mAChR). The various steps in neuronal signalling and signal transduction provide central targets that are affected by several amines

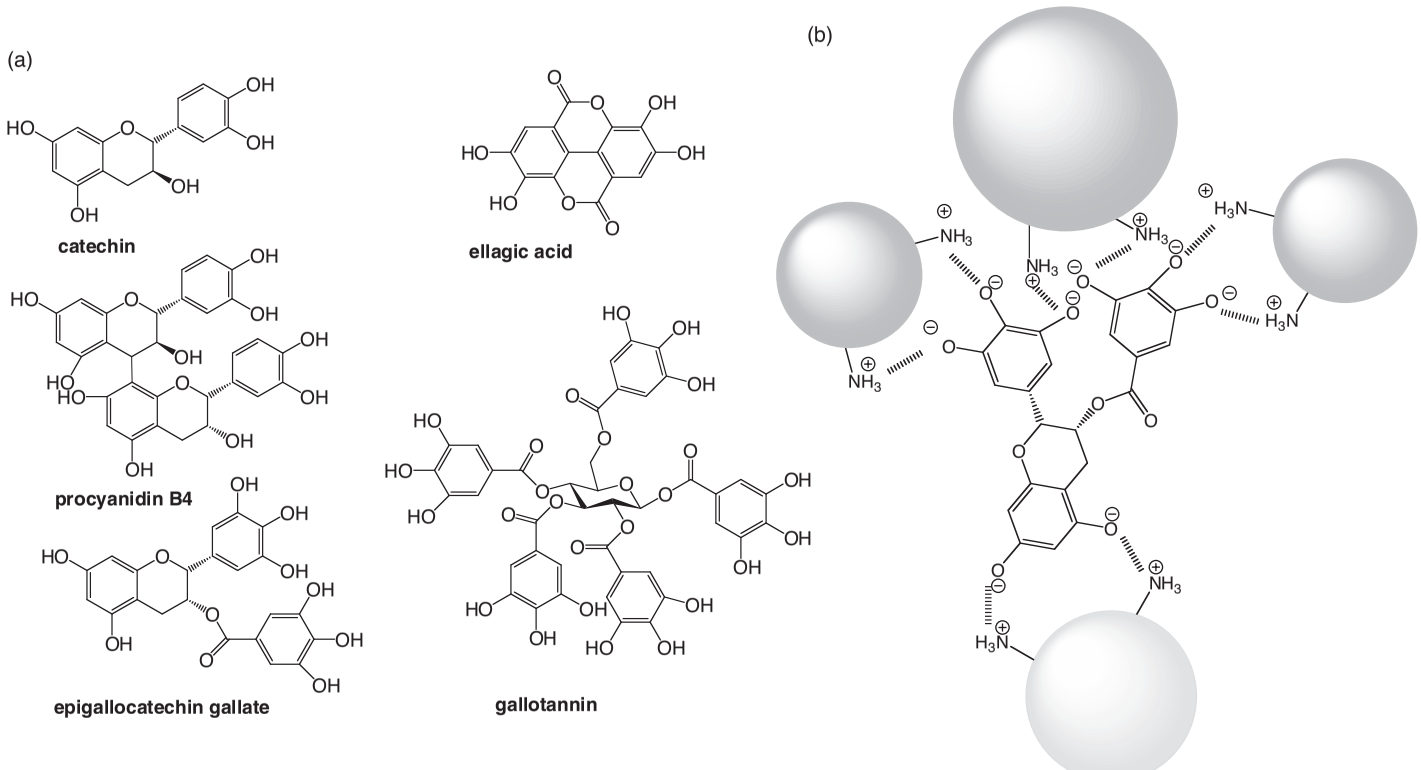

Figure 2.2 Examples of common polyphenols found in plants (a). Epigallocatechin gallate (EGCG) as a typical polyphenol can modify proteins by forming non-covalent bonds with one or several proteins (mainly ionic bonds) (b). (Abbas and Wink, 2009). (See Plate 9 in colour plate section.) (© Bentham Science Publishers Ltd.)

and alkaloids in a specific way; they are discussed in more detail in Section 2.2.3. SM interacting with neuroreceptors and neuronal signalling are often strong poisons or stimulants/hallucinogens (Roberts and Wink, 1998; Wink and Van Wyk, 2008).

Another important class of selective toxins, the cardiac glycosides, inhibits Na^+, K^+-ATPase, one of the most important targets in animal cells, responsible for the maintenance of Na^+ and K^+ gradients in all cells, especially in nerve cells. Cardiac glycosides bind to an extracellular loop of the ion pump and inhibit it. A number of diterpenes are infamous for their toxic properties (cytotoxicity, inflammation), such as phorbol esters of Euphorbiaceae and Thymelaeaceae. They specifically activate protein kinase C, which is an important key regulatory protein in animal cells. Or another diterpene forskolin acts as a potent activator of adenynyl cyclase.

Microtubule formation (polymerization step) is another specific target in cells. Microtubule inhibitors include the alkaloids vinblastine (from *Catharanthus roseus*), colchicine (*Colchicum autumnale*), maytansine (from *Maytenus ovatus*, *Putterlickia verrucosa*; Celastraceae) or the lignan podophyllotoxin (from *Podophyllum* and several *Linum* species). Taxol is a diterpene alkaloid (paclitaxel, taxol®) that can be isolated from several yew species (including the North American *Taxus brevifolia* and the European *Taxus baccata*). Taxol stabilizes microtubules and thus blocks cell division in the late G_2 phase; because of these properties, taxol has been used for almost 10 years with great success in the chemotherapy of various tumours. The cytotoxic SM cause immunosuppression since they also block the multiplication of immune cells. They also disturb microtubule formation in neuronal axons causing neuronal disturbances.

Specific protein inhibitors can be found in the class of NPAAs that often figure as antinutrients or antimetabolites in many plants (e.g. in Fabaceae). Many NPAAs mimic 1 of the 20 protein amino acids and interfere with almost any enzyme or transporter involved in the biochemistry and physiology of protein amino acids.

Another example for a more specific inhibitor is HCN released from cyanogenic glucosides that are common SM in plants and some invertebrates. HCN is highly toxic for animals or microorganisms due to the inhibition of enzymes of the respiratory chain (i.e. cytochrome oxidase) because it blocks the essential ATP production. HCN also binds to other enzymes containing heavy metal ions.

2.2.1.2 Fluidity and permeability of the biomembrane

The **biomembrane** surrounds every living cell and functions as a permeation barrier. It prevents polar molecules leaking out of the cell or unwanted molecules entering a cell. Several SM exist in nature that interfere with membrane permeability (Table 1.3). Most famous are saponins that occur widely in the plant kingdom and less commonly in animals; monodesmosidic saponins are **amphiphilic** and function basically as detergents that can solubilize or

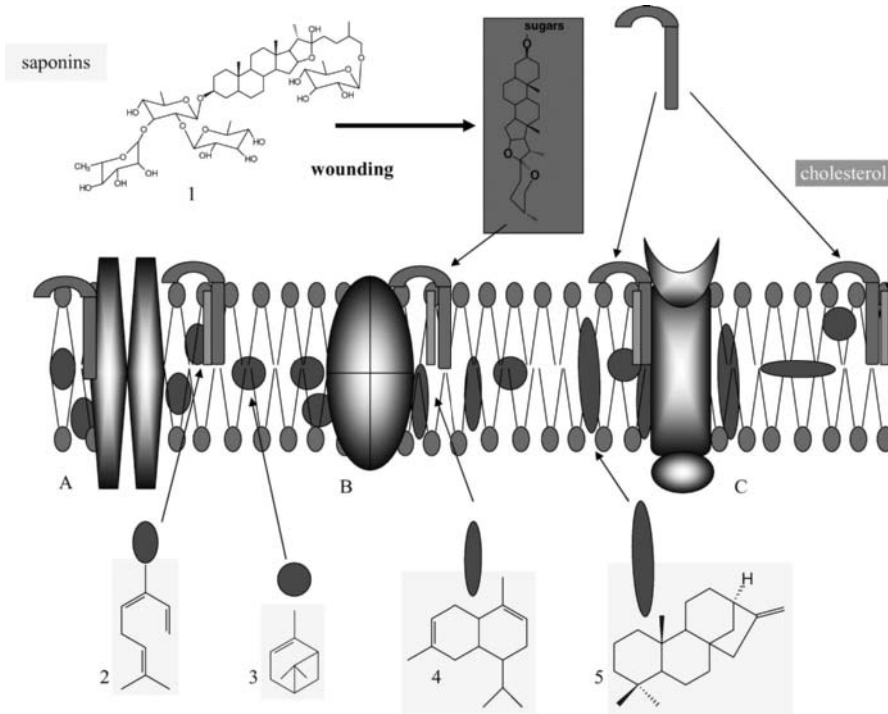

Figure 2.3 Examples of secondary metabolites that can modulate membrane permeability and the conformation of membrane proteins. A = ion channels, B = transporters; C = receptors; 1 = bidesmosidic steroidal saponin that becomes a monodesmosidic saponin after hydrolysis; 2 = example for a simple monoterpene, 3 = example for a simple cyclic monoterpene; 4 = example for a simple sesquiterpene; 5 = example for a diterpene. (See Plate 10 in colour plate section.)

destabilize biomembranes. With their lipophilic moiety they are anchored in the lipophilic membrane bilayer (often complexing with cholesterol), whereas the hydrophilic sugar part remains outside and interacts with other glycoproteins or glycolipids (Fig. 2.3). As a result, transient or permanent pores are generated in the membrane and make it leaky. This property can be shown easily with red blood cells. If saponins are present, a haemolysis takes place, i.e. haemoglobin flows out of the cells.

Also other lipophilic SM, such as mono-, sesqui- and diterpenes (which are hardly soluble in water) bind to biomembranes and can disturb membrane fluidity and permeability at higher concentrations. Such a membrane disturbance is unselective and therefore SM with such properties are not only toxic to animal cells but several of them also affect bacterial, fungal and viral membranes.

Biomembranes carry a wide set of **membrane proteins**, including ion channels, pumps and transporters for nutrients and intermediates, receptors

and proteins of signal transduction and the cytoskeleton. These proteins can only work properly, if their room structure is in the right conformation. Membrane proteins with transmembrane domains are stabilized by the surrounding lipids (phospholipids and cholesterol). If lipophilic SM dive into the biomembrane, they disturb the close interaction between membrane lipids and proteins thus changing the protein conformation. Usually a loss of function is the consequence. This property is known from anaesthetics that are small and lipophilic compounds. They inactivate ion channels and neuroreceptors and thus block signal transduction (and in consequence pain and conscience). Several of the small terpenoids can react in a comparable way; plants with essential oils are often employed in medicine as carminative drugs, i.e. a drug that relieves intestinal spasms. We suggest that small lipophilic SM inactivate ion channels and receptors that lead to relaxation of smooth muscles in the intestinal tissues. Or they are compounds that can induce mind-altering effects. The inner core of proteins often constitutes a hydrophobic environment. Therefore, small lipophilic SM can diffuse into proteins and thus disturb protein conformation.

It should be noted that animal cells express ABC transporters (Pg-p, MDR protein) that actively pump out lipophilic compounds that have entered a cell by free diffusion. These pumps probably evolved in animals as an answer to lipophilic poisonous compounds produced by plants. Together with detoxifying enzymes in the liver (especially the cytochrome oxidase P_{450}), they help the body to overcome noxious chemicals (see Fig. 2.3).

2.2.1.3 DNA, RNA and related targets

The genetic information of most organisms is encrypted in DNA (with the exception of some viruses that have RNA in their genome). Since DNA encodes all RNAs and, via messenger ribonucleic acid (mRNA), proteins and enzymes that are important for the metabolism and development of an organism, DNA is a highly vulnerable target. It is not surprising that, during evolution, a number of SM have been selected which interact with DNA, DNA-processing enzymes and other DNA-related targets.

Protein synthesis can be affected by low-molecular-weight compounds but more prominently by several macromolecular peptides, including lectins (which are common storage proteins in several seeds; important toxins are ricin and abrin) and haemagglutinins. Inhibitors of replication, transcription and translation are often active against a wide range of organisms, such as bacteria, fungi and animal cells.

The DNA itself can be modified by compounds with reactive functional groups, such as epoxide and aldehyde groups or cyclopropane rings (Fig. 2.4) which can covalently bind to functional groups of the DNA bases (so-called alkylation). Infamous are pyrrolizidine alkaloids (PAs), aristolochic acid (AA), cycasin, furanocoumarins, ptaquiloside and SM with epoxy groups (sometimes produced in the liver as a detoxification reaction). Some alkylants (such as furanocoumarins) are able to cross-link both DNA strands. Covalent

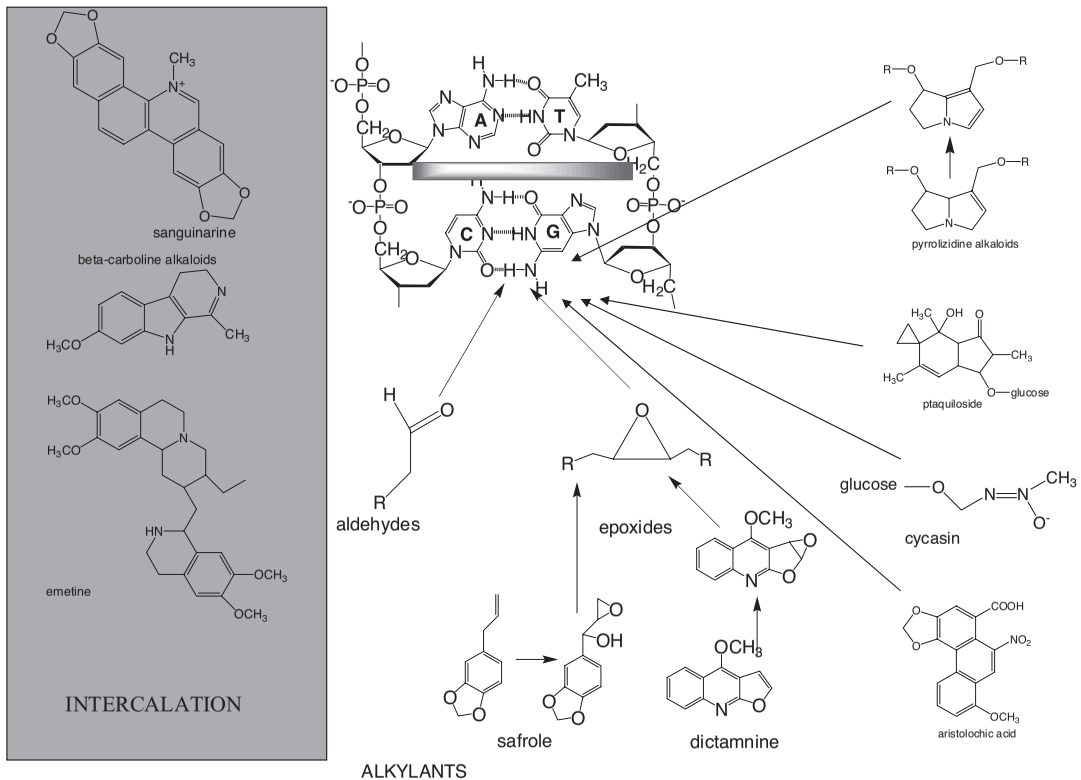

Figure 2.4 Examples of secondary metabolites that can interact with DNA: alkylants and intercalating agents. (See Plate 11 in colour plate section.)

modifications can lead to point mutations and deletion of single bases or several bases if the modified bases are not exchanged by repair enzymes.

Other SM with aromatic rings and lipophilic properties intercalate DNA, which can lead to frameshift mutations (Fig. 2.4). Some alkaloids are known to bind or to intercalate with DNA (Krey and Hahn, 1969; Maiti *et al.*, 1982; Nandi and Maiti, 1985; Wink and Latz-Brüning, 1995; Schmeller *et al.*, 1997b; reviewed in Wink, 1993a; Wink *et al.*, 1998a,b). Many of these molecules are planar, hydrophobic molecules, which fit between the planar stacks of adenine–thymine (AT) and guanine–cytosine (GC) base pairs. Important alkaloids that fall into this group include sanguinarine, harmine, berberine, berbamine, ergometrine, harmaline, emetine, quinidine, quinine, cinchonidine, cinchonine, boldine, norharman, solanine, canadine, chelidonine, lobeline, ajmalicine and, possibly, ajmaline (Wink and Latz-Brüning, 1995; Wink *et al.*, 1998a,b). Furanocoumarins can intercalate DNA and upon illumination with UV light can form cross-links with DNA bases, but also with proteins. They are therefore mutagenic and possibly carcinogenic. The degree of DNA intercalation is strongly and positively correlated with inhibition of DNA and RNA processing enzymes, such as DNA polymerase I and reverse transcriptase (RT) (Wink and Latz-Brüning, 1995; Wink *et al.*, 1998a,b). Other alkaloids act at the level of DNA and RNA polymerases, DNA topoisomerase, or even telomerase thus impairing the processes of replication, DNA repair and transcription.

Because frameshift mutations and non-synonymous base, exchanges in protein coding genes alter the amino acid sequence in proteins such mutations are usually deleterious for the corresponding cell. If they occur in germ line cells, such as oocytes and sperm cells even the next generation is negatively influenced through either malformations of newborn animals or protein malfunctions responsible for certain kinds of inheritable health disorders or illnesses. Mutagenic agents can cause cancer (when applied over a longer period), as has been reported for PAs, AA, cycasin and ptaquiloside; this topic is explored in more detail in the next paragraph.

Interference with DNA, protein biosynthesis and related enzymes can induce complex chain reactions in cells. Among them, **apoptosis**, that leads to programmed cell death, is a very important process. Several alkaloids, flavonoids, allicin and cardiac glycosides have been shown to induce apoptosis in primary and tumour cell lines (reviewed in Wink, 2007a; see Table 2.6) and even in some single cell organisms, such as trypanosomes (Rosenkranz and Wink, 2008).

2.2.2 Cytotoxic properties of SM

The interaction of SM with a central target of cells, such as DNA/RNA, protein biosynthesis, energy generation or cell division can lead to cell death, especially apoptosis.

2.2.2.1 Secondary plant metabolites with mutagenic and carcinogenic properties

Mutagenic and carcinogenic properties of secondary plant metabolites have, for a long time, been considered to be merely a curious exception in the spectrum of biological activities of naturally occurring plant products. However, since the discovery of the mutagenicity of some PAs in *Drosophila melanogaster* (Clark, 1960), our knowledge in the field of plant mutagens and carcinogens capable of displaying DNA-damaging activity in prokaryotic and eukaryotic cells and organisms has vastly increased. It is now certain that compounds with mutagenic potential are widely distributed throughout the plant kingdom. They have been isolated from bacteria, fungi, algae and lichens, but also from ferns and from many members of families belonging to the spermatophytes.

In the past, such compounds were discovered mainly as a result of outbreaks of disease in agricultural livestock. During the last two decades, however, screening programmes have been developed with the specific aim of identifying natural mutagens in our environment. This was initiated by the understanding that mutagenicity is associated with carcinogenicity. It was also established that mutagenicity is characteristically connected with considerable cytotoxicity. It was, therefore, assumed that mutagenic properties reflect particular aspects of the intrinsic toxicity and have not been evolved as a direct basis upon which selection might act.

The term 'mutagenicity' is often used in a more strict sense in order to distinguish it from the term 'clastogenicity', which means the ability to induce structural or numerical chromosomal aberrations, e.g. breaks, exchanges and gaps. Loss of chromosomal material is also an indication of genetic damage and can be detected by special methods, e.g. the micronucleus test and the comet assay. In addition, DNA damage is indicated by the induction of sister chromatid exchange (SCE) and, indirectly, by lethality in recombination repair-deficient bacterial strains. But the most widely employed in vitro mutagenicity test is the Ames assay. It is performed with *Salmonella typhimurium* strains, which are constructed to detect base pair and frameshift mutagens. Independently of the genetic endpoint tested, DNA-damaging agents are generally termed 'genotoxic agents'.

The purpose of the present review is to discuss the current status of our knowledge of genotoxic plant metabolites, their interaction with DNA and the evidence of their association with tumour formation in experimental animals and with human cancer.

Table 2.1 is not a complete list of the presently known natural mutagens but it gives an overall impression of the great variability in the chemical structures involved and the abundance of such compounds. However, in the present review, it is not possible to discuss all of the mutagens listed. It should also be pointed out that the mutagenic potential of certain genotoxic agents has not yet been evaluated accurately or has been associated with in vitro conditions. Although genotoxic carcinogens are apparently the main cause of

Table 2.1 Secondary metabolites with genotoxic properties

Producing organisms	Type of compounds	Examples
Bacteria	'**Antibiotics**'	Streptozotocin, azaserin, daunomycin, adriamycin, mitomycin C, bleomycin
Fungi	**Mycotoxins**	Aflatoxins, sterigmatocystins, ochratoxin A, luteoskyrin, patulin, sporidesmin, griseofulvin, citrinin, fusarin C, alternariolmethylether, gyromitrin, nivalenol, isovelleral, illudin S, necatorin, ergotamine and other ergot alkaloids
Lichens		Physodalic acid
Algae		Plocamenon
Ferns		Ptaquiloside, hypoloside A and C
Spermatophytes	**Alkaloids**	
	Acridone alkaloids	Rutacridone, rutacridone epoxide, isogravacridonchlorine
	Quinolizidine alkaloids	Cryptopleurine
	Furoquinoline alkaloids	Dictamnine, γ-agarine, maculine, evolitrine, kokusaginine, pteleine
	Indole alkaloids	Ellipticine, vincristine, voacristine, strychnine
	Quinoline alkaloids	Camptothecin
	Isoquinoline alkaloids	Liriodenine, roemerine, lysicamine, noscapine, tetrandrine
	Cephalotaxine alkaloids	Harringtonine, homoharringtonine
	Piperidine alkaloids	Arecoline, arecaidine
	Pyrrolizidine alkaloids	Clivorine, heliotrine, monocrotaline, senecionine, senkirkine, seneciphylline, retrorsine, echimidine, fulvine, jacobine, ligularidine, lycopsamine, intermedine, petasitenine
	Phenylalkylamines	Cathinone
	Purines	Caffeine, theobromine, theophylline
	β-Carboline alkaloids	Harman, harmine, brevicolline, harmalol, harmol
	Further *N*-containing plant metabolites	Capsaicin, benzoxazinones
	Nitro aromatic compounds and related lactams	Aristolochic acid I, II, IV, methoxytariacuripyrone, aristolactam I, II
	N-containing glycosides	Cycasin, neocycasins, macrozamin
	Glucosinolates and mustard oils	Sinigrin, allylisothiocyanate, thiourea, phenethylisothiocyanate
	Anthranoids	Aloe-emodin, emodin, physcion, lucidin, purpurin, rubiadin
	Flavonoids	Quercetin, kaempferol, galangin, wogonin, norwogonin

Table 2.1 (*Continued*)

Producing organisms	Type of compounds	Examples
	Furanocoumarins	Bergapten, heraclenin, imperatorin, xanthotoxin
	Phenols	Gossypol, hydroquinone
	Phenylpropanoids	Estragole, safrole, isosafrole, β-asarone, (6)-gingerole, (6)-shogaol, caffeic acid, cinnamaldehyde
	Terpenoids	Citronellal, menthone, catalpin, costunolide, hymenoxon, steviol, valepotriates
	Xanthones	Gentisin, isogentisin, swertianin, bellidifolin, methylbellidifolin, desmethylbellidifolin.

tumour induction in animals, tumour incidence can also be forced by tumour promoters, which occur in plants and microorganisms. Tumour promoters, such as phorbol esters of many Euphorbiaceae, exert their action through non-genotoxic mechanisms by activating protein kinase C (PKC) or affecting cell proliferation. It has been shown that some genotoxic agents also possess tumour-promoting properties.

2.2.2.2 Mode of action

Genotoxic agents can damage DNA and chromosomes through very different mechanisms. Many naturally occurring mutagens do not show genotoxic properties per se. They need metabolic activation by mammalian enzymes. In vitro, the exogenous metabolization is achieved with rat liver enzyme preparations used as an S9 fraction or S9 mix. If the bacterial enzymes are not capable of activating a compound to become a mutagen and mammalian enzymes are necessary for induction of mutation, the genotoxin is characterized as a promutagen or an indirect-acting mutagen.

Promutagens are metabolized into the ultimate mutagens from which the electrophilic intermediates are formed. These are detoxified or react with nucleophilic biopolymers, e.g. nucleic acids, forming DNA adducts. Safrole and aflatoxin B_1 are known to be promutagens. Aflatoxins need microsomal monooxygenases (cytochrome P_{450} enzymes) to be activated; safrole is converted into the ultimate mutagen through cytochrome P_{450} enzymes and a sulfotransferase.

Naturally occurring glycosides, e.g. cycasin and rutin, are also inactive per se in mutagenicity tests. But when they are hydrolyzed by the action of bacterial glycosidases, stable or unstable aglycones with mutagenic properties are formed. In mammals, hydrolysis is mediated by the intestinal microflora.

A considerable number of plant mutagens have been found to be frameshift mutagens (intercalation; Fig. 2.4). This has been suggested for molecules with a planar structure, e.g. quercetin. Planarity facilitates the intercalation within the DNA. After intercalation, the compound can interact further with the DNA. The nature of the binding and the binding properties are important for the consequences of intercalation and DNA complexation.

More potent mutagens result from irreversible bonds with DNA (alkylation; Fig. 2.4). Very strong effects are expected when a mutagen is capable of forming cross-links, i.e. irreversible bonds with both DNA strands. The PAs and some furanocoumarins belong to this mutagen-type. Several plant metabolites interfere in the process of mitosis (e.g. vinblastine, colchicine, taxol, podophyllotoxin), damaging the function and the structure of the spindle apparatus. This can lead to aneuploidy, as evidenced from experiments with noscapine.

Finally, some genotoxic compounds can affect the function of the topoisomerases. The importance of this mechanism of action for genotoxicity has now been established for several mutagens with cytostatic properties, e.g. ellipticine and camptothecin (Anderson and Berger, 1994). Detailed references for Section 2.2.2.2 can be found in the review of Clark (1982), Hirono (1987b), Lai and Woo (1987), Ishidate and co-workers (1988), Stich (1991), and in the following sections (2.2.2.3 and 2.2.2.4).

2.2.2.3 Significant endogenous mutagens/carcinogens

Mycotoxins. Aflatoxins (AFs) are chemically related compounds with a common difuranocoumarin structural element. They are produced by several strains of *Aspergillus flavus*. AF B_1 (Fig. 2.5) is the most potent carcinogen of

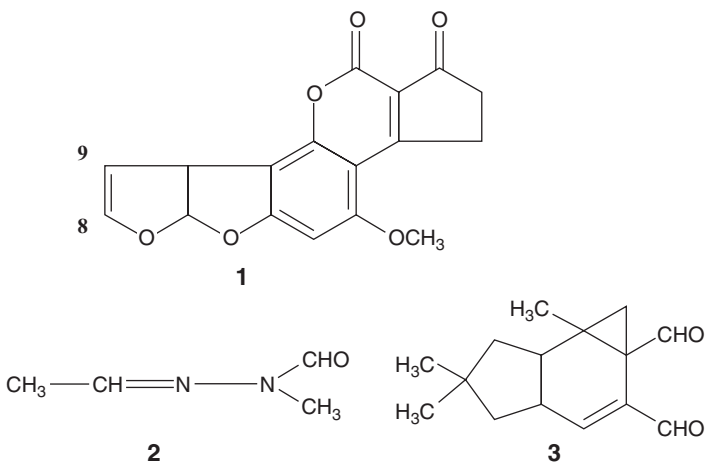

Figure 2.5 Structure of the mycotoxins aflatoxin B_1 (1), gyromitrin (2) and isovelleral (3).

the group. AF B_1 shows mutagenic, clastogenic and recombinogenic activity in most test systems. In the Ames assay, metabolic activation is needed for mutagenicity. This leads to the formation of an unstable epoxide, which is further converted into an electrophile intermediate. The guanosine moieties are the reactive sites, where the intermediate is covalently bound. The major DNA adduct in vivo was the 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 (Groopman and Cain, 1990). Formation of DNA adducts correlated to mutagenicity, SCE induction, clastogenicity and recombinogenic effects. Recent data support the hypothesis that oncogenes may have been mutated as a result of AF–DNA adduct formation. The most important results of AF research can be found in an excellent review by Chu (1991).

Experiments with rats, fish and non-human primates have produced evidence that AF B_1 is a potent hepatocarcinogen. But neoplastic formations in other organs were also induced. Details, such as the sensitivity of animals, dose, route of administration and the role of the factors, can be found in reviews by Tazima (1982) and Groopman and Cain (1990). Circumstantial evidence from epidemiological studies indicates a causal relationship between the incidence of primary liver cancer in humans and the mean daily intake of AF B_1 (Tazima, 1982). Shen and Ong (1996) examined more than 1500 human hepatocellular carcinoma samples and found evidence that oncogenes are critical molecular targets for AF B_1.

Brief mention can be made of several other mycotoxins that might have genetic significance to herbivores and man. Whereas extensive studies have been made of AF, little is known about the mode of action of other mycotoxins produced by *Aspergillus* and *Penicillium* species. Most of them have carcinogenic properties in vivo but show different responses in mutagenicity tests in vitro (Table 2.2). Fusarin C, an SM of *Fusarium moniliforme*, showed mutagenicity comparable to that of AF B_1 in the Ames assay. It remains to be determined whether the presence of fusarin C is associated with the known carcinogenicity of *F. moniliforme* isolates. Very recently, additional Fusarium mycotoxins were investigated with respect to their genotoxicity in bacteria and rat hepatocytes (Knasmüller *et al.*, 1997). Nivalenol and mycotoxins with trichothecene structure also showed mutagenic effects but their carcinogenic potential has not been tested (Chu, 1991).

Gyromitrin. In 1967, gyromitrin (Fig. 2.6) was isolated from *Gyromitra esculenta*, false morel (Pezizales, Ascomycetes), and was found to be the main toxic principle of this mushroom. Under acidic conditions, gyromitrin forms *N*-methyl-*N*-formylhydrazine. In the stomach, it is converted to *N*-methylhydrazine. *N*-methyl-*N*-formylhydrazine can be oxidized to the *N*-nitroso derivative by liver cytochrome Paso enzymes. Positive results were obtained with *N*-methyl-*N*-formylhydrazine in the Ames assay. A stronger effect was found after metabolic activation (Von der Hude and Braun, 1983). The authors postulated the formation of the *N*-nitroso derivative to explain the genotoxic effect. *N*-methylhydrazine showed a positive effect in *Escherichia coli*. The high bactericidal activity made it difficult to

Table 2.2 Mutagenicity and carcinogenicity of selected mycotoxins (combined data from Tazima, 1982)

Compound	Carcinogenicity	Mutagenicity in vitro Ames assay	Bacterial recombinant assay	Mammalian cell cultures
Aflatoxin B_1	+ (rat, trout, mouse)	+	+	+
Sterigmatocystin	+ (rat, mouse)	+	+	+
Patulin	+ (rat)	−	+	+
Citrinin	+ (rat)	−	+	?
Luteoskyrin	+ (mouse)	−	+	−
Ochratoxin A	+ (mouse)	−	−	−

evaluate the genotoxic potential of *N*-methylhydrazine accurately (IARC, 1983).

A summary of data reported for carcinogenicity in experimental animals and evaluation of such data is presented in the review by Natori (1987) and the IARC (1983). The results provide sufficient evidence for the carcinogenicity of gyromitrin and its metabolites in animals.

Mutagens in larger fungi. Screening experiments have resulted in the discovery of some compounds present in *Lactarius* species that showed weak but significant mutagenicity in the Ames assay. Isovelleral (Fig. 2.6) and hydroxyisovelleral were mutagenic after metabolic activation. The activity is possibly connected with the unsaturated dialdehyde structure of the molecules

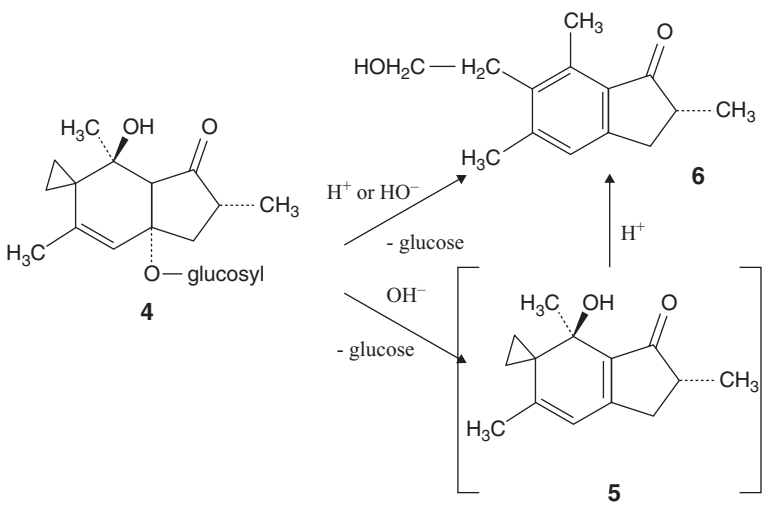

Figure 2.6 Ptaquiloside (4) and its conversion into an unstable dienone (5) and pterosin B (6) as end-product. (Redrawn from Hirono and Yamada, 1987).

(Sterner *et al.*, 1987). Their role in chemical defence was discussed by Sterner and co-workers (1985).

Fern toxins: *ptaquiloside.* Ptaquiloside (Fig. 2.6) is an *O*-glycoside with a norsesquiterpene aglycone. It has a planar structure and is relatively unstable under heat, light and acidic or alkaline conditions. In alkaline aqueous solution, it is converted into pterosin B via an unstable conjugated dienone (Fig. 2.6) (Hirono and Yamada, 1987). Ptaquiloside was first isolated from *Pteridium aquilinum* (L.) Kuhn (syn. *Pteris aquilina*) but more recently it was also detected in *Pteris cretica* and other ferns (Saito *et al.*, 1990). *P. aquilinum* (bracken fern) is widely distributed in many parts of the world. The hypolosides are related compounds and were isolated from other pteridophyta (Saito *et al.*, 1990). Ptaquiloside showed marked mutagenicity in a modified Ames assay after preincubation at pH 8.5 (Nagao *et al.*, 1989). It proved to be a direct-acting mutagen, with an activity comparable to that of the illudins, metabolites of certain basidiomycetes. Ptaquiloside induced chromosomal aberrations in cultured Chinese hamster lung cells (Matsuoka *et al.*, 1989), as did the illudins and the hypolosides. The clastogenic effect was pH-dependent.

Two possibilities have been discussed for the mechanism of action: an electrophilic intermediate may be formed either via the highly reactive cyclopropane ring or via the cyclopropylcarbinol structure (Hirono and Yamada, 1987). In 1965, the carcinogenicity of bracken fern was clearly demonstrated by experiments with rats. The tumour-inducing effect of a bracken fern diet was confirmed by many research groups in the following decades. After being fed a diet containing bracken powder, tumours were observed in mice, rats, hamsters, guinea pigs and cattle. Target organs were the urinary bladder and the intestinal tract. The carcinogenicity of ptaquiloside was shown in female circular dichroism (CD) rats. For details of the carcinogenicity experiments, see the review by Hirono and Yamada (1987).

Cycasin and related azoxyglycosides. Cycasin (Fig. 2.4) was first isolated from *Cycas revoluta* and *C. circinalis*. Subsequently, macrozamin and the neocycasins were described as metabolites of other Cycads. They differ in their sugar moiety from cycasin. Cycads, which represent a group of ancient gymnosperms, occur in tropical and subtropical zones. Azoxyglycosides are present in higher amounts in the seeds, which are used as a source of food starch. The glycosides are hydrolyzed by glycosidases in herbivores and humans, forming methylazoxymethanol (MAM), an aglycone that causes acute intoxications as well as mutations and tumour initiation.

Cycasin was inactive in the Ames assay because the standard S9 mix from rat liver lacks the appropriate β-glucosidase. MAM, however, induced genetic alterations in *S. typhimurium* and *Bacillus subtilis*. Gene mutations and chromosomal aberrations were observed in yeast, plant cells, *D. melanogaster* and mammalian cells. The genotoxicity was possibly due to the spontaneous decomposition of MAM into alkylating intermediates, presumably diazomethane or, more likely, into another methyl donor. After administration

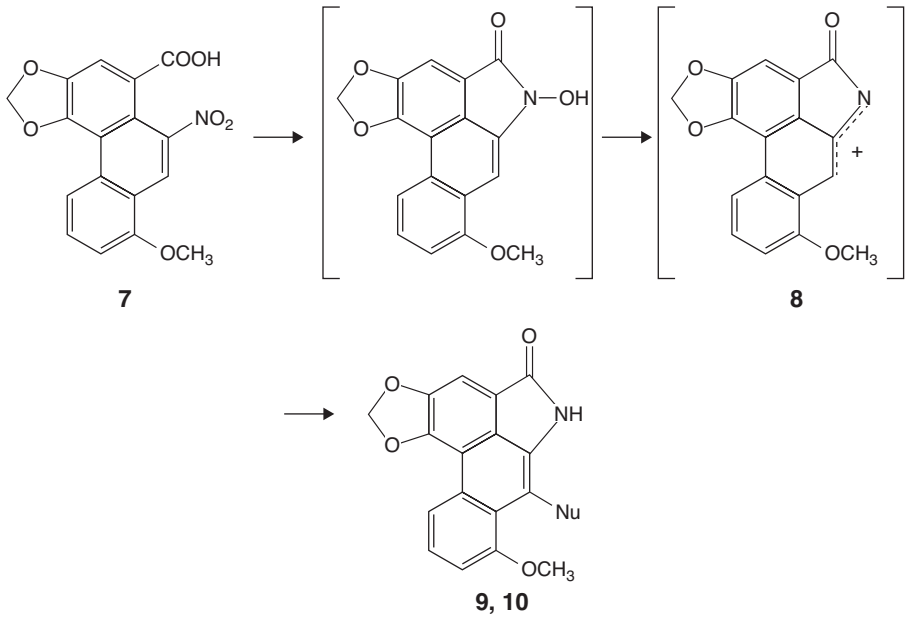

Figure 2.7 Proposed mechanism of activation of aristolochic acid 1 (7) via a pentacyclic nitrenium ion (8). The two DNA adducts identified were deoxyguanosine-N^2-yl-aristolactam and deoxyadenosine-N^6-yl-aristolactam (9,10). (Redrawn from Pfau *et al.*, 1990c).

of cycasin, N^7-methylguanine was discovered in rat DNA in vitro and in vivo. The carcinogenicity of cycasin and the other azoxyglycosides has been demonstrated in mice, rats, hamsters, guinea pigs, rabbits, fish and monkeys (Hoffman and Morgan (1984); Hirono, 1987).

Aristolochic acids and related compounds. AAs occur in the roots and in the aerial parts of many members of the genus *Aristolochia*, e.g. *Aristolochia clematitis*. AAs are often accompanied by aristolactams or related nitro aromatic compounds (Mix *et al.*, 1982; Achenbach *et al.*, 1992). In *Aristolochia* species, AAs are normally present as a mixture of at least six compounds. The main ingredients are the AAs I and II (Fig. 2.7). AAs have been employed in a number of in vitro mutagenicity tests. A mixture of AA I and II proved to be mutagenic in the Ames test with *S. typhimurium* strains (Robisch *et al.*, 1983; Schmeiser *et al.*, 1984). Subsequently, it was established that AA II was more active in *S. typhimurium* than the acids I and IV (Götzl and Schimmer, 1993; Pistelli *et al.*, 1993). The aristolactams, which occur naturally but are also formed metabolically after oral ingestion of the AAs (Krumbiegel *et al.*, 1987), showed weak mutagenic activities after metabolic activation by liver enzymes in the Ames assay (Schmeiser *et al.*, 1986). Aristolactams were also produced in vitro when AAs were incubated with S9 mix (Schmeiser *et al.*, 1986).

AAs are direct-acting mutagens in bacteria (Robisch *et al.*, 1983; Schmeiser *et al.*, 1984; Pezzuto *et al.*, 1988). They were activated by bacterial nitroreductases analogous to the activation of other nitro aromatic compounds. A cyclic nitrenium ion is formed via hydroxylamine (Fig. 2.7). Delocalization of the positive charge finally leads to DNA binding via the C7-position. The intermediates are covalently bound at the exocyclic amino groups of the purines (Pfau *et al.*, 1990a). AA II can also be activated by the cytosolic fraction of liver homogenates from Wistar rats (Schimmer and Drewello, 1994). In *S. typhimurium*, the microsomal fraction was capable of detoxification and reduced the mutagenic effect. It is therefore suggested that, in the presence of mammalian liver enzymes, activation and deactivation processes take place concurrently and at a comparable level.

The important role of the nitro group for activation could be established using nitroreductase overproducing strains (Götzl and Schimmer, 1993). This was also deduced from results obtained with nitrophenanthrene derivatives (Pfau *et al.*, 1990c). The lower activity of AA I compared to acid II was attributed to the methoxy group, which possibly produced steric hindrance for binding of the genetically active intermediate to DNA or for binding of the substrate to the active site of the enzyme(s).

Many positive results are available concerning the mutagenicity of AAs in eukaryotic test systems. They induced point mutations and recombinations in *D. melanogaster* (Frei *et al.*, 1985) and caused gaps and chromosome breaks and induced SCE in human lymphocytes in vitro (Abel and Schimmer, 1983). AAs also induced point mutations in V79 Chinese hamster cells at the HPGRT locus (Manolache *et al.*, 1985). Positive results were obtained in the point mutation test on L5178Y/TK$^{+/-}$ mouse lymphoma cells, in the DNA repair test on rat hepatocytes, and in the cell transformation test with BALB 3T3 cells (Puri and Milller, 1985). In the granuloma pouch assay, which detects gene mutations in vivo, AAs were more potent at equimolar doses than *N*-methyl-*N*-nitro-*N*-nitrosoguanidine, a mutagen that has been known for its strong alkylating potency (Maier *et al.*, 1985). AA was found to be mutagenic in further in vivo mutagenicity tests. Genotoxic effects on bone marrow cells of mice were reported by Mengs and Klein (1988), who used the micronucleus test system.

During the last two decades, experiments in vitro and in vivo have been performed to elucidate the nature of DNA adducts and their relevance for carcinogenicity. Using the ^{32}P-postlabelling assay, it could be shown that AA I forms covalent DNA adducts upon metabolic activation in vitro (Pfau *et al.*, 1990b). Incubation of AA I or II with rat liver S9 and calf thymus DNA also gave rise to DNA adduct formation. The aristolactams I and II (corresponding to the acids I and II) did not form DNA adducts in the presence of S9 (Schmeiser *et al.*, 1988). However, evidence exists that aristolactams can bind to natural and synthetic DNA by a mechanism of intercalation and exhibit considerable specificity towards alternating GC polymers (Nandi *et al.*, 1991). When AA I or II was administered orally to male Wistar rats and

the DNA from different target and non-target tissues was analyzed for DNA adducts, the patterns were similar to those obtained from in vitro incubations (Schmeiser *et al.*, 1988).

Several papers have been published during the last 5 years on the chemical nature of the adducts and the molecular consequences. The two main adducts were identified as 7-(deoxyguanosin-N^2-yl)-aristolactam and 7-(deoxyadenosin-N^6-yl)-aristolactam (Pfau *et al.*, 1991; Fernando *et al.*, 1992) (Fig. 2.7). Further experiments showed that irrespective of the AA used to induce DNA adducts, deoxyadenosine was the major target for chemical carcinogenesis of these compounds (Stiborova *et al.*, 1994). In a study with synthetic oligonucleotides, it was demonstrated that all purine adducts provided severe blocks to DNA replication but the guanine adducts may not be very efficient mutagenic lesions. The adenine adducts, however, exhibited a distinct mutagenic potential, resulting from deoxyadenylate adenosine monophosphate (dAMP) incorporation by polymerase.

AT→TA transversions would be the mutagenic consequences of adenine adducts (Broschard *et al.*, 1994). This is consistent with the detection of transversion mutations in *c-ras* genes in the analysis of AA-induced tumours in rodents (Schmeiser *et al.*, 1990, 1991). In this context, it is noteworthy that DNA adducts formed by AAs were also identified in renal tissues from patients with Chinese herbs nephropathy (Schmeiser *et al.*, 1996).

The carcinogenicity of AAs was first recognized in rodents even before mutagenicity was proved. Carcinogenicity in rats was first reported by Mengs and co-workers (1982), who found that AAs caused gastric carcinomas with a short latency period. Further reports on carcinogenicity with respect to organ or tissue specificity followed (Mengs, 1988; Schmeiser *et al.*, 1990). The animals developed papillomas or squamous cell carcinomas in the forestomach, renal pelvis and urinary bladder. Liver cell carcinogenesis was initiated after partial hepatectomy (Rossiello *et al.*, 1993). AAs are potent inhibitors of seed germination (Watanabe *et al.*, 1988). They also have insect chemosterilant activity (Mathur *et al.*, 1980; Watanabe *et al.*, 1988). This may be discussed as a particular evolutionary aspect of these secondary plant products.

9-Methoxytariacuripyrone, another naturally occurring and closely related nitro aromatic compound showed much stronger mutagenicity than AA II in the Ames assay (Schimmer and Drewello, 1994).

Pyrrolizidine alkaloids. PAs occur mainly in members of the families Boraginaceae (e.g. *Symphytum*, *Echium*, *Anchusa*, *Heliotropium*), Asteraceae (e.g. *Senecio*, *Adenostyles*) and Fabaceae (mainly tribe Crotalarieae) (Wink and Van Wyk, 2008). Many representatives of these families are used for medicinal purposes (Röder, 1995). More than 6000 species contain alkaloids with this basic structure.

The hepatotoxicity and the mutagenicity of PAs have been known for more than 30 years. The toxicity and carcinogenicity are connected with certain structural elements: a 1,2-double-bond in the pyrrolizidine ring and branched chain acids, esterifying a 9-hydroxyl and preferably also the 7-hydroxyl

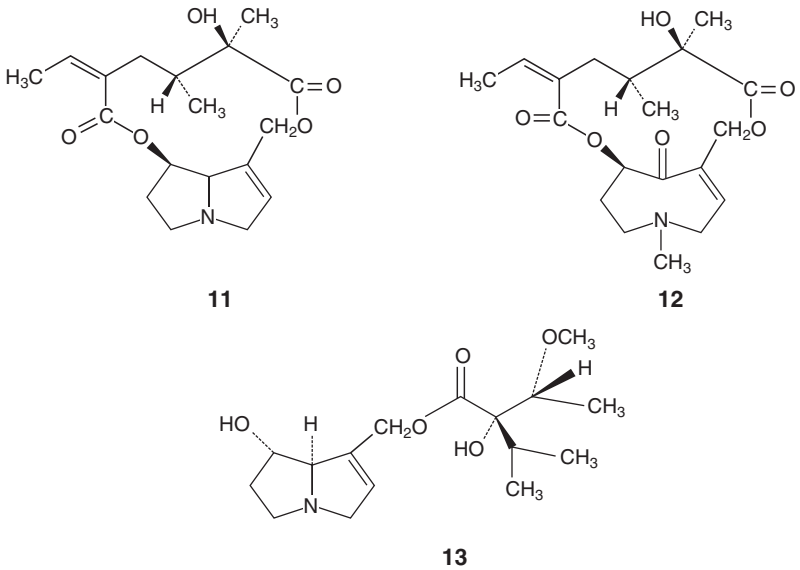

Figure 2.8 Pyrrolizidine alkaloids with cyclic diester and monoester structures, senecionine (11), senkirkine (12) and heliotrine (13).

substituent. PAs with a saturated ring system are inactive. The structures of three active PAs are presented in Fig. 2.8. Other active compounds are listed in Table 2.1. The alkaloids occur as free bases and *N*-oxides. *N*-oxides can be reduced to the free bases by the intestinal microflora and then exhibit activities similar to those of the free bases in animals. In the liver, the bases are metabolized into pyrrole derivatives (dehydroalkaloids) by cytochrome P_{450} enzymes (monooxygenases). They can produce electrophilic intermediates and then act as monofunctional or as bifunctional alkylating agents (Fig. 2.9). The pyrrole derivatives are considered to be the ultimate genotoxic compounds. Their rate of formation varies greatly from PA to PA and may, in part, explain the variation in activity. The pyrrolic alcohol metabolites show antimitotic effects, which are relevant for hepatotoxicity. PAs cross the placental barrier and are transferred into the milk of mammals fed with plants containing PAs.

Most PAs with a 1,2-double-bond in their pyrrolizidine ring exhibit only weak or insignificant mutagenicity in *S. typhimurium*. A slight increase in mutants was observed under preincubation conditions and in the presence of S9 mix (Rubiolo *et al.*, 1992). More pronounced effects were obtained in *D. melanogaster*. Chromosomal aberrations were also induced in plant cells, fungi and mammalian cells. Positive effects were reported with the SCE assay, the micronucleus test and DNA repair test. Earlier results from genotoxicity experiments were reviewed by Mattocks (1986) and Furuya and co-workers (1987). The activity of the individual PAs varied greatly from assay to assay.

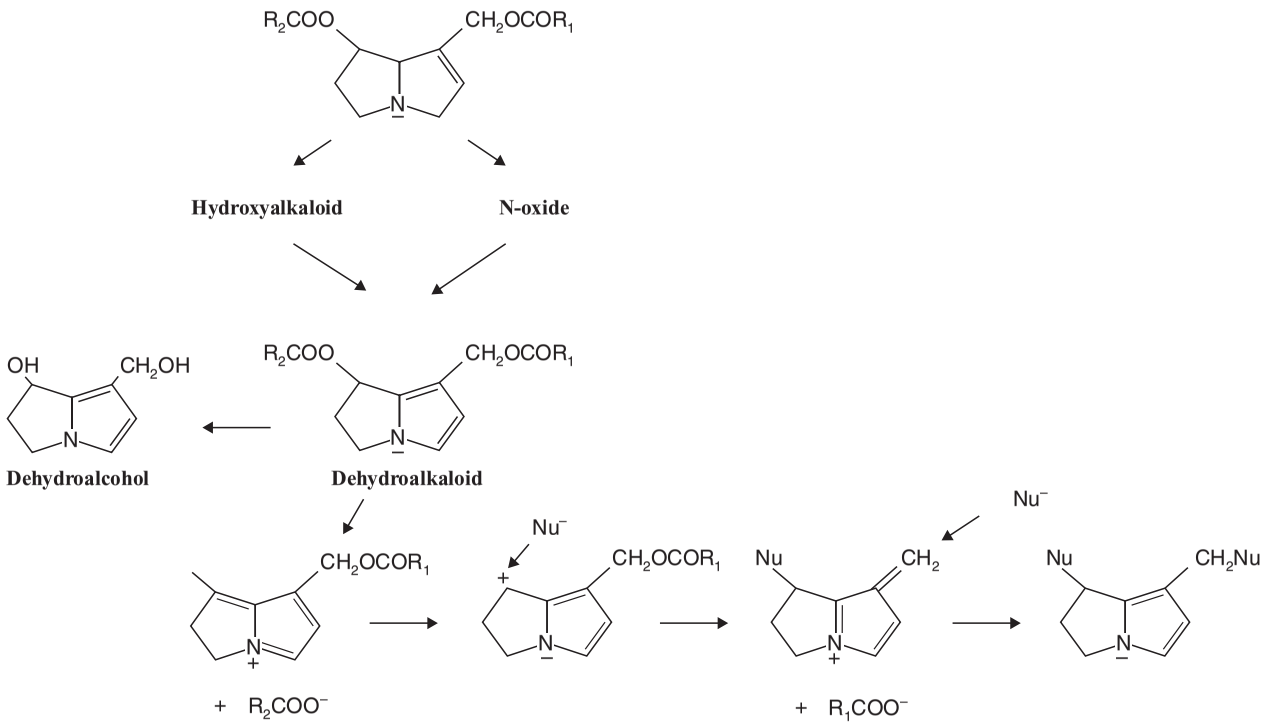

Figure 2.9 Metabolism of 1,2-unsaturated pyrrolizidine alkaloid (PA). Formation of monofunctional and bifunctional pyrrolic intermediates.

In human lymphocytes in vitro, heliotrine was capable of inducing chromosomal aberrations but senkirkine was not (Kraus *et al.*, 1985). SCE and chromosomal breaks were also induced by crude extracts from the roots of *Symphytum officinale*, which contained the monoester lycopsamine and intermedine (Behninger *et al.*, 1989). In vitro, both the dehydroalkaloids and the dehydroaminoalcohols interacted with DNA. In *E. coli* and rat liver, DNA cross-links were formed, indicating the bifunctional nature of these metabolites. The DNA adducts formed with dehydroretronecine were identified as derivatives with a covalent bond between the C7 position of the PA and the N^2 position of deoxyguanosine.

The carcinogenicity of PAs appears to parallel their mutagenicity. The most active PAs belong to the macrocyclic diester and the open diester type, in which the amino alcohol part is retronecine, heliotridine or otonecine. Crude plant extracts and numerous PAs and metabolites have been tested for carcinogenicity. The studies were primarily carried out on rats. Lasiocarpine produced the largest yield of tumours. Hepatocellular carcinomas and haemangiosarcoma were the most common tumour types. The carcinogenicity studies of PAs in experimental animals were extensively reviewed by Mattocks (1986), Furuya and co-workers (1987) and the World Health Organization (WHO, 1988).

Isoquinoline alkaloids. Isoquinoline alkaloids with an aporphine structure (Fig. 2.10) occur mainly in genera belonging to the families Menispermaceae and Annonaceae. Nozaka and co-workers (1987) researched the

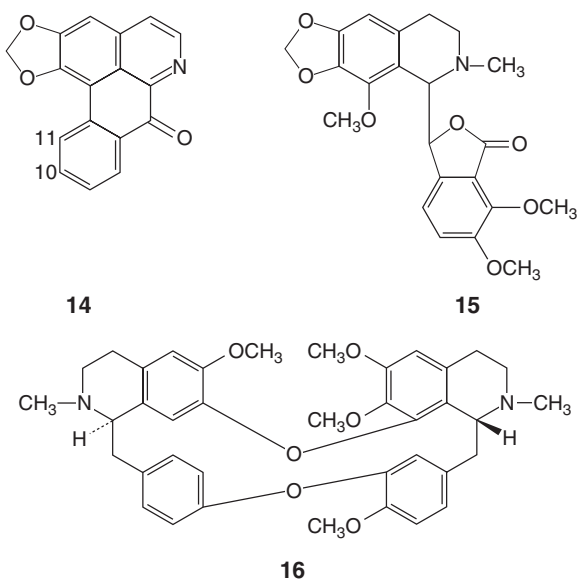

Figure 2.10 Structure of the isoquinoline alkaloids, liriodenine (14), noscapine (15) and tetrandrine (16).

mutagenic principles in *Sinomenum acutum* (*Sinomeni caulis et rhizoma*). They isolated *N*-demethyl-*N*-formyl-dehydronuciferine as the compound responsible for mutagenicity in *S. typhimurium*. Subsequently, 44 alkaloids of this type were screened for mutagenicity in *S. typhimurium* (Nozaka *et al.*, 1990). Most aporphine-type alkaloids, e.g. dicentrine, nornuciferine, roemerine, liriodenine and lysicamine, were reported to be positive. Liriodenine was found to be the most active alkaloid. The same research group investigated the clastogenicity of 18 related aporphines in the chromosomal aberration test in vitro, using a Chinese hamster lung cell line (Tadaki *et al.*, 1991). Although the alkaloids showed mutagenicity to Salmonella strains only in the presence of S9 mix, many of the compounds tested induced chromosomal aberrations in the absence as well as in the presence of rat liver enzymes. Among these compounds, liriodenine was the most potent clastogen. Liriodenine and roemerine also induced polyploidy in the Chinese hamster lung cells. The authors postulated that the mutagenicity may be due to an epoxide that is formed at the C10–C11 position (see Fig. 2.10). Boldine, however, did not exhibit mutagenicity in the Ames assay but showed weak activity in inducing mitochondrial mutants in *Saccharomyces cerevisiae* (Moreno *et al.*, 1991).

Among the biosynthetically related phthalide isoquinoline alkaloids, noscapine (Fig. 2.10), a centrally acting antitussive agent, has been shown to induce polyploidy in Chinese hamster lung cells, in V79 Chinese hamster cells and in human lymphocytes in vitro (Gatehouse *et al.*, 1991). In addition, spindle damage was observed in V79 cells and human skin fibroblasts using concentrations of 30 and 60 mg/mL. From these studies and further preliminary results, it appears that noscapine might induce an increase in chromosome loss and polyploidy through effects upon spindle structure and function.

The investigation of the bisbenzyl isoquinoline alkaloid, tetrandrine (Fig. 2.10), resulted in particularly interesting results. From the molecular structure, it has been predicted that the alkaloid would be a genotoxic carcinogen (Rosenkranz and Klopman, 1990). Using the ^{32}P-postlabelling assay, it was recently shown that tetrandrine is capable of adduct formation with DNA (Schmeiser, H.H., DKFZ Heidelberg, personal communication). From in vitro experiments, however, only weak effects were observed in Salmonella and in Chinese hamster lung cells and tetrandrine was found to be a weak indirect-acting genotoxicant (Whong *et al.*, 1989; Xing *et al.*, 1989). Nonetheless, the alkaloid was a potent enhancer of the mutagenicity of benzo(a)pyrene, 2-aminoanthracene, mitomycin C and cigarette smoke condensate (Whong *et al.*, 1989; Xing *et al.*, 1989).

Furanoquinoline alkaloids. Furanoquinoline alkaloids are characteristic SM of the Rutaceae, in which they co-occur with the chemically related furanocoumarins. Dictamnine (Fig. 2.11) was isolated from the roots of *Dictamnus albus* and was detected in the herb of *Ruta graveolens*. Both plants have been used in the past for medical purposes.

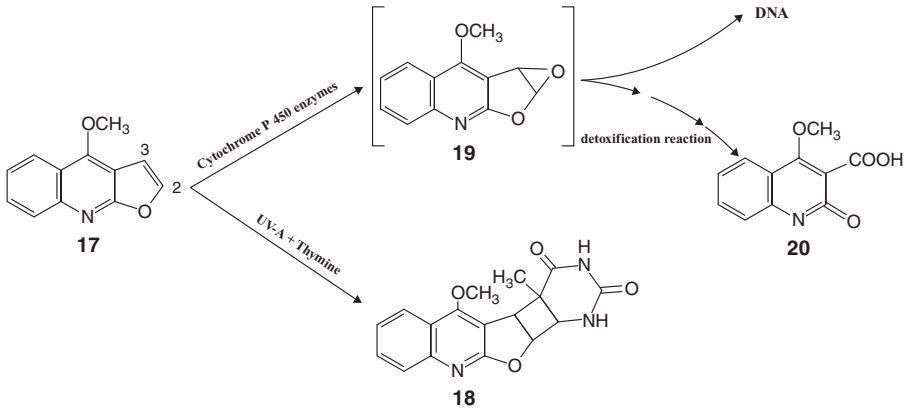

Figure 2.11 Dictamnine (17) and its enzymatic and UV-A light activation. Cyclobutane adduct with thymine (18), (*syn-cis*-configuration not shown), dictamnine oxide (19) and the non-active dictamnic acid (20).

Several reports have been published on the biological activity of dictamnine. The activities of the mono- and dimethoxy derivatives, e.g. γ-fagarine and skimmianine, were investigated to a lesser extent (Table 2.3). The mutagenicity of dictamnine after metabolic activation with rat liver microsomes was first reported by Mizuta and Kanamori (1985) using *S. typhimurium* as the indicator organism. Paulini and co-workers (1987) and Häfele and Schimmer (1988) confirmed its activity in the Ames assay and investigated further naturally occurring furoquinolines. Structure–mutagenicity relationships were tested with a series of 11 furoquinolines using base pair and frameshift indicator strains of *S. typhimurium* (Paulini *et al.*, 1989). Klier and co-workers (1990) suggested that dictamnine is metabolized by the liver microsomes via an unstable 2,3-epoxide to an electrophilic intermediate which is covalently bound to DNA, analogous to the mechanism of aflatoxin B_1 (Fig. 2.11).

In *E. coli*, dictamnine was reported to be a direct-acting mutagen producing bacterial frameshift mutations in the dark (Ashwood-Smith *et al.*, 1982). Schimmer and Leimeister (1989), who studied the SCE-inducing potency of γ-fagarine in human lymphocytes in vitro, also showed a direct effect in this system, which could not be enhanced after addition of liver microsomes.

Besides exhibiting genotoxic activity in the dark, furoquinolines were also capable of inducing genetic damage in the light. When prokaryotic and eukaryotic cells were irradiated with long-wave ultraviolet (UV-A) in the presence of dictamnine, the furanoquinoline was covalently bound to DNA. Monoadducts were formed with the DNA bases, particularly with the thymine moieties (Pfyffer and Towers, 1982a; Pfyffer *et al.*, 1982b). Monoadducts were formed in vitro as well as in vivo. The photobinding of dictamnine to DNA is thought to be the reason for its phototoxicity and photomutagenicity. The sites in the DNA for the photobinding of dictamnine are probably identical with those for monoadducts of furanocoumarins (Pfyffer

Table 2.3 Summary of published mutagenicity data for dictamnine and gamma-fagarine

Compounds	Organism tested	Assay	Reference
Dictamnine	*Escherichia coli*	Reversion (WP 2 lac)	Ashwood-Smith *et al.* (1982)
	Salmonella typhimurium	Ames assay	Mizuta and Kanamori (1985), Paulini *et al.* (1987) Häfele and Schimmer (1988)
	Human cell cultures	SCE/chromosomal aberrations	Schimmer (unpublished)
Dictamnine plus UV-A	*E. coli*	Reversion	Ashwood-Smith *et al.* (1982)
	CHO cell cultures	SCE	Ashwood-Smith *et al.* (1982)
	CHO cell cultures	Chromosomal aberrations	Towers and Abramowski (1983)
	Chlamydomonas reinhardtii	Reversion (arg-1)	Schimmer and Kühne (1991)
Gamma-fagarine	*Salmonella typhimurium*	Ames assay	Mizuta and Kanamori (1985) and Paulini *et al.* (1987)
	Human cell cultures	SCE	Schimmer and Leimeister (1989)

SCE, sister chromatid exchange; UV-A, long wave ultraviolet; CHO, Chinese hamster ovary.

et al., 1982b). Since furanocoumarins react with their furan 2,3-double-bond, the furan ring appears to be the crucial reactive site for the induction of light-dependent mutations. If this were confirmed, it would mean that both mechanisms of mutagenic activation take place at the same site of the furanoquinoline molecule (Fig. 2.11).

Photomutagenic effects were found in *E. coli* (Ashwood-Smith *et al.*, 1982; Fujita and Kakishima, 1989) and in the green alga, *Chlamydomonas reinhardtii* (Schimmer and Kühne, 1991). In the alga, dictamnine had the strongest effect among the furanoquinolines tested. But the activity was lower than that of bergapten, the most active furanocoumarin. This may be due to the lower photobinding to DNA (Pfyffer *et al.*, 1982b). In Chinese hamster ovary cells, chromosome aberrations were induced by dictamnine and skimmianine (Towers and Abramowski, 1983).

Recently, it was established with newly synthesized furanoquinolines that, after intercalation and subsequent UV-A irradiation, only monoadducts with thymine with *cis-syn* configurations were formed; in the lower energy conformation, the furan ring was turned towards the minor groove of the polynucleotide, in such a way that photoreaction of this ring with thymine

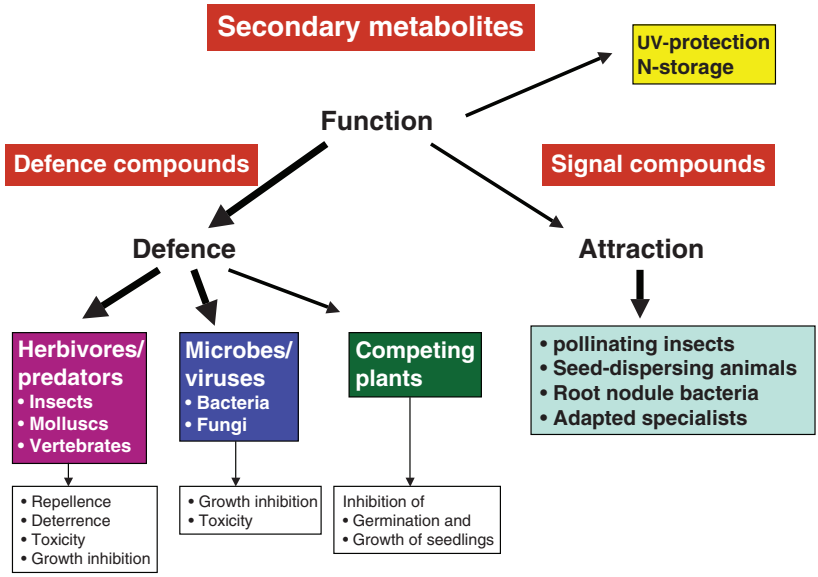

Plate 1 Ecological and physiological functions of plant secondary metabolites.

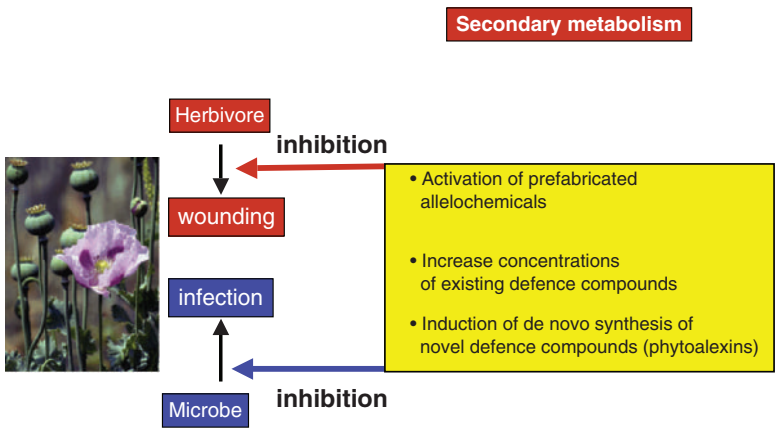

Plate 2 Examples of induced defence in plants.

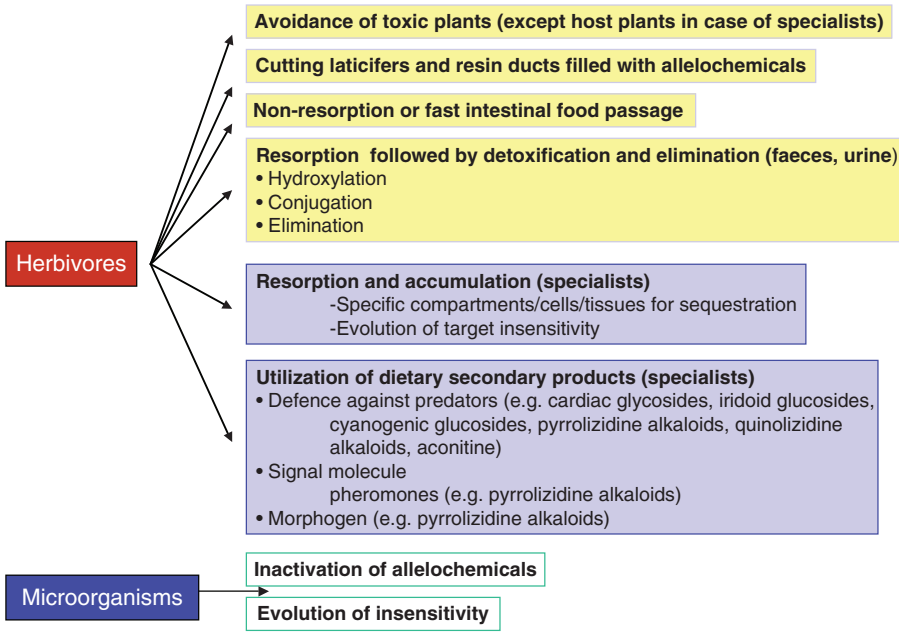

Plate 3 Adaptations of specialist herbivores and pathogens.

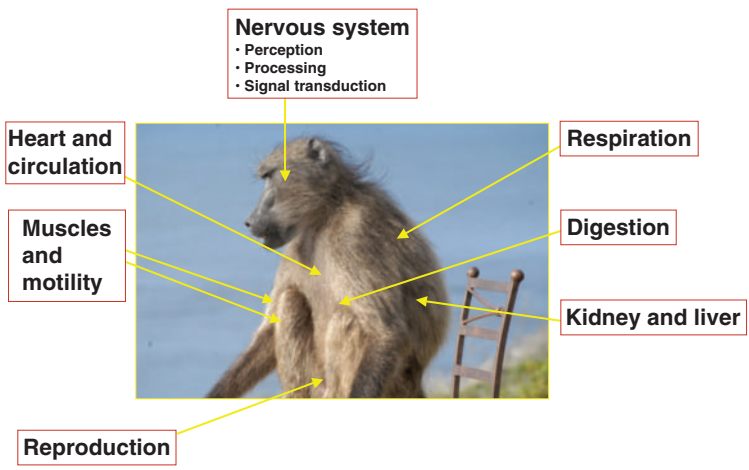

Plate 4 Targets for allelochemicals in animals.

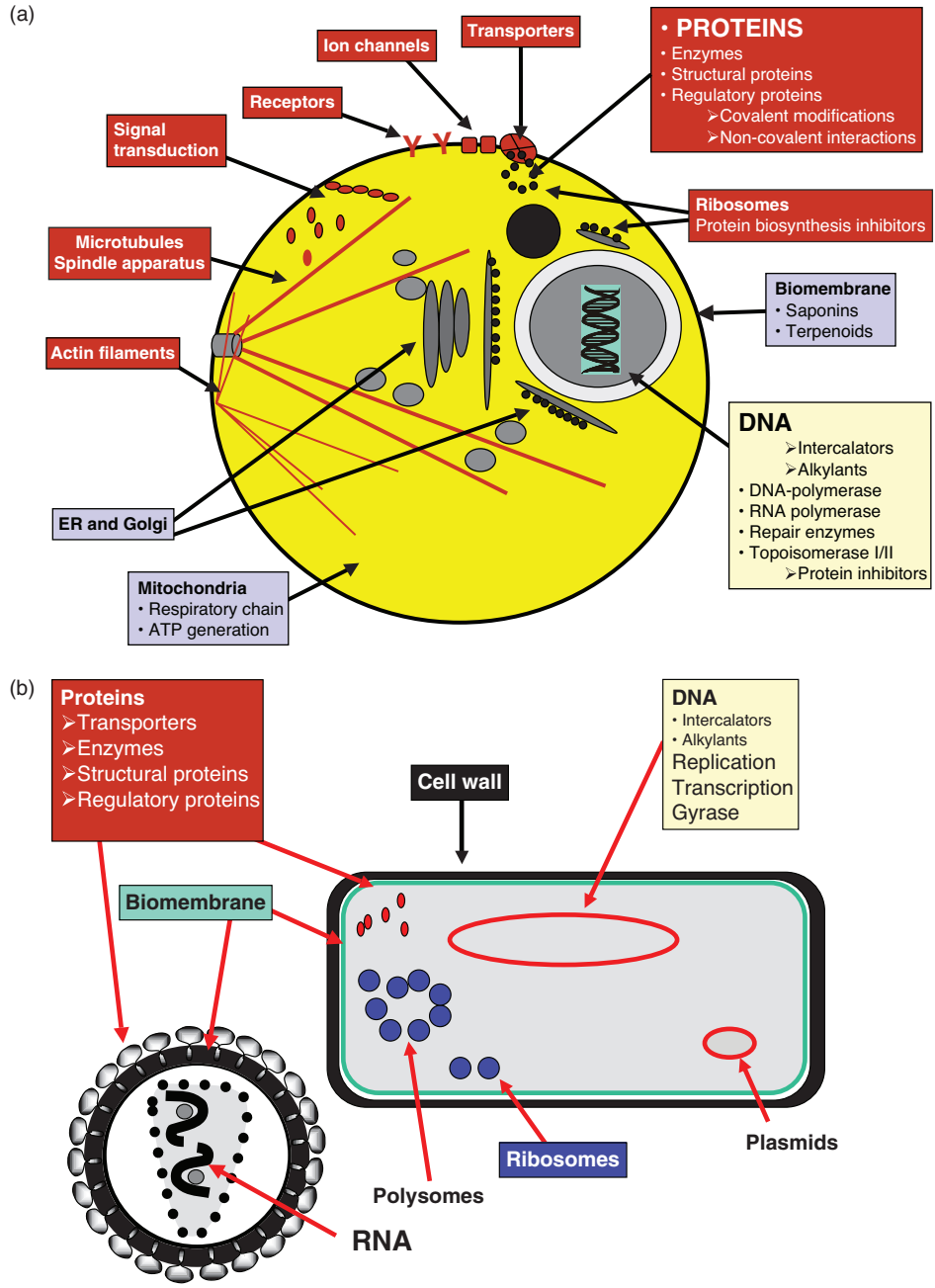

Plate 5 Molecular targets of defence chemicals in animal cells (a, b).

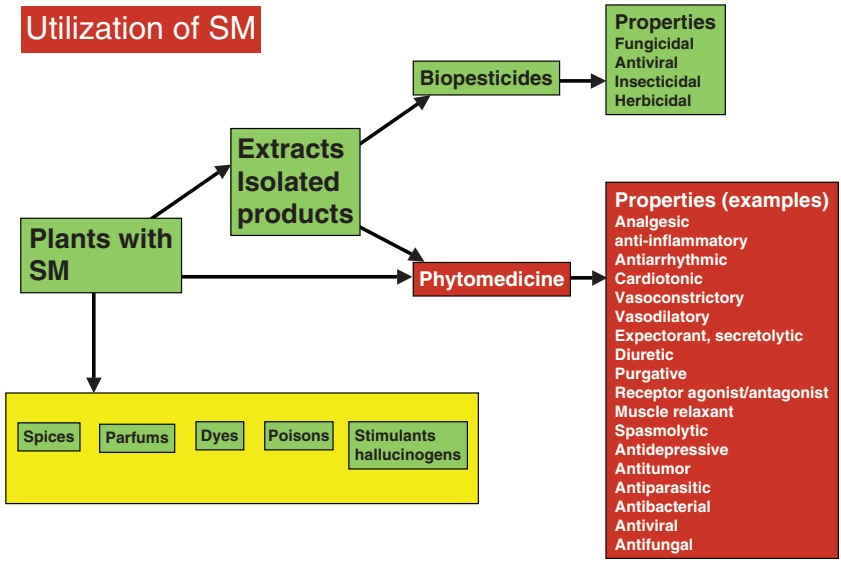

Plate 6 Utilization of secondary metabolites (SM) in biotechnology.

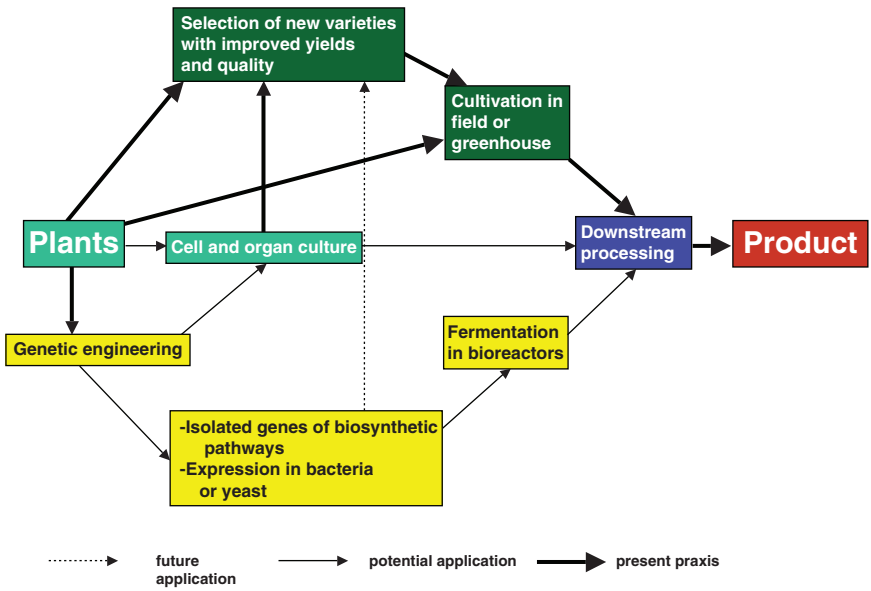

Plate 7 Strategies for the production of secondary metabolites.

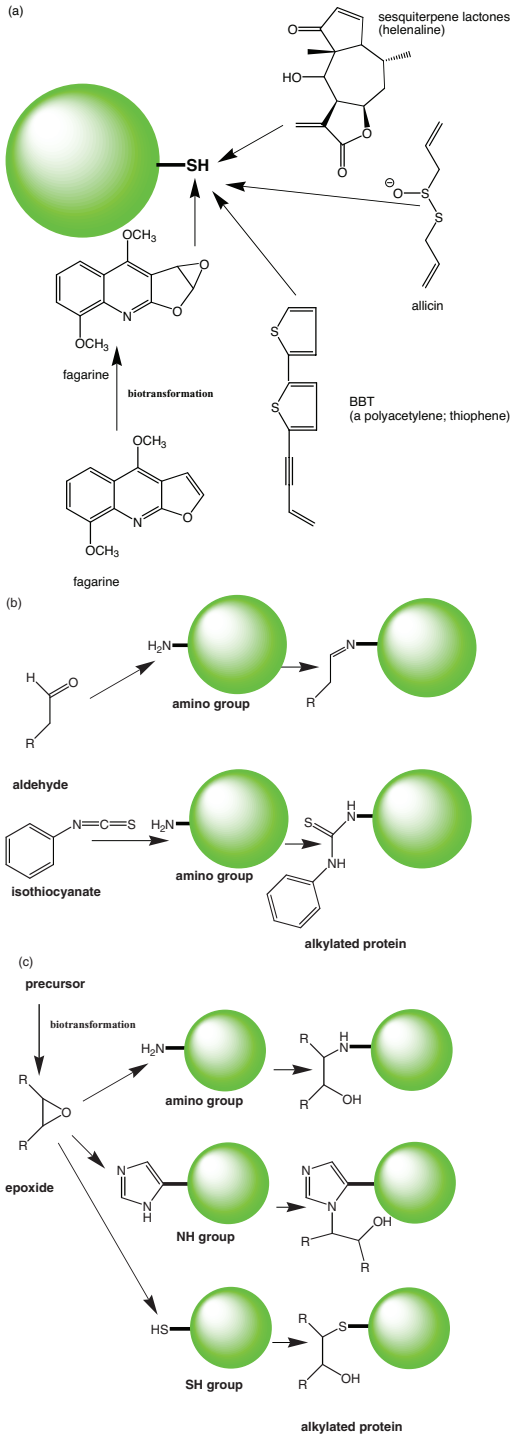

Plate 8 Examples of secondary metabolites that can modify proteins by forming covalent bonds. (a) Reactions with SH-groups of proteins; (b) reactions of aldehydes with amino acids of proteins; (c) reactions of epoxides wuth amino groups of proteins.

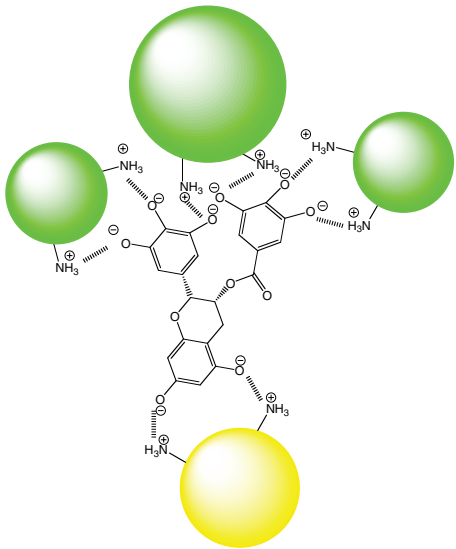

Plate 9 Epigallocatechin gallate (EGCG) as a typical polyphenol can modify proteins by forming non-covalent bonds with one or several proteins (mainly ionic bonds).

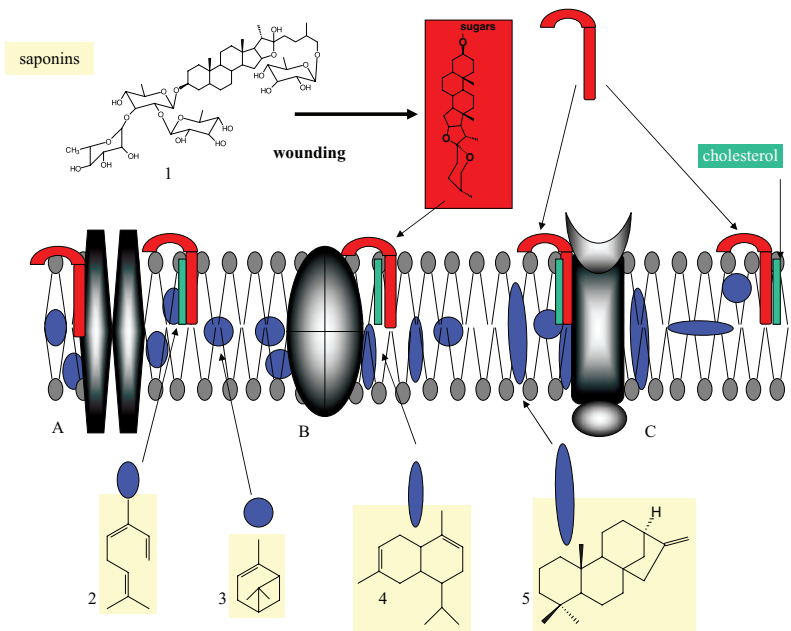

Plate 10 Examples of secondary metabolites that can modulate membrane permeability and the conformation of membrane proteins. A = ion channels, B = transporters; C = receptors; 1 = bidesmosidic steroidal saponin that becomes a monodesmosidic saponin after hydrolysis; 2 = example for a simple monoterpene, 3 = example for a simple cyclic monoterpene; 4 = example for a simple sesquiterpene; 5 = example for a diterpene.

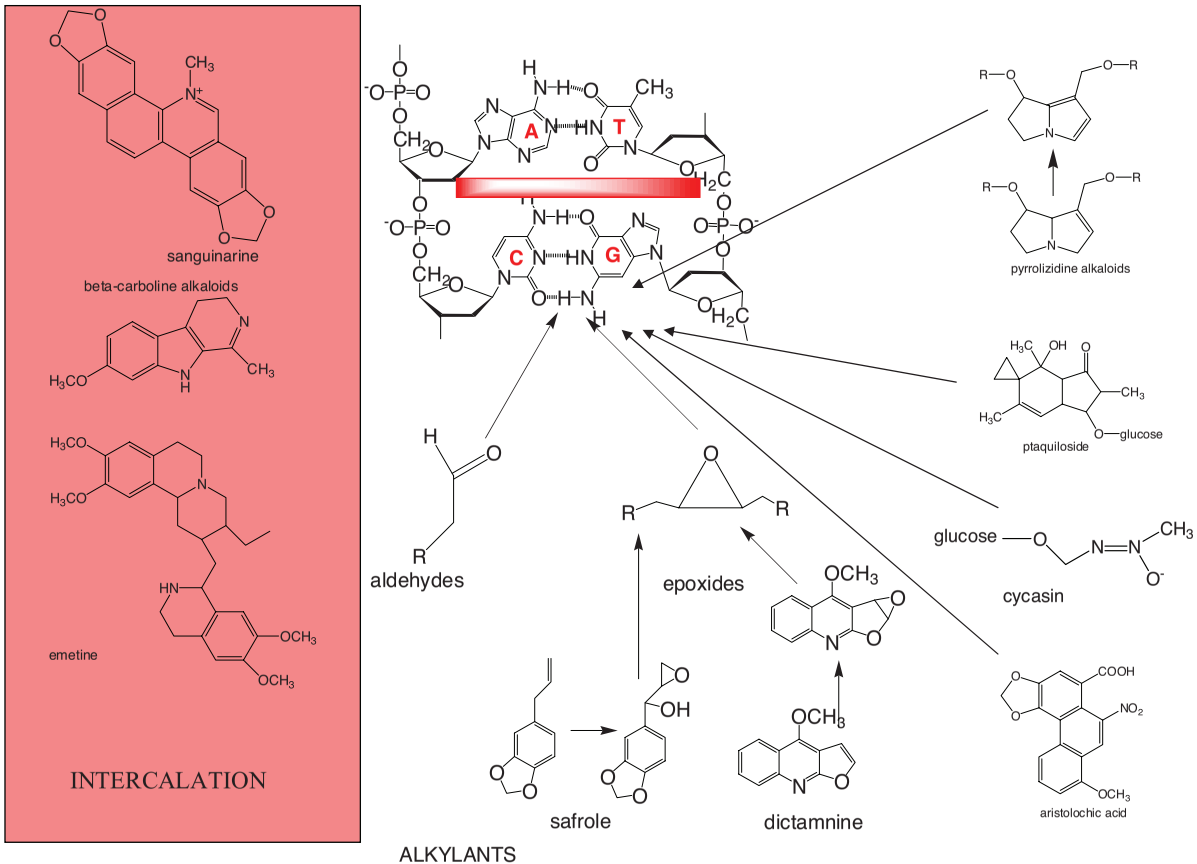

Plate 11 Examples of secondary metabolites that can interact with DNA: alkylants and intercalating agents.

was favoured (Rodighiero *et al.*, 1996). No information was found in the literature concerning the carcinogenic potential of this group of alkaloids. However, it appears to be significant that dictamnine has ovicidal properties against spider mites (Tanaka *et al.*, 1985) and possibly participates in the antifertility effect of the roots of *D. albus* (Woo *et al.*, 1987).

Furanocoumarins. Furanocoumarins are present in many members of the family, Apiaceae, and are also accumulated in some representatives of the families Rutaceae and Fabaceae. They are found in roots and are more concentrated in fruits and leaves, where they are usually stored in resin ducts as components of the essential oil.

The most outstanding property of furanocoumarins is their great ability to sensitize cells to visible light, sunlight and, especially, near-ultraviolet light. This results in strong toxicity, mutagenicity and possibly carcinogenicity. The mechanism of action is well known. After intercalation into the double helix of the DNA and molecular complexing, the light-activated furanocoumarins react with the pyrimidine bases, especially with thymine. The reactive sites for the covalent photobinding are the 2′3′-furan double bond and the 3,4-pyrone double bond. This leads to the formation of monoadducts and cross-links. Both reactions contribute to mutagenicity. The linear furanocoumarins, e.g. bergapten (5-methoxypsoralen) and xanthotoxin (8-methoxypsoralen), were more effective in mutation induction than the angular compounds or those linear furanocoumarins, which have bulky side chains. Bergapten and xanthotoxin were capable of inducing mutations in bacteria, yeasts, algae and mammalian cells in vitro. Positive reactions were obtained in the SCE test, the cell transformation assay and in most other conventional test systems.

Without UV light, furanocoumarins showed only weak effects in *E. coli* (Clarke and Wade, 1975) and in human lymphocytes in vitro (Abel *et al.*, 1985). Heraclenin, a linear furanocoumarin with an epoxidic structure in its side chain, was a weak mutagen in bacteria (Ivie *et al.*, 1980) but a strong clastogen in human lymphocytes (Abel and Schimmer, 1986). The photobiology and the genetic basis of furanocoumarin reactions were reviewed by Scott and co-workers (1976), Song and Tapley (1979), Averbeck (1989) and in an IARC report (1986).

Two recently published observations have received special attention. It has long been known that treatment with furanocoumarins plus UV-A (PUVA) induces skin cancer in mice. It has now been shown that PUVA-induced mouse skin cancers display carcinogen-specific mutations in the p53 tumour suppressor gene (Nataraj *et al.*, 1996). Calsou et al. (1996) investigated the role of mono- and biadducts for mutagenicity. They found further evidence that the monoadducts persist for a longer time, whereas the cross-links are rapidly repaired via excision. Since furanocoumarins are strong phototoxic compounds, their presence in a plant may be indicative and has been demonstrated to be a protective mechanism against phytopathogenic microorganisms and herbivores.

Flavonoids. The flavonoids constitute one of the largest groups of secondary plant metabolites universally distributed among vascular plants. Although some occur naturally in a sugar-free form, the majority exist in glycosidic form. The glycosides themselves are probably not mutagenic but they can be hydrolyzed enzymatically to liberate the aglycones, which may show mutagenicity. Whereas the flavone and the flavonol glycosides were inactive in short-term mutagenicity tests, several aglycones, e.g. quercetin, kaempferol and galangin, showed moderate mutagenic effects in bacteria and yeasts. Among the flavone group, wogonin and norwogonin showed mutagenicity comparable to that of quercetin. Their mutagenicity spectrum, however, differed from that of quercetin by a difference in mechanism of activation. A few compounds belonging to other flavonoid groups also showed weak activities in bacteria. The genotoxic effect of the various flavonoids in microorganisms with and without metabolic activation was reviewed by Brown (1980), Nagao and co-workers (1981) and Elliger et al. (1984).

The mutagenicity of quercetin has been established in many experiments using prokaryotic and eukaryotic organisms. In the Ames assay, quercetin was the most active flavonoid. Mutagenicity was shown without metabolic activation but was strongly increased when microsomes from rat liver were added. Quercetin possibly acts via intercalation into DNA, inducing frameshift mutations. However, using biophysical methods, Solimani (1996, 1997) suggested that a frameshift mutagenicity of quercetin is highly improbable.

After metabolic activation, quercetin may be enzymatically oxidized to quinoidic intermediates, which could be responsible for the stronger effect observed in the experiment with rat liver enzymes. However, the possible role of such oxidation products for mutation induction is not yet clear.

From further results, it has been suggested that quercetin might exert DNA damage via more than one mechanism (Rueff *et al.*, 1986). Quercetin induced chromosomal aberrations, SCEs and micronuclei in mammalian cells in vitro without using metabolic activation systems. Quercetin was also capable of inducing single DNA strand breaks and of transforming hamster embryo cells. Micronuclei and polyploidy were induced in human lymphocytes (Popp and Schimmer, 1991). It seems quite clear that quercetin is mutagenic in vitro. In vivo, quercetin was inactive in most assays. The published mutagenicity data from in vivo and in vitro experiments have been summarized by Müller and co-workers (1991). In further papers, the divergent reactivity of quercetin in vitro and in vivo was confirmed, even when the same model organism or cell line was used (Caria *et al.*, 1995). Müller and co-workers (1992) explained the lack of genotoxicity of quercetin in vivo with detoxification reactions or low absorption capacity. Two papers recently published reported studies in which the role of cytochrome P_{450} enzymes in the bioactivation of kaempferol and galangin and its relevance to genotoxicity were investigated (Silva *et al.*, 1997a,b).

Most carcinogenicity tests with experimental animals have produced negative results (Natori and Ueno, 1987; Ito *et al.*, 1989). The lack of mutagenicity

effects in vivo correlates with the negative carcinogenicity tests and is an indication that the metabolic competence of the organisms may play an important role. Flavonoids are scarcely absorbed from the intestinal tract of mammals. Furthermore, the microflora of the large intestine is capable of decomposing flavonols very rapidly. However, it must be pointed out that quercetin can occasionally act as enhancer of the activity of mutagens/carcinogens (Ogawa *et al.*, 1986).

Anthranoids. Anthranoids are secondary plant metabolites with a basic anthrone or anthraquinone structure. In the intact plant, they normally occur as glycosides. The 1,8-dihydroxy derivatives are of primary ecological and pharmaceutical interest owing to their laxative properties. They occur in many members of the families Liliaceae, Polygonaceae, Rhamnaceae and Caesalpinioideae. A second type of anthranoid without laxative activity is present in the family Rubiaceae. Only free anthraquinones, e.g. luteoskyrin (Table 2.12), were synthesized in *Penicillium* and *Aspergillus* species. Free aglycones are formed from the glycosides in higher plants by the action of enzymes during drying and storage. The genotoxic data of 80 phenolic anthraquinones were summarized in an early review by Brown (1980). In the *S. typhimurium* assay, lucidin showed the strongest mutagenicity; aloeemodin, emodin and physcion were less active and needed metabolic activation. A frameshift mechanism was postulated from these results and it was suggested that a non-specific free radical mechanism may be involved in the DNA damage elicited by these agents. A great deal of information on in vitro genotoxicity of anthranoids in bacterial and mammalian systems was presented in a review by Westendorf (1993). Positive results were obtained with aloe-emodin, emodin and with the rubiadin-type anthraquinones, lucidin, purpurin and rubiadin, in the Ames assay. Various anthraquinones induced gene mutations in Chinese hamster V79 cells, in the DNA repair test and in the transformation assay with mouse fibroblasts, but controversial results in V79 cells were also reported (Bruggeman and Van Der Hoeven, 1984; Heidemann *et al.*, 1996). Heidemann and co-workers discussed the genotoxicity data of aloe-emodin in detail and referred to the negative in vivo results. However, in vitro, the DNA-damaging activity of several anthraquinones was again confirmed in the micronucleus assay and the newly developed comet assay (Miller *et al.*, 1996). From these results, it was concluded that the genotoxicity of aloe-emodin is caused by interaction with the topoisomerase II activity.

No evidence was found that emodin or emodin metabolites were covalently bound to rat liver DNA or Salmonella DNA (Bösch *et al.*, 1987). 2-Hydroxyemodin formed in vitro with hepatic microsomes was the most active mutagen in Salmonella (Masuda and Ueno, 1984). The metabolic activation of emodin was shown to proceed through the cytochrome P_{450} system (Tanaka *et al.*, 1987). Emodin and chrysophanol were not capable of producing oxidative damage in DNA, in comparison to luteoskyrin, which enhanced the number of 8-hydroxyguanosine sites in the DNA of hepatoma cells (Akuzawa *et al.*, 1992). In contrast, the clastogenicity of the anthraquinones was not mediated by cytochrome P_{450} enzymes (Simi *et al.*, 1995). It was, therefore,

concluded that anthraquinones are active as intercalative agents due to their planar structure and do not need to be metabolized in mammalian cells in vitro.

Lucidin, however, was shown to exhibit its genotoxicity through a different mechanism. As indicated from results obtained with the ^{32}P-postlabelling method, lucidin forms adducts with DNA (Poginsky *et al.*, 1991). After feeding mice and rats with lucidin, DNA adducts were observed in the intestinal tissue, liver and kidney.

The carcinogenic effects of 1,8-dihydroxyanthraquinone and 1-hydroxyanthraquinone, which was identified as a metabolite of alizarin primeveroside (Blömeke *et al.*, 1992), were demonstrated after rats and mice were fed with these compounds over a longer period of time (Mori *et al.*, 1985, 1986, 1990). The carcinogenic risk of anthranoids for humans is the subject of controversy. It should be mentioned, however, that hydroxyanthraquinones and anthrones have tumour-promoting properties in vitro (Di Giovanni *et al.*, 1985; Wölfle *et al.*, 1991) and lead to a considerable increase in the proliferation of intestinal cells in vivo, after oral administration (Kleibeuker *et al.*, 1995). It should also be noted that 1-hydroxyanthraquinone acts synergistically on MAM-induced carcinogenesis in rats (Mori *et al.*, 1991).

Phenylpropanoids. Among the genotoxic phenylpropanoids, safrole and the chemically related estragole and β-asarone, are the most important DNA-damaging agents. Safrole (Fig. 2.12) and estragole are characterized by an

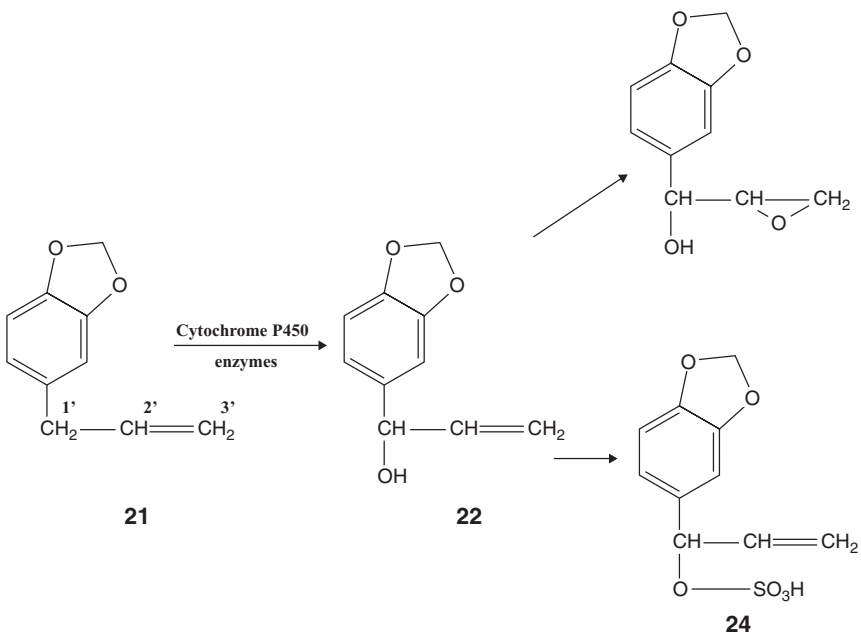

Figure 2.12 Metabolic activation of safrole (21). Metabolites with carcinogenic properties are 1′-hydroxy-safrole (22), 1′-hydroxy-2′,3′-safrole oxide (23) and 1′-sulfoxy safrole (24).

allylic side chain, in contrast to β-asarone, which bears a propenylic substituent. The allylic structure is apparently important for the metabolic activation and the formation of ultimate mutagens. Safrole is the main component in the essential oil of *Sassafras officinale* and occurs in small amounts in some species of the genera *Illicium*, *Asarum* and *Cinnamomum*. Estragole is a component of the essential oil of *Artemisia dracunculus* and β-asarone is present in the oil of *Acorus calamus*.

In the standard *S. typhimurium* test, safrole and estragole were inactive with or without added rat liver preparations. The known or possible metabolites 1′-hydroxysafrole, 1′-hydroxyestragole, the 2′, 3′oxides and the esters were mutagenic (Enomoto, 1987) (Fig. 2.12). Beta-asarone showed dose-dependent mutagenicity only with rat liver enzymes (Göggelmann and Schimmer, 1983), and was also active in inducing chromosomal aberrations and SCE in human lymphocytes in vitro (Abel, 1987). Safrole and estragole showed low clastogenic effects in vitro and in vivo. In Chinese hamster cells, a slight increase in SCE was observed by safrole. The effect was enhanced with rat liver preparations (Tayama, 1996). Recently, the cytogenetic effect and DNA adduct formation induced by safrole in Chinese hamster lung cells were analyzed (Daimon *et al.*, 1997). Schiestl and co-workers (1989) reported that safrole induced intrachromosomal recombination in yeasts in a dose-related response.

Safrole and estragole were found to produce liver tumours in rats. The 1′-hydroxy derivatives, the 2′,3′-oxides and the 1′-acetoxy derivatives were also tumorigenic. In vivo, the sulfoxy derivative may be the ultimate carcinogen. Sulfotransferase catalyzes the formation of the sulfuric ester of 1′-hydroxysafrole. The enzyme was detected in rat liver cytosol. Inhibition of the enzyme represses hepatic tumour formation.

DNA adduct formation and DNA binding properties have been extensively investigated. Safrole and estragole exhibited strong binding to mouse liver DNA. Several DNA adducts were isolated and confirmed by nuclear magnetic resonance (NMR) and CD spectroscopy. The adducts persist for a long time. However, the possible carcinogenic relevance of the long-term presence of such unrepaired adducts is unknown. They have been detected in target as well as in non-target tissues. Abundant information on metabolic activation, DNA adduct formation and tumour incidence in experimental animals is available from reviews by Groopman and Cain (1990) and Enomoto (1987).

Beta-asarone was also active in inducing liver tumours, but the hepatocarcinogenicity was not inhibited by sulfotransferase inhibitors. Nothing is known at present of DNA binding and DNA adduct formation. Although the carcinogenic potential of safrole, estragole and β-asarone does not appear to be high, since their effects in animals are relatively weak, we need more information to calculate the health risk of these compounds for humans.

2.2.2.4 Other endogenous mutagens in plants

Numerous plant metabolites have been assayed for mutagenicity in only a few in vitro test systems. Thus, their genotoxic potential cannot be critically assessed at present.

Xanthones. Free and glycosidic xanthone derivatives have been identified as SM of several members of the Gentianaceae (genera *Gentiana*, *Swertia*). Some of them, listed in Table 2.1, showed mutagenic effects after metabolic activation in certain *S. typhimurium* strains. The corresponding glycosides were inactive when β-glucosidase was absent in the system (Morimoto *et al.*, 1983; Kanamori *et al.*, 1984; Matsushima *et al.*, 1985).

Hymenoxon. The sesquiterpene lactone, hymenoxon (isolated from *Hymenoxis odorata*, Asteraceae), was found to be a direct-acting mutagen in the Salmonella Ames assay but did not produce lethal DNA damage in recombination repair deficient *E. coli* and *B. subtilis* strains (Jones and Kim, 1981). Alkylation of deoxyguanosine was reported (Sylvia *et al.*, 1985). Evidence was obtained that hymenoxon is capable of cross-linking the DNA strands (Sylvia *et al.*, 1987).

Cathinone. The mutagenicity of (−)cathinone has been evaluated in the germ cells of male albino mice using the dominant lethal test. Reduction in fertility and dose-dependent postimplantation loss but no dominant lethality was observed (Qureshi *et al.*, 1988). However, the alkaloid produced chromosomal aberrations in rats (De Hondt *et al.*, 1984) and in somatic cells of mice (Tariq *et al.*, 1987). The mechanism of action is unknown at present.

Sanguinarine. Sanguinarine is the main alkaloid in the seeds of *Argemone mexicana* and *Sanguinaria canadensis* but is also present in other members of the Papaveraceae. Chromosomal breaks in plant cells induced by the seed oil were attributed to the presence of sanguinarine (Subramanyam *et al.*, 1974).

Nandi and Maiti (1985) showed that sanguinarine has a high specificity to bind on GC-rich DNA in vitro. They concluded that the alkaloid preferentially binds to the GC pairs in the DNA template. Further data confirmed the intercalation model for sanguinarine (Faddejeva *et al.*, 1984; Schmeller *et al.*, 1997b). Single doses of 10 mg/kg increased the activity of certain liver enzymes in rats and caused a significant loss of microsomal cytochrome P_{450}. It was, therefore, suggested that sanguinarine was a potential hepatotoxic compound (Dalvi, 1985) but its role as a potential mutagen remains to be clarified. Farnsworth and co-workers (1976) reported that sanguinarine was carcinogenic in rats, guinea pigs and hamsters, but this result has not yet been confirmed.

Ellipticine. Ellipticine is an indole alkaloid with intercalating properties. It induced mutations in *S. typhimurium*, especially in the frameshift indicator strains (Ashby *et al.*, 1980), mitochondrial mutations in *S. cerevisiae* (Pinto *et al.*, 1982) and gene mutations in mouse lymphoma cells (Moore *et al.*, 1987). In addition, it showed SCE-inducing capacity and chromosome-breaking activity in human lymphocytes in vitro and clastogenic potency on bone marrow cells of Wistar rats in vivo (Sakamoto-Hojo *et al.*, 1988). The authors assumed that the chromosomal damage and the SCE formations may have been the result of interaction with topoisomerase enzymes. Additional information is available from Anderson and Berger (1994).

Acridone alkaloids. Only a few reports were found in the literature concerning the evaluation of genotoxicity of acridone alkaloids. Rutacridone epoxide was characterized as a direct-acting mutagen with high activity in *S. typhimurium*. Rutacridone was less active in this assay and needed metabolic activation (Paulini and Schimmer, 1989). Isogravacridonchlorine was shown to induce frameshift mutations via a reactive intercalation mechanism (Paulini *et al.*, 1991). The occurrence of acridone alkaloids and their biological properties were described in a review by Gröger (1988).

Hydroquinone. Hydroquinone was detected in higher amounts in the basidiomycete, *Agaricus hondensis*, where it was assumed to be responsible for the toxicity of this species (Jovel *et al.*, 1996). In higher plants, it occurs in glycosidic forms, e.g. as arbutoside, which is a characteristic metabolite of the Ericaceae family.

Endogenous glycosidases or bacterial enzymes produced by the intestinal microflora are capable of hydrolyzing the glycosides and of releasing free hydroquinone. The first report concerning the genotoxicity of hydroquinone was by Boyland and co-workers (1964). The authors described the appearance of carcinomas after implantation of the compound into the urinary bladder of mice. Walles (1990) studied the effect of hydroquinone in primary hepatocyte cultures. DNA single-strand breaks were detected with the alkaline elution technique. Evidence was found that OH radicals were involved in the DNA-damaging effect of hydroquinone. Marrazzini and co-workers (1991) tested the genotoxicity of hydroquinone in bone marrow cells of mice. Micronucleated erythrocytes, structural chromosomal aberrations and aneuploidy were induced in this system. This indicates that hydroquinone may represent a risk to both somatic cells (carcinogenic) and/or germ cells (aneuploidy). Recently, Tsutsui and co-workers (1997) studied the genotoxic activity of hydroquinone in Syrian hamster embryo cells, using methods with different genetic endpoints.

Isothiocyanates and related compounds. Isothiocyanates are characteristic SM of Brassicaceae, Tropaeolaceae and other members of the order Capparales (Wink and Waterman, 1999). In intact plants, they occur in their *S*-glycosidic form, as glucosinolates. Hydrolysis by the endogenous thioglucosidases leads to an unstable aglycone, which is rapidly converted into isothiocyanate and several byproducts, e.g. thiocyanate, and nitrile. Allylisothiocyanate is formed in this way from sinigrin. Allylisothiocyanate was one of the first natural mutagens to be studied (Auerbach and Robson, 1944). The compound is highly toxic to bacteria and fungi. After local application, it causes severe skin irritation in humans.

Allylisothiocyanate showed weak genotoxic effects in *D. melanogaster* and fungi. Chromosomal damage was induced in onion root tip cell (reviewed by Clark, 1982). Neudecker and Henschler (1985) found that allylisothiocyanate was an indirect-acting mutagen in *S. typhimurium*. The effect could be reduced when higher amounts of mammalian liver enzymes were present. Yamaguchi (1980) tested various isothiocyanates, thiourea compounds and the glycoside

sinigrin under preincubation conditions. Most substances showed positive effects in the absence of any exogenous metabolic activation. In studies by Neudecker and Henschler (1985) and by Yamaguchi (1980), long preincubation times were required to express mutagenicity in *S. typhimurium*.

The clastogenicity of allylisothiocyanate was investigated in Chinese hamster cells by Kamasaki and co-workers (1982) and later by Musk and co-workers (1995). Whereas, Kamasaki and co-workers (1982) observed chromosomal aberrations in a B241 cell line, Musk and co-workers (1995) found that the compound was inactive in Chinese hamster ovary cells. Phenethylisothiocyanate and – at higher doses – sinigrin and gluconasturtiin, the parent compounds of the isothiocyanates tested, were active in the chromosomal aberration test and in the SCE test.

Carcinogenicity experiments with allylisothiocyanate were performed by Dunnick and co-workers (1982). After long-term administration of the substance, transitional cell papillomas and epithelial hyperplasia were induced in the urinary blatter of male rats. An important property of glucosinolates and several of their decomposition products is the ability to modulate chemically induced carcinogenesis (Morse *et al.*, 1988). There is some evidence that administration of these compounds during the initiation period of carcinogenesis may cause the inhibition of tumour production; administration during the promotion, however, may enhance the carcinogenic effect (McDanell *et al.*, 1988).

2.2.2.5 Outlook

There can be little doubt that further screening of secondary plant metabolites will bring to light many more examples of endogenous mutagens (Table 2.4 lists recent results for alkaloids). Considering the numerous mutagens on which insufficient preliminary studies were performed, it will be necessary to fill the gaps in our knowledge first before being able to assess their genotoxic potential. This will include study of genetic and biochemical aspects as well as tissue specificities and deactivation mechanisms. We can expect that the role of intercalation in the process of mutagenicity/carcinogenicity will become clearer. The consequences of frameshift mutations for the eukaryotic cell, in particular, have to be elucidated. This would enable us to evaluate the health risk of intercalators, such as sanguinarine and berberine.

Fruitful future research might be the study of the interaction between mutagens co-occurring in the same plant or mutagens with different mechanisms of action. Under certain conditions, the first mutagen can act as an antimutagen, inhibiting the metabolic activation of the second mutagen or scavenging its mutagenic intermediate. Such interactions have already been demonstrated (Schimmer *et al.*, 1991).

2.2.2.6 Interaction with the cytoskeleton

Many cellular activities, such as motility, endo- and exocytosis and cell division, are mediated through elements of the cytoskeleton, including

Table 2.4 Interaction of alkaloids with DNA, RNA and associated targets

Alkaloid	Source	Effect
Alkaloids derived from tryptophan		
Camptothecin	*Camptotheca acuminata* (Cornaceae)	DNA intercalator; topoisomerase I inhibitor; stabilization of topo I/DNA/CPT complex; cell cycle arrest in G_2/M phase; apoptosis induction
Cinchonine	*Cinchona* sp. (Rubiaceae)	DNA intercalation; inhibition of DNA polymerase and reverse transcriptase
Cinchonidine	*Cinchona* sp. (Rubiaceae)	DNA intercalation; inhibition of DNA polymerase and reverse transcriptase
Cryptolepine	*Cryptolepis sanguinolenta* (Asclepiadaceae)	Intercalation of GC rich DNA, inhibition of topoisomerase II, stabilizes topo II-DNA complexes; stimulates DNA cleavage, cell cycle arrest; apoptotic; forms G-quadruplexes with telomeric DNA
Dictamine	*Dictamnus* (Rutaceae)	DNA intercalation; mutagenic
Ellipticine	*Ochrosia elliptica* (Apocynaceae)	DNA intercalator; inhibition of topoisomerase II and telomerase; apoptotic; mutagenic
Evolitrine	Rutaceae	DNA intercalation; mutagenic
γ-Fagarine	Rutaceae	DNA intercalation; mutagenic
Harmine	*Peganum harmala* (Zygophyllaceae)	DNA intercalation; inhibition of topoisomerase I; antitumour activity; induction of DNA strand breaks; inhibition of DNA polymerase and reverse transcriptase
Harmaline	*Peganum harmala* (Zygophyllaceae)	DNA intercalation; inhibition of DNA polymerase and reverse transcriptase
10-Hydroxy-camptothecin	*Camptotheca acuminata* (Cornaceae)	Topoisomerase I inhibitor; cell cycle arrest in G_2/M phase; apoptosis induction
Kokusaginine	Rutaceae	DNA intercalation; mutagenic
Luotonine A	*Peganum nigellastrum* (Zygophyllaceae)	Inhibitor of topoisomerase I; cytotoxic
Maculine	Rutaceae	DNA intercalation; mutagenic
Matadine	*Strychnos gossweileri* (Loganiaceae)	DNA intercalation; topoisomerase II inhibition
Neocryptolepine	*Cryptolepis sanguinolenta* (Asclepiadaceae)	Intercalation of GC rich DNA; inhibition of topoisomerase II; cytotoxic, antibacterial, antiparasitic
Norharman	*Peganum harmala* (Zygophyllaceae)	DNA intercalation; inhibition of DNA polymerase and reverse transcriptase
Quinine	*Cinchona* sp. (Rubiaceae)	DNA intercalation; inhibition of DNA polymerase and reverse transcriptase
Quinidine	*Cinchona* sp. (Rubiaceae)	DNA intercalation; inhibition of DNA polymerase and reverse transcriptase
Serpentine	*Rauvolfia serpentina* (Apocynaceae)	DNA intercalation; topoisomerase II inhibition

(*Continued*)

Table 2.4 (*Continued*)

Alkaloid	Source	Effect
Skimmianine	Rutaceae	DNA intercalation; mutagenic
Tangutorine	*Nitraria tangutorum* (Zygophyllaceae)	Induction of cyclin kinase inhibitor p21; inhibition of topoisomerase II expression
Usambarensine	*Strychnos usambarensis* (Loganiaceae)	DNA intercalator; apoptotic; cytotoxic, antiparasitic
Alkaloids derived from phenylalanine/tyrosine		
Actinodaphnidine	*Cassytha filiformes* (Lauraceae)	DNA intercalation; inhibition of topoisomerases; cytotoxic, antitrypanosomal activity
Aristolactam glucoside	*Aristolochia* sp. (Aristolochiaceae)	DNA intercalator
Aristolochic acid	*Aristolochia* sp. (Aristolochiaceae)	Activation by NADPH:CYP reductase leading to formation of DNA adducts; mutagenic and carcinogenic
Berbamine	*Berberis* sp. (Berberidaceae)	DNA intercalation; inhibition of DNA polymerase and reverse transcriptase
Berberine	*Berberis* sp. (Berberidaceae)	DNA intercalation; binding to single stranded poly (rA); inhibitor of topoisomerase I and II; inhibition of DNA polymerase and reverse transcriptase
Berberrubine	*Berberis* sp. (Berberidaceae)	DNA intercalator; topoisomerase II inhibitor (stabilizing topo II cleavable complexes); inhibition of catalytic activity
Boldine	*Peumus boldo* (Monimiaceae)	DNA intercalation; inhibition of DNA polymerase and reverse transcriptase
Bulbocapnine	*Corydalis* sp. (Fumariaceae)	DNA intercalation
Burasaine	*Burasaia* sp. (Menispermaceae)	Probably DNA intercalating; cytotoxic
Cassythine	*Cassytha filiformes* (Lauraceae)	DNA intercalation; inhibition of topoisomerases; cytotoxic; antitrypanosomal activity
Chelerythrine	*Chelidonium majus* (Papaveraceae)	When activated by CYP, DNA adducts can be generated
Coptisine	Ranunculaceae	DNA intercalator
Coralyne	Papaveraceae	DNA intercalator (GC rich sequences), topoisomerase I inhibitor
Dicentrine	*Dicentra* sp. (Fumariaceae)	Minor groove DNA intercalator; inhibition of topisomerase II; mutagenic
Dicentrinone	*Ocotea leucoxylon* (Lauraceae)	Inhibitor of topoisomerase I
Emetine	*Psychotria ipecacuanha* (Rubiaceae)	DNA intercalation; inhibition of DNA polymerase and reverse transcriptase, and protein biosynthesis; apoptotic
Epiberberine	*Coptis chinensis* (Ranunculaceae)	Inhibitor of topoisomerase I

Table 2.4 (*Continued*)

Alkaloid	Source	Effect
Fagaronine	*Fagara zanthoxyloides* (Rutaceae)	Major groove DNA intercalator; inhibition of topoisomerase I and II; antitumour compound
Glaucine	Papaveraceae	DNA intercalation
Groenlandicine	*Coptis chinensis* (Ranunculaceae)	Inhibitor of topoisomerase I
Haemanthamine	*Lycoris radiata* (Amaryllidaceae)	Complex formation with RNA
Isocorydine	Papaveraceae	DNA intercalation
Jatrorrhizine	Ranunculaceae	DNA intercalator
Liriodenine	*Cananga odorata* (Annonaceae)	Inhibitor of topoisomerase II (catalytic inhibitor); mutagenic
Lycobetaine	Amaryllidaceae	Inhibitor of topoisomerase II; cytotoxic
Lycorine	*Narcissus* sp. (Amaryllidaceae)	Complex formation with RNA
Nitidine	*Toddalia asiatica* (Rutaceae)	DNA intercalator; inhibition of topoisomerase I and II; antitumour compound
Palmatine	Ranunculaceae	DNA intercalator
Roemerine	*Roemeria* sp. (Papaveraceae)	Mutagenic
Salsolinol	*Salsola* sp. (Chenopodiaceae)	In combination with Cu(II) causes DNA strand breaks; ROS formation; cytotoxic
Sanguinarine	*Sanguinaria canadensis* (Papaveraceae)	DNA intercalator; intercalation of double stranded GC-rich RNA; when activated by CYP, DNA adducts can be generated; inhibition of DNA polymerase and reverse transcriptase; clastogenic; mutagenic
Tetrandrine	*Stephania tetrandra* (Menispermaceae)	DNA alkylating; mutagenic; carcinogenic; apoptotic
Alkaloids derived from ornithine/arginine		
Clivorine	*Ligularia hodgsonii* (Asteraceae)	Metabolic activation by CYP3A1 and CYP3A2;
Monocrotaline	*Crotalaria* sp. (Fabaceae)	CYP3A substrate; CYP oxidation to dehydromonocrotaline which forms DNA-DNA interstrand and DNA-protein cross-links
Pyrrolizidine alkaloids	Asteraceae, Boraginaceae	Metabolic activation by CYP3A; generation of reactive dehydropyrrolizidines that can alkylate DNA, formation of DNA-DNA interstrand and DNA-protein cross-links; mutagenic, carcinogenic
Retrorsine	*Senecio* sp. (Asteraceae)	Induction of CYP1A1, 1A2, 2EI, 2B1/2
Miscellaneous alkaloids		
Acronycine	*Acronychia baueri* (Rutaceae)	DNA alkylation; intercalator; carcinogen, apoptotic

(*Continued*)

Table 2.4 (*Continued*)

Alkaloid	Source	Effect
Cycasin	*Cycas* and related Cycadaceae	Activated methylazoxymethanol is a DNA methylating agent, therefore mutagenic and carcinogenic
Lobeline	*Lobelia* sp. (Campanulaceae)	DNA intercalation; inhibition of DNA polymerase and reverse transcriptase
Mahanine, murrayanol	*Murraya koeningii* (Rutaceae)	Inhibition of topoisomerase I and II; antimcrobial and molluscicidal activities

Note: For earlier papers and references, see Wink (1993a, 2000, 2007a).

microfilaments and microtubules (for an overview, see Alberts *et al.*, 2008; Lodish *et al.*, 1995) (Fig. 2.13; Table 2.5). In plants and fungi, a number of allelochemicals have been identified that can interfere with tubulin and microtubules (e.g., colchicine, *Vinca* alkaloids, maytansine, maytansinine, taxol and the lignan podophyllotoxin).

Cell stability, phagocytosis, cell–cell interactions and cell movements are also controlled by actin filaments, which are rapidly assembled or disassembled from actin monomers. Cytochalasin B, an alkaloid produced by a number of moulds, binds to the plus end of a growing actin filament, preventing the

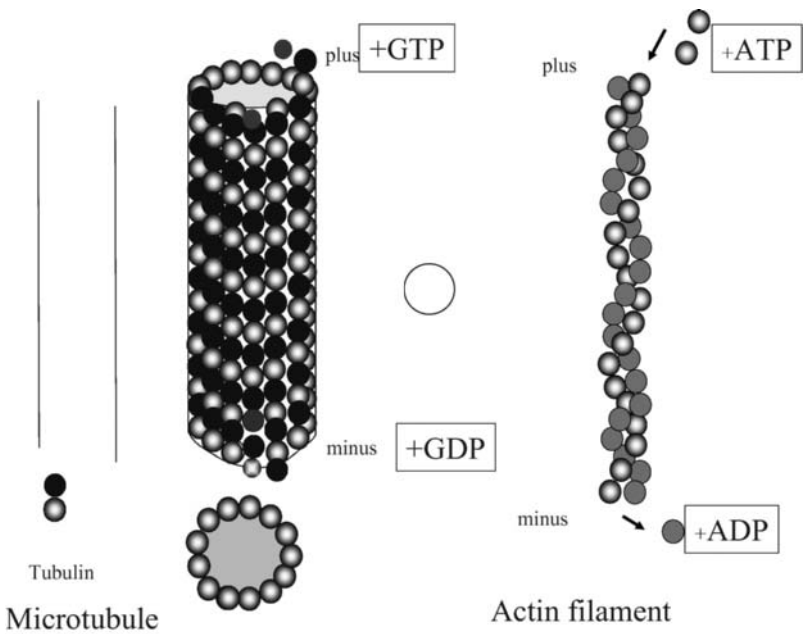

Figure 2.13 Microtubules and actin filaments as targets for secondary metabolites. (Adapted from Wink, 2007©). (See Plate 12 in colour plate section.)

Table 2.5 Interference of alkaloids with the assembly and disassembly of microtubules and actin filaments

Alkaloid	Source	Effect
Chelidonine	*Chelidonium majus* (Papaveraceae)	DNA intercalation; spindle poison; activation of caspase-3; DNA fragmentation; depolarization of mitochondrial membranes, apoptotic
Colchicine	*Colchicum* sp. (Colchicaceae)	Inhibition of S–S cross-links between tubulin units
Evodiamine	*Euodia hortensis* (Rutaceae)	Inhibition of tubulin polymerization; induction of cell cycle arrest in G_2/M phase
Maytansine	*Maytenus* sp. (Celastraceae)	Inhibition of S-S cross-links between tubulin units; antimitotic, anticancer drug
Noscapine	*Papaver somniferum* (Papaveraceae)	Tubulin binding; inhibition of microtubule assembly; induction of polyploidy
Paclitaxel/taxol	*Taxus* sp. (Taxaceae)	Inhibitor of microtubule depolymerization; activation of polymerization
Sanguinarine	Papaveraceae	Interaction with cytoskeleton
Vincristine, vinblastine	*Catharanthus roseus* (Apocynaceae)	Inhibition of S–S cross-links between tubulin units; forming spiral filaments of tubulin

Source: After Wink (2007a).

addition of actin monomers at that point. Latrunculin B from *Latrunculia magnifica* (a marine organism) is 10- to 100-fold more potent than cytochalasins in the inhibition of microfilament organization. Phalloidin, produced by the fatally poisonous toadstool, *Amanita phalloides*, stabilizes actin filaments and inhibits their depolymerization. Any allelochemical that impairs the function of microtubules or microfilaments is likely to be toxic and, from the point of view of defence, a well-designed molecule (reviewed in Wink, 1993a, 1998; Wink and Van Wyk, 2008).

2.2.2.7 Protein biosynthesis

Protein biosynthesis is essential for all cells and, thus, provides another important target. Indeed, a number of allelochemicals have been detected (although not many have been studied in this context) that inhibit protein biosynthesis in vitro. Emetine, from *Cephaelis ipecacuanha* (Rubiaceae), is the most potent plant constituent. Other alkaloids with the same ability include harringtonine, homoharringtonine, cryptopleurine, tubulosine, hemanthamine, lycorine, narciclasine, pretazettine, pseudolycorine, tylocrepine and tylophorine (Wink, 1993a). A weaker inhibition was observed for ajmaline, berberine, boldine, cinchonine, cinchonidine, harmaline, 'harmine', lobeline, norharman, papaverine, quinidine, quinine, salsoline, sanguinarine, solanine and yohimbine (Wink and Latz-Brüning, 1995; Wink *et al.*, 1998a,b). Tannins

inhibit protein biosynthesis effectively in vitro; if they are resorbed, activity is also likely in vivo.

Most compounds that substantially affect DNA, DNA polymerase I and RT are also active as translation inhibitors; a significant ($p < 0.01$) correlation can be established between the degree of intercalation and inhibition of translation (Wink *et al.*, 1998a,b). Interaction of these alkaloids with ribosomal nucleic acids, e.g. ribosomal ribonucleic acid (rRNA), tRNA) or mRNA, is likely, in addition to interactions with ribosomal proteins (Wink and Twardowski, 1992). On the other hand, most compounds (such as aconitine, caffeine, colchicine, cytisine, gramine, hyoscyamine, lupanine, narcotine, scopolamine, sparteine or strychnine) that do not intercalate do not have a substantial influence on translation (Wink *et al.*, 1998a,b). Inhibition of protein biosynthesis shows a significant correlation with inhibition of bacterial and radicle growth in *Lepidium sativum* and insect and vertebrate toxicity, indicating the importance of this molecular target (Wink *et al.*, 1998a,b).

Quinolizidine alkaloids (QAs), such as sparteine, lupanine and cytisine, are relatively weak inhibitors of this target (they strongly affect ACh receptors and Na^+-channels) (see Tables 2.7 and 2.8). The stages that are inhibited are the loading of aminoacyl-tRNA with amino acids and the elongation step. The inhibitory activity was visible in heterologous systems but protein biosynthesis in the producing plants (in this case lupins) was not affected (Wink and Twardowski, 1992).

A number of antibiotics (from *Streptomyces* and other bacteria or fungi) inhibit protein biosynthesis at specific steps, such as initiation, peptidyl transferase or elongation. Depending on their affinity for prokaryotic or eukaryotic ribosomes, some of the antibiotics selectively inhibit microbial systems. Since mitochondria also contain ribosomes of prokaryotic origin, effects can also occur in animals. These compounds have apparently evolved as chemical defence compounds of fungi or bacteria against other microorganisms.

2.2.2.8 Electron chains

The respiratory chain and ATP-synthesis in mitochondria or photophosphorylation in chloroplasts demand the controlled flux of electrons and protons. These targets seem to be attacked by sanguinarine, ellipticine, gramine, alpinigenine, capsaicin, HCN, rotenone, nicotine and a few other allelochemicals (Wink, 1993a).

2.2.2.9 Induction of apoptosis

Apoptosis, which is a natural mechanism in the development of organisms, can be induced by a number of toxins and appears to be the major mechanism of cytotoxicity caused by SM (Wink, 2007a,b,c; Wink and Van Wyk, 2008). Many polyphenols, terpenoids, saponins and also alkaloids apparently can induce apoptosis.

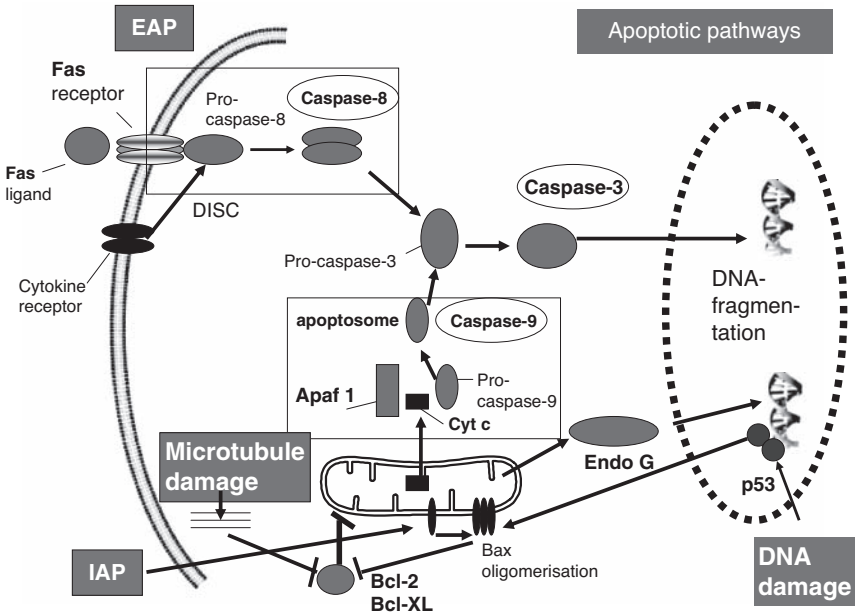

Figure 2.14 Schematic illustration of apoptoses with a focus on the extrinsic and intrinsic pathways. (See Plate 12 in colour plate section.) EAP = extrinsic apoptotic pathway; IAP = intrinsic apoptotic pathway

Apoptosis can be induced by two pathways: the extrinsic pathway starts with an activation of death receptors on the cell surface, which leads to the activation of caspases (Fig. 2.14). Death receptors are a subgroup of the tumour necrosis factor (TNF) receptor family that have an intracellular death domain. Death receptors include CD95, TRAIL-R1 and TRAIL-R2 (TNF-related apoptosis inducing ligand). The stimulated death receptors activate an adaptor protein FADD (Fas-associated death domain protein), which in turn activates the inactive form of caspase-8 (*c*ysteine-*a*spartyl-*s*pecific *p*rote*ase*s). Caspase-8 activates pro-caspase-3 and also the protein Bid (a member of the Bcl-2 family) that can stimulate and amplify the intrinsic pathway.

The intrinsic pathway is triggered by the permeabilization of mitochondrial membranes releasing cytochrome c and reducing ATP levels. Cytochrome c and other apoptotic factors form a complex with Apaf-1 (apoptotic protease activating factor) and inactive caspase-9. This complex has been termed apoptosome and leads to the activation of caspase-9. Activated caspases-8 and -9 can activate pro-caspase-3 to caspase-3. The activated caspases cleave cellular proteins (e.g. proteins of the cytoskeleton) via caspase-activated DNAse (CAD) and also chromatin. As a consequence, the sequence of morphological and biochemical degradation steps sets in. During apoptosis a DNAse, Endo G, a caspase-independent apoptotic protein, is activated. If the DNA of apoptotic cells is analyzed by gel electrophoresis, a typical ladder pattern of fragmented chromosomes can be observed.

Bcl-2 is an antiapoptotic protein that keeps caspases in an inactive state and regulates the mitochondrial pathway. Overexpression of the *bcl-2* gene may confer resistance to chemotherapeutic drugs. A down-regulation of Bcl-2 and Bcl-XL enhances apoptosis. Agents that interfere with microtubules (such as spindle poisons) apparently inactivate Bcl-2.

Some alkaloids have oxidizing properties and lead to the production of reactive oxygen species (ROS). The formation ROS is associated with DNA damage, cell cycle and mitochondrial disturbance. DNA damage, which can also be caused by alkylating and intercalating compounds or gamma-irradiation and other signals, can stimulate the tumour suppressor gene p53. In consequence, Apaf-1 becomes activated, triggering apoptosis. P53 is a stress sensor that is modified by posttranslational modifications. Activated p53 reacts as a transcription factor for pro-apoptotic proteins, including BAX, and death receptors (CD95, TRAIL). Loss of p53 function is associated with tumour aggressiveness and resistance to anticancer treatments.

Upon inhibition of the apoptotic pathway, as by loss of p53 or Apaf-1 function, pre-cancerous cells survive and proliferate, and eventually form a tumour. A number of alkaloids have been described recently that exhibit antiapoptotic effects. Huperzine A from *Huperzia serrata* (Lycopodiaceae) blocks apoptosis via the inhibition of ROS formation and caspase-3 activation. In case of sampangine, the apoptotic effects could be blocked by administration of ROS quencher, such as *N*-acetylcysteine, vitamin C and vitamin E. Apoptosis is a very complex process in which many factors and interactions are still unknown. Therefore, the scheme drawn in this review (Fig. 2.14) must necessarily be incomplete.

Searching for new drugs with anticancer activities, those SM with apoptotic properties are of major interest. Table 2.6 lists these alkaloids that have been reported to have apoptotic properties. Not all alkaloids are apoptotic; in a more systematic approach, 70 alkaloids from several biogenetic groups were tested in this laboratory with leukaemia cells (HL-60; Jurkat T-cells): Several protoberberine and benzophenanthridine alkaloids (berberine, chelerythrine, chelidonine, sanguinarine), homoharringtonine, noscapine, indole alkaloids (harmine, quinine, vincristine, vinblastine, ellipticine), emetine, piperine and colchicines apparently induce apoptosis, whereas the tropane, quinolizidine, piperine, pyridine, purine, steroidal, diterpene alkaloids were not active up to a concentration of 100 μM (Rosenkranz and Wink, 2007). The active alkaloids have in common that they intercalate DNA, and in consequence inhibit DNA and RNA polymerase, topoisomerases and even ribosomal protein biosynthesis, or bind to tubulin/microtubules, thus acting as spindle poisons.

2.2.3 Interference of SM with neuronal signalling

In the present chapter, special emphasis has been laid on interactions between nitrogen-containing SM and major neuroreceptors, such as cholinergic, adrenergic, serotonergic and GABAergic neuroreceptors, and Na^+-, K^+-, Cl^-- and

Table 2.6 Induction of apoptosis by alkaloids

Alkaloid	Source	Effect
Alkaloids derived from tryptophan		
Camptothecin	*Camptotheca acuminata* (Cornaceae)	Topoisomerase I inhibitor; apoptosis induction in S-phase
Cinchonidine	*Cinchona pubescens* (Rubiaceae)	Slight DNA intercalation; activation of caspase-3; DNA fragmentation; depolarization of mitochondrial membranes; apoptotic
Cryptolepine	*Cryptolepis sanguinolenta* (Asclepiadaceae)	Cell cycle arrest in G_2/M phase; PARP cleavage; cytochrome c release; upregulation of p53, DNA intercalation; activation of caspase-3, -9; PARP cleavage; DNA fragmentation; apoptotic; clastogenic potential
Ellipticine	*Ochrosia elliptica* (Apocynaceae)	Inhibition of cell cycling (G_2/M arrest); activation of Fas/Fas ligand pathway; induction of mitochondrial apoptotic pathway with caspase-9 activation; activation of caspase-3; DNA fragmentation; depolarization of mitochondrial membranes; inhibitor of protein kinases which phosphorylate p53; bax activation; apoptotic
Evocarpine	*Evodia rutaecarpa* (Rutaceae)	Apoptotic
Evodiamine	*Evodia rutaecarpa* (Rutaceae)	Induction of cell cycle arrest and apoptosis; tubulin inhibitor
Harmine	*Peganum harmala* (Zygophyllaceae)	Inhibitor of cyclin-dependent kinases; inhibition of Bcl-2 expression; upregulation of death receptor Fas and some DNA intercalation; activation of caspase-3; DNA fragmentation; depolarization of mitochondrial membranes, apoptotic
9-Hydroxyellipticine	*Ochrosia elliptica* (Apocynaceae)	Apoptotic; inhibition of p53 phosphorylation; inhibition of cdk2 kinase
10-Hydroxycamptothecin	*Camptotheca acuminata* (Cornaceae)	Topoisomerase I inhibitor; cell cycle arrest in G_2/M phase; apoptosis induction
Isostrychnopentamine	*Strychnos usambarensis* (Loganiaceae)	Caspase-3, -9 activation, cell cycle arrest in G_2/M phase, p21 induction,
Neocryptolepine	*Cryptolepis sanguinolenta* (Asclepiadaceae)	Cell cycle arrest in G_2/M phase; PARP cleavage; cytochrome c release; upregulation of p53
Quinine	*Cinchona pubescens* (Rubiaceae)	Weak DNA intercalation; cell cycle arrest; activation of caspase-3; DNA fragmentation; depolarization of mitochondrial membranes, apoptotic

(*Continued*)

Table 2.6 (*Continued*)

Alkaloid	Source	Effect
Sungucine	*Strychnos icaja* (Loganiaceae)	Caspase-3, -9 activation, cell cycle arrest in G_1; PARP cleavage;
Usambarensine	*Strychnos usambarensis* (Loganiaceae)	Caspase activation; apoptotic
Vinblastine, vinblastine	*Catharanthus roseus* (Apocynaceae)	DNA intercalation; spindle poison; phosphorylation of Bcl-2 and Bcl-XL by JNK; induction of p53; activation of caspase-3; DNA fragmentation; apoptotic; mitochondrial pathway; ROS production
Alkaloids derived from phenylalanine/tyrosine		
Antofine and related alk	*Cynanchum* sp. (Asclepiadaceae)	Cell cycle arrest in G_2/M phase, cytotoxicity
Berbamine	Ranunculaceae	Apoptotic
Berberine	*Berberis vulgaris* (Berberidaceae)	DNA intercalator; enzyme inhibitor; apoptotic, cell cycle arrest in G_0–G_1 phase; caspase-3 activation; depolarization of mitochondrial membranes; downregulation of telomerase; antineoplastic, antimalarial
Cepheranthine	*Stephania cepharantha* (Menispermaceae)	Caspase-3 activation; induction of apoptosis; protection against apoptosis in some cells radical scavenger
Chelerythrine	*Chelidonium majus* (Papaveraceae)	DNA intercalation; release of cytochrome c; Bcl-XL inhibitor; activation of caspase-3; DNA fragmentation; depolarization
Chelidonine	*Chelidonium majus* (Papaveraceae)	Spindle poison; activation of caspase-3; DNA fragmentation; depolarization of mitochondrial membranes, apoptotic
Colchicine	*Colchicum autumnale* (Colchicaceae)	Spindle poison; activation of caspase-3; DNA fragmentation; apoptotic; activation of JNK/SAPK; ROS production; inhibition of mitochondrial electron chain;
Emetine	*Psychotria ipecacuanha* (Rubicaeae)	Inhibitor of protein, DNA and RNA synthesis; DNA intercalation; induction of apoptosis; activation of caspase-3; DNA fragmentation; depolarization of mitochondrial membranes
Harringtonine	*Cephalotaxus hainanensis* (Cephalotaxaceae)	Apoptosis induction
Homoharringtonine	*Cephalotaxus harringtonia* (Cephalotaxaceae)	Apoptotic antileukaemia drug; DNA fragmentation; caspase-3 activation; cytochrome c release; cleavage of poly(ADP-ribose) polymerase (PARP); bax upregulation, Bcl-2 downregulation
Isoharringtonine	*Cephalotaxus hainanensis* (Cephalotaxaceae)	Apoptosis induction , Bcl-2 downregulation

Table 2.6 (*Continued*)

Alkaloid	Source	Effect
Liriodenine	*Liriodendron* sp. (Magnoliaceae)	Cell cycle arrest in G_2/M phase; reduction of cyclin-dependent kinases; caspase activation, apoptotic
Lycorine	*Narcissus* sp. (Amaryllidaceae)	Cell cycle arrest in G_2/M phase, activation of caspase-8, -9, -3; downregulation of Bcl-2
6-Methoxydihydro-Sanguinarine	*Hylomecon hylomeconoides* (Papaveraceae)	Activation of caspase-8, -9, -3; PARP cleavage; release of cyt c; downregulation of Bcl-2; upregulation of p53 and Bax; ROS generation, apoptotic
Noscapine	*Papaver somniferum* (Papaveraceae)	Tubulin binding; inhibition of microtubule assembly; cell cycle arrest; activation of c-Jun NH2-terminal kinases (JNK); DNA fragmentation of mitochondrial membranes, apoptotic; ROS production
Pancratistatin	*Pancratium littorale* (Amaryllidaceae)	Apoptotic via mitochondria; cell cycle arrest
Papaverine	*Papaver* sp. (Papaveraceae)	Low activation of caspase-3; DNA fragmentation; apoptotic
Piperine	*Piper nigrum* (Piperaceae)	Activation of caspase-3; DNA fragmentation; depolarization of mitochondrial membranes, apoptotic
Protopine	*Chelidonium majus* (Papaveraceae)	Activation of caspase-3; DNA fragmentation; slightly apoptotic (1 μM)
Sampangine	*Cananga odorata* (Annonaceae)	Induction of ROS formation, which disturb cell cycle and mitochondria, leading to apoptosis; antiparasitic
Sanguinarine	*Sanguinaria canadensis* (Papaveraceae)	Cell cycle arrest in G_0–G_1 phase; induction of apoptosis in mitochondrial pathway, downregulation of cycline and cycline-dependant kinase; PARP cleavage; caspase activation; glutathione depletion; ROS production; depolarization of mitochondrial membranes; activation of caspase-3
Sinoculine	*Stephania sutchuensis* (Menispermaceae)	Cytotoxic, apoptotic
Tetrandrine	*Stephania tetrandra* (Menispermaceae)	Activation of caspase-3-dependent mitochondrial apoptosis pathway; activation of Endo G; PARP cleavage
Thaliblastine	*Thalictrum* sp. (Ranunculaceae)	Apoptotic DNA fragmentation
Alkaloids derived from ornithine/arginine		
Monocrotaline	*Crotalaria* sp. (Fabaceae)	Mutagenic; apoptotic;
Retrorsine	*Senecio* sp. (Asteraceae)	Apoptotic in liver cells, bax upregulation; Bcl-xL downregulation; cytochrome c release

(*Continued*)

Table 2.6 (*Continued*)

Alkaloid	Source	Effect
Alkaloids derived from lysine		
Matrine	*Sophora* sp. (Fabaceae)	Inhibition of DNA synthesis; cell arrest in G_1 phase; induction of apoptosis
Alkaloids with a terpenoid backbone		
Cyclopamine	*Veratrum album* (Liliaceae)	Blocks intracellular Shh signalling; apoptotic
Paclitaxel	*Taxus* sp. (Taxaceae)	Cell cycle arrest in G_2/M phase; apoptotic; Bcl-XL phosphorylation; caspase-3 activation; PARP cleavage; caspase-8 activation (CD95/CD95-L independent)
Solamargine	*Solanum* sp. (Solanaceae)	Cytotoxicity; apoptotic;
Miscellaneous alkaloids		
Acronycine	*Acronychia baueri* (Rutaceae)	DNA alkylation; intercalator; carcinogen, apoptotic
Caffeine	*Coffea arabica* (Rubiaceae)	Slightly apoptotic; cell cycle arrest; p53 dependence
Capsaicin	*Capsicum frutescens* (Solanaceae)	Apoptotic; Bcl dependent
Mahanine	*Micromelum minutum* (Rutaceae)	Induction of apoptosis by activating mitochondrial pathway and caspase-3; -8,-8 and -9; PARB cleavage; cytochrome c release; generation of ROS;
1-Methoxy-canthin-6-one	*Ailanthus altissima* (Simaroubaceae)	Induction of apoptosis
Theophylline	*Camellia sinensis* (Theaceae)	Slightly apoptotic, downregulation of Bcl-2;

Source: After Wink (2007a).

Ca^{2+}-channels, although other elements of the neuronal signal transduction (ACh esterase [ACE], monoamine oxidase [MAO], adenylyl cyclase, phosphodiesterase, phospholipase, protein kinase and neurotransmitter transport) have also been addressed. Since any substantial interference at the neuroreceptors (e.g. competitive inhibition of ligand binding by antagonistic SM or agonistic receptor activation by a defence substance with structural similarity to the natural ligand) will influence neuronal signal transduction (including muscular and central nervous system [CNS] activity), the intake of a larger dose should lead to substantial physiological disturbance (Wink and van Wyk, 2008).

It is outside the scope of the present chapter to provide a complete overview of all the relevant interactions that have been published. Rather, one aim is to highlight the body of information that has accumulated, especially during the last decade, due to a number of technical breakthroughs, such as patch clamp techniques (Neher and Sakman, 1992) allowing direct measurements of ion

channel activities; and receptor ligand assays (Yamamura and Snyder, 1974) with a wide number of cloned receptors and specifically labelled ligands.

The nervous system consists of the central (brain and spinal cord) and the peripheral system (afferent sensory and efferent motor nerves); it regulates all aspects of bodily function and is staggering in its complexity. Another distinction is between the somatic and the autonomic nervous system, which regulates heart and blood circulation, respiration, motility of the gastrointestinal tract, smooth muscles of the gall and urinary bladder, ureter and uterus and also glandular secretion. The somatic nervous system innervates the skeletal muscles. The autonomic nervous system is further divided into a sympathetic and parasympathetic part, which often regulate the same organ in opposite ways. The basic elements of the nervous systems are neurons, which communicate with each other via chemical synapses. Whereas somatic nerves are usually monosynaptic, two neurons, which communicate via a ganglionic synapse, are found in sympathetic and parasympathetic nerves. When the postsynaptic cell is a muscle cell, the synapse is called a neuromuscular junction or motor end-plate.

The presynaptic terminal contains vesicles that are filled with neurotransmitters. Presynapse and postsynapse are separated by a narrow synaptic cleft, into which the neurotransmitters are released from the vesicles via exocytosis. Transmitters diffuse across the synaptic cleft and bind to a neuroreceptor on the postsynaptic cell. The ion permeability of the postsynaptic membrane is changed in the next step, causing a sudden change in the corresponding membrane potential. In neurons, this electric disturbance can induce an action potential. At the motor end-plate, the change of membrane potential leads to muscle contraction; in gland cells, it may induce hormone secretion. Many nerve and most nerve–muscle synapses are excitatory. Binding of neurotransmitters to inhibitory receptors on the postsynapse causes an opening of K^+- and Cl^--channels that hyperpolarize the membrane and, thus, block the generation of an action potential. Neuroreceptors are found at the post- and presynaptic membrane. Activation of presynaptic receptors usually leads to an inhibition of neurotransmitter release, whereas their inhibition results in an enhanced release of neurotransmitters.

Thus, neurotransmitters and neuroreceptors are the basic elements for signal transduction in synapses of the central and peripheral nervous system and in neuromuscular junctions. The corresponding neurotransmitters (ligands) include ACh, NA, adrenaline, serotonin (i.e. 5-hydroxytryptamine, 5-HT), dopamine, histamine, glycine, glutamate/aspartate, GABA, ATP and several peptides. Two classes of membrane-residing neuroreceptors can be distinguished: a fast ligand-gated channel (class I) and a slower G protein linked receptor (class II), which are very similar across a wide range of animals. The class I neuroreceptor is part of an ion channel complex (Fig. 2.15). When a neurotransmitter binds, a conformational change induces the opening of an ion channel. According to the geometry and polarity of the 'gate', a selective permeability is achieved for Na^+, K^+, Ca^{2+} and Cl^- ions. The driving force is

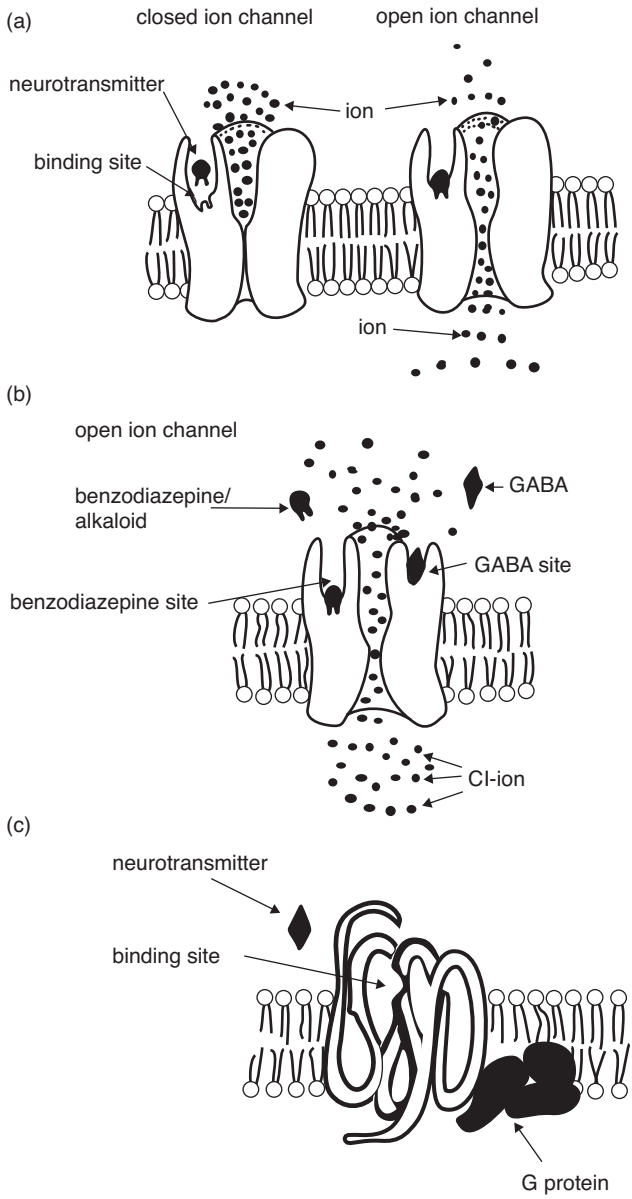

Figure 2.15 Schematic illustration of ligand-gated ion channels and G-protein linked neuroreceptors. (a) Could be a nicotinic acetylcholine receptor; (b) a gamma-aminobutyric acid (GABA) receptor; and (c) a dopamine or noradrenaline receptor.

provided via the ion concentration inside the cell or the extracellular space. Class I ligandoperated ion channels include the excitatory nicotinic ACh, glutamate/aspartate, ATP_{P2z} and the $5\text{-}HT_3$ receptor and the inhibitory glycine and $GABA_A$ receptor. In the well-studied nicotinic ACh-receptor, the five subunits, consisting of two α-subunits that bind ACh and of one β-, γ- and δ-subunit each, form the ligand-gated ion channel (Changeux, 1993) (Fig. 2.15).

The G protein-linked receptors (GPCR, class II) (Fig. 2.15) are far more numerous and more complex than the ligand-operated ion channels. They include muscarinic ACh, adenosine, adrenergic, serotonergic (except $5\text{-}HT_3$; see above), $GABA_B$, glutamate (mGluR), histamine and opiate receptors. They share a common architecture, having seven transmembrane domains and three internal and three external loops each (Fig. 2.15). When the corresponding neurotransmitter binds, the receptor changes its conformation, inducing a conformational change in an adjacent G protein molecule, consisting of three subunits α, β and γ. G proteins function as an on/off switch, which is off when the α-subunit binds guanosine diphosphate (GDP). Binding of a ligand to the receptor causes the G protein to release its bound GDP and to bind guanosine triphosphate (GTP), converting the α-subunit to the 'on' state. The α-subunit dissociates and then either interacts with an ion channel or activates/inhibits the enzymes of second messenger formation (Fig. 2.16), such

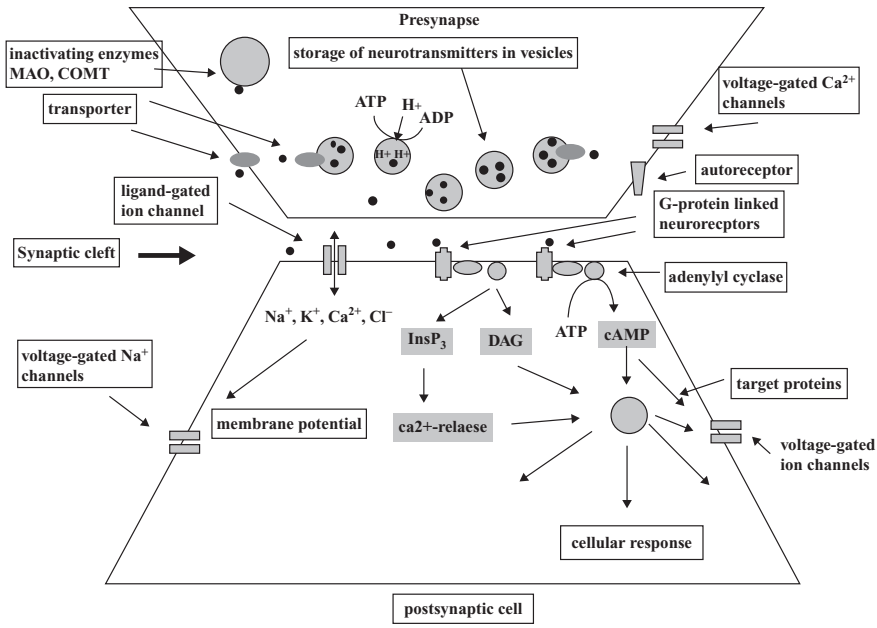

Figure 2.16 Schematic illustration of signalling in neuronal synapses (for explanation see text). ATP, adenosine triphosphate; ADP, adenosine diphosphate; cAMP, cyclic adenosine monophosphate; MAO, monoamine oxidase; COMT, catecholamine-*O*-methyltransferase; IP_3, inositol-1,4,5-triphosphate; DAG, diacylglycerol.

as adenylyl cyclase (making cyclic adenosine monophosphate [camp], an allosteric regulator of protein kinases and other proteins), or phospholipase C (splitting phosphatidylinositol-4,5-diphosphate [PIP2] into inositol-1,4,5-triphosphate [IP_3]) (which activates Ca^{2+} release channels in the endoplasmic reticulum (ER) setting free the second messenger Ca^{2+}) and diacylglycerol (DAG), which activates proteinkinase C (PKC). Whereas the hydrolysis of GTP (bound to the α-subunit) switches the G protein back to the inactive ('off') state, the second messenger can then regulate various ion channels, protein kinases and other proteins (Fig. 2.16). The second messengers, cAMP and cyclic guanosine monophosphate (cGMP), are inactivated by specific phosphodiesterases (PDE), IP_3 through hydrolysis to the inactive inositol 1,4-biphosphate or in some cells via phosphorylation and hydrolysis to the inactive inositol 1,3,4-triphosphate.

Voltage-gated ion channels are also integral parts of the signal pathway. When an action potential approaches the axon terminal, voltage-gated Ca^{2+}-channels (N-type) open and Ca^{2+} enters the presynapse. Ca^{2+} ions bind to proteins that connect the synaptic vesicle with the plasma membrane (acronym SNAP25 or t-SNARE), inducing membrane fusion and, consequently, exocytosis of the neurotransmitter, ACh, into the synaptic cleft. ACh activates the nAChR, resulting in a rapid influx of Na^+ in the postsynapse, which open voltage-gated Na^+-channels. This event generates an action potential, which spreads along the membrane via voltage-gated Na^+-channels (Friedrich *et al.*, 2007).

At the neuromuscular junction, the change in membrane potential induces a conformational change in the dihydropyrine-coupled ryanodine receptor, so that Ca^{2+} ions are released from the sarcoplasmic reticulum (SR) into the cytosol. The released Ca^{2+} ions activate the actin–myosin system of the muscle cell, leading to muscle contraction. The ER membranes in cardiac muscle cells and neurons also contain ryanodine-sensitive Ca^{2+} release channels that do not associate directly with a receptor in the cell membrane. In these cells, depolarization of the cell membrane leads to a small influx of Ca^{2+} through voltage-gated Ca^{2+}-channels (L-type). Binding of these Ca^{2+} ions to ryanodine sensitive Ca^{2+}-channels induces a rapid release of Ca^{2+} from the ER or other intracellular Ca^{2+} stores. Another Ca^{2+} release channel in the ER/SR is regulated by IP_3.

Specific Ca^{2+}-ATPases in the plasma membrane and in the SR (only in muscle cells) or ER pump Ca^{2+} into the SR/ER or into the extracellular space. The Na^+ and K^+ gradients are restored by Na^+-, K^+-ATPase.

Whereas ACh is degraded by a membrane-anchored ACE in the synaptic cleft (choline is taken up presynaptically), the biogenic amines, adrenaline, NA, serotonin and dopamine, are taken up by the presynaptic membrane via transporters. These transporters have similar structure and contain 12 transmembrane regions. Once in the presynapse, the neurotransmitters are either degraded by MAO or catecholamine *O*-methyltransferase (COMT) or taken up by synaptic vesicles. This may occur via diffusion of the free bases

into the vesicles, where they become protonated and concentrated by an 'ion trap' mechanism, or the amines are taken up and concentrated via specific proton-coupled antiporters.

2.2.3.1 Interactions of allelochemicals with individual steps of neurosignalling

In Fig. 2.16, the various steps in neuronal signalling and signal transduction, which were discussed above, have been schematically summarized. The following targets are affected by several allelochemicals, some of which are listed in Tables 2.7–2.17:

- The neuroreceptor itself (Tables 2.7–2.15); agonists mimic the function of a signal compound by binding to its receptor and causing the normal response, whereas antagonists also bind to the receptor but do not activate the transmitter-induced effects. Thus, an antagonist acts as an inhibitor of the natural ligand by competing for binding sites on the receptor and, thereby, blocking the physiological response (antagonists are, therefore, often called 'blockers').
- Voltage-gated Na^+-, K^+- and Ca^{2+}-channels (Table 2.16).
- The enzymes which deactivate neurotransmitters after they have bound to a receptor (Table 2.17), such as ACE, MAO and COMT.
- Transport processes (Table 2.17), which are important for the uptake and release of the neurotransmitters in the presynapse or synaptic vesicles. Na^+-, K^+- and Ca^{2+}-ATPases, which restore the ion gradients, must also be considered in this category.
- Modulation of key enzymes of signal pathways (Table 2.17):
  1. adenylyl cyclase (producing cAMP);
  2. phosphodiesterase (inactivating cAMP);
  3. phospholipase C (PLC) (releasing inositol phosphates, such as IP_3 and DAG); all phospholipases have been considered (not only PLC, which produces IP_3 and DAG);
  4. several protein kinases, such as protein kinase C or tyrosine kinase (activating other regulatory proteins or ion channels).

2.2.3.2 Cholinergic receptors

Agonists at the nAChR (Table 2.7) include tetramethylammonium hydroxide which is of simple structure, and small alkaloids, such as anabasine, nicotine, cotinine, coniine, pseudopelletierine and epibatidine. These alkaloids carry a secondary or tertiary nitrogen atom in a pyrrolidine or piperidine ring, which becomes protonated under physiological hydrogen ion concentrations in animals (Fig. 2.15).

Often the N is methylated. If a second ring structure is present, it is not coupled via an ester or ether bond and does not carry bulky oxygen substituents,

Table 2.7 Examples of alkaloids which bind to nicotinic acetylcholine receptors (nAChRs) (natural ligand: acetylcholine)

Alkaloid	Type	Occurrence	Activity	Reference
Acetylcholine	Amine	Endogenous neurotransmitter, several plants and in venom of Hymenoptera	nAChR agonist	Teuscher and Lindequist (1994), Buckingham (1996)
Ammodendrine	Piperidine	*Lupinus*, *Cytisus*, *Genista* and other Fabaceae	nAChR binding	Schmeller *et al.* (1994), Wink *et al.* (1998b)
Anabasine	Pyridine	*Anabasis aphylla* (Chenopodiaceae), *Nicotiana* spp., *Duboisia* spp. (Solanaceae)	nAChR agonist	Buckingham (1996), Wink *et al.* (1998b)
Arborine	Quinazolinone	*Glycosmis arborea*, *Ruta graveolens*, *Zanthoxylum budrunga* (Rutaceae)	ACh blocker	Buckingham (1996)
Arecoline	Piperidine	*Areca catechu* (Palmae)	Binding to nAChR	Wink *et al.* (1998b)
Berberine	Protoberberine	*Berberis* spp., *Mahonia* spp. (Berberidaceae) and other families	Binding to nAChR	Schmeller *et al.* (1997b)
Boldine	Aporphine	*Peumus boldo*	nAChR antagonist	Hue *et al.* (1994) and Wink *et al.* (1998b)
Brucine	Indole	*Strychnos nux-vomica* and many other *Strychnos* spp. (Loganiaceae)	Binding to nAChR	Wink *et al.* (1998b)
C-Toxiferine I, II (calebassine, curarine)	Bis-Indole	*Strychnos divaricans* and several other *Strychnos* spp. (Loganiaceae)	nAChR antagonist (muscle, neuronal)	Buckingham (1996)
Codeine	Morphinane	*Papaver somniferum* (Papaveraceae)	Non-competitive nACh agonist	Maelicke *et al.* (1995), Storch *et al.* (1995)
Coniine	Piperidine	*Conium maculatum* (Apiaceae)	nAChR agonist	Forsyth *et al.* (1996) and Wink *et al.* (1998b)
Cytisine and related alpha-pyridone alkaloids	Quinolizidine	*Cytisus*, *Genista*, *Laburnum*, *Baptisia*, *Thermopsis* and several other Fabaceae	Neuronal nAChR agonist (muscle, neuronal)	Papke and Heinemann (1994), Schmeller *et al.* (1994)
Dihydro-β-erythroidine	Erythrina	*Erythrina* spp. (Fabaceae)	nAChR antagonist (muscle, neuronal)	Williams and Robinson (1984)

Epibatidine	Pyridine	*Epipedobates tricolour* (Dendrobatidae)	Potent agonist at neuronal nAChR	Daly *et al.* (1993), Badio and Daly (1994), Elguero *et al.* (1996)
Erysodine	Erythrina	*Erythrina* spp. (Fabaceae)	Very potent neuronal nAChR antagonist	Decker *et al.* (1995)
Galanthamine	Lycorine	*Galanthus* spp. and many other Amaryllidaceae	Non-competitive nACh agonist	Maelicke *et al.* (1995), Storch *et al.* (1995)
Gramine	Indole	Poaceae, Fabaceae, Aceraccae	Binding to nAChR	Wink *et al.* (1998b)
Hirsuteine and related alkaloids	Indole	*Mitragyna* spp., *Uncaria* spp.	Antagonist of nicotine-evoked dopamine release	Watano *et al.* (1993)
Histrionicotoxin	Piperidine	Skin of *Dendrobates* spp. (Dendrobatidae) and other frogs	Non-competitive nAChR blocker	Daly *et al.* (1993)
Lobeline	Piperidine	*Lobelia* spp., *Campanula medium* (Campanulaceae)	nAChR binding	Buckingham (1996)
Lupanine and other quinolizidines	Quinolizidine	*Lupinus*, *Cytisus*, *Genista* and other Fabaceae	nAChR binding	Schmeller *et al.* (1994), Wink *et al.* (1998b)
Methyllycaconitine	Norditerpenoid	*Delphinium* spp., *Consolida* spp. (Ranunculaceae)	Potent antagonist at neuronal, α-bungarotoxinsensitive nAChR	Coates *et al.* (1995), Hardick *et al.* (1995)
Nicotine and other *Nicotiana* alkaloids	Pyridine	*Nicotiana* spp. (Solanaceae), *Asclepias syriaca* (Asclepiadaceae) and many other families	Potent nAChR agonist (muscle, neuronal)	Buckingham (1996)

(*Continued*)

Table 2.7 (*Continued*)

Alkaloid	Type	Occurrence	Activity	Reference
Physostigmine	Indole	*Physostigma venenosum* (Fabaceae)	Non-competitive nACh agonist	Schrattenholz *et al.* (1993), Maelicke *et al.* (1995), Storch *et al.* (1995)
Pseudopelletierine	Piperidine	*Punica granatum* (Punicaceae)	Strong binding to nAChR	Wink *et al.* (1998b)
Pumiliotoxin-C	Quinoline	*Dendrobates* spp., *Epipedobates* spp., *Phyllobates* spp., *Melanophryniscus* spp.	Non-competive nAChR blocker	Daly *et al.* (1993)
Sanguinarine	Benzophenanthridine	Several Papaveraceae, Fumariaceae	Binding to nAChR	Schmeller *et al.* (1997b), Wink *et al.* (1998b)
Strychnine	Indole	*Strychnos* spp. (Apocynaceae)	Binding to nAChR	Wink *et al.* (1998b)
D-Tubocurarine	Bis-isoquinoline	*Chondodendron tomentosum* (Menispermaceae)	nAChR antagonist (muscle, neuronal)	Buckingham (1996)

which could mimic an ester group as in ACh. It is obviously not pure chance that an additional pyridine ring is often found in nAChR agonists, since a pyridine tetramer, nemertilline, exhibits agonistic properties alone. Several QAs, such as cytisine or *N*-methylcytisine, are strong nAChR agonists; although these molecules are much bigger, they still share the important characteristics described for the simpler nAChR agonists, i.e. a protonable quaternary N and an α-pyridone ring (which resembles a pyridine ring) at the correct distance.

Antagonist of nAChR also carries a tertiary nitrogen, which becomes protonated under physiological conditions. In a few powerful antagonists, the N is permanently quaternary, as in toxiferine or tubocurarine, which thus closely mimic the terminal quaternary N in ACh. Because the dimeric alkaloids have two ligand sites, they can cross-link both alpha subunits of the nAChR. A much greater structural diversity is apparent in nAChR antagonists than in agonists. In addition to a tertiary or quaternary nitrogen in piperidine- or indolizidine-type rings, bulky ring structures, such as indoles or benzyls, are found in the vicinity (e.g. in boldine, toxiferine, tubocurarine, dihydro-β-erythroidine, erysodine, hirsutine or methyllycaconitine). Smaller molecules are esters with long alkyl substituents, as in pahutoxin, or smaller N- and O-containing rings, as in murexine. A few bulky peptides from animal venoms, such as α-bungarotoxin or α-conotoxin, also block the ACh binding site. These compounds appear to interact with other functional groups of the receptor, so that a conformational change (to open the cation channel) is no longer possible.

At mAChR (Table 2.8), a similar situation is generally found. Agonists are usually small alkaloids with piperidine or imidazol rings (as in arecaidine, arecoline or pilocarpine), or they can be regarded as structural analogues of ACh (such as tetramethylammonium hydroxide or muscarine). The agonists carry a quaternary N under physiological conditions but also an oxygen substituent at a distance similar to the ester oxygen in ACh (Fig. 2.17).

Muscarinic AChR antagonists are much bigger alkaloids with a tertiary nitrogen, being present as *N*-methylpiperidine, *N*-methylpyrrolidine, indolizidine or quinolizidine skeletons as in all tropane alkaloids (anisodine, hyoscyamine, littorine, scopolamine or 3-tigloyltropine), in cryptolepine, gephyrotoxin, gindarine, imperialine or usambarensine (Fig. 2.17). Again, this nitrogen is quaternary under physiological conditions, like that of ACh. In addition, these alkaloids have either an ester group, as in tropane alkaloids, or hexane, indole, benzyl or other bulky rings or alkane chains in the vicinity, as in cyclostellettamines, gephyrotoxins, gindarine, himandravine, himbacine or usambarensine. These compounds appear to bind to the ACh binding site but interact with other functional groups of the receptor, so that a conformational change (to activate the adjacent G protein) is no longer possible.

In conclusion, ACh agonists are rather small alkaloids, which competitively bind to the AChR and induce conformational changes, whereas antagonist share the quaternary N for binding but inhibit the necessary conformational

Table 2.8 Examples of alkaloids which bind to muscarinic acetylcholine receptors (mAChR) (natural ligand: acetylcholine)

Alkaloid	Type	Occurrence	Activity	Reference
Acetylcholine	Amine	Endogenous neurotransmitter, several plants and in venom of Hymenoptera	mAChR agonist	Teuscher and Lindequist (1994), Buckingham (1996)
Acetylheliosupine and related alkaloids	Pyrrolizidine	Boraginaceae	Binding to mAChR	Schmeller *et al.* (1997a), Wink *et al.* (1998b)
Aconitine	Diterpene	*Aconitum* spp. (Ranunculaceae)	Binding to mAChR	Wink *et al.* (1998b)
Angustifoline and related alkaloids	Quinolizidine	*Lupinus* spp. (Fabaceae)	Binding to mAChR	Schmeller *et al.* (1994), Wink *et al.* (1998b)
Arecoline/arecaidine	Piperidine	*Areca catechu* (Palmae)	mAChR agonist	Buckingham (1996), Wink *et al.* (1998b)
Berbamine	Bis-isoquinoline	Berberidaceae, Menispermaceae, Ranunculaceae	Binding to mAChR	Hou and Liu (1988)
Berberine, palmatine and related alkaloids	Protoberberine	*Jateorhiza* spp. (Menispermaceae), *Berberis* spp., *Mahonia* spp. (Berberidaceae)	Binding to mAChR	Schmeller *et al.* (1997b)
Boldine	Aporphine	*Peumus boldo*	Binding to mAChR	Wink *et al.* (1998b)
Brucine/strychnine	Indole	*Strychnos nux-vomica* and many other *Strychnos* spp. (Loganiaceae)	Binding to mAChR	Wink *et al.* (1998b)
Cocaine	Tropane	*Erythroxylum* spp. (Erythroxylaceae)	Binding to mAChR	Schmeller *et al.* (1995), Wink *et al.* (1998b)
Cryptolepine	Indole	*Cryptolepis sanguinolenta* (Periplocaceae)	M1, M2, M3 antagonist	Rauwald *et al.* (1992)
Dicentrine	Aporphine	Menispermaceae, Lauraceae, Fumariaceae, Papaveraceae	Binding to mAChR	Liu *et al.* (1989b)
Ebeinone	Homosteroidal	*Fritillaria imperialis* (Liliaceae)	M2 receptor antagonist	Gilani *et al.* (1997)

Echihumiline *N*-oxide	Pyrrolizidine	Boraginaceae	Binding to mAChR	Schmeller *et al.* (1997a), Wink *et al.* (1998b)
Emetine	Isoquinoline	*Alangium* spp., *Psychotria* (*Cephaelis*) spp. (Alangiaceae, Rubiaceae)	Binding to mAChR	Wink *et al.* (1998b)
Harmaline and related alkaloids	β-Carboline	*Peganum harmala* (Zygophyllaceae), *Banisteriopsis* spp. (Malpighiaceae)	Binding to mAChR	Wink *et al.* (1998b)
Himandravine	Piperine	*Himantandra* spp. (Himantandraceae)	mAChR antagonist	Darroch *et al.* (1990)
Himbacine	Piperidine	*Himantandra* spp. (Himantandraceae)	mAChR antagonist	Kozikowski *et al.* (1992), Wess *et al.* (1992)
13-Hydroxylupanine and related alkaloids	Quinolizidine	*Lupinus*, *Cytisus*, *Genista* and other Fabaceae	Weak binding to mAChR	Schmeller *et al.* (1994), Wink *et al.* (1998b)
Ibogaine	Indole	*Tabernanthe iboga*, *Voacanga thouarsii*, *Tabernaemontana* spp. (Apocynaceae)	Binding to alpha 1 and 2 receptors	Sweetnam *et al.* (1995)
Imperialine	Homosteroidal	*Fritillaria* spp., *Petilium* spp., *Rhinopetalum* spp. (Liliaceae)	(Cardioselective) M2 antagonist	Eglen *et al.* (1992)
Laudanosine	Isoquinoline	*Papaver somniferum*, *Argemone grandiflora* (Papaveraceae)	Binding to mAChR	Wink *et al.* (1998b)
Lupinine	Quinolizidine	*Lupinus* spp. and other Fabaceae	Weak binding to mAChR	Schmeller *et al.* (1994), Wink *et al.* (1998b)
Martinelline	Pyrroloquinoline	*Martinella iquitosensis* (Bignoniaceae)	Affinity for mAChR	Witherup *et al.* (1995)
Multiflorine and related alkaloids	Quinolizidine	*Lupinus* spp. and other Fabaceae	Binding to mAChR	Schmeller *et al.* (1994), Wink *et al.* (1998b)
Muscarine	Furan	*Amanita muscaria*, *Inocybe* spp., *Clitocybe* spp. and other fungi	mAChR agonist (M1-M5)	Buckingham (1996)

(*Continued*)

Table 2.8 (*Continued*)

Alkaloid	Type	Occurrence	Activity	Reference
Palmatine	Protoberberine	*Jateorhiza palmata* (Menispermaceae), *Berberis* spp., *Mahonia* spp. (Berberidaceae), but also many families	Binding to mAChR	Wink *et al.* (1998b)
Physostigmine	Indole	*Physostigma venenosum* (Fabaceae)	Binding to mAChR	Wink *et al.* (1998b)
Pilocarpine	Imidazole	*Pilocarpus* spp. (Rutaccae)	mAChR agonist (M1-M5)	Wink *et al.* (1998b)
Quinine and related alkaloids	Quinoline	*Cinchona* spp. (Rubiaceae)	Binding to mAChR	Wink *et al.* (1998b)
Sanguinarine	Benzophenanthridine	Several Papaveraceae, Fumariaceae	Binding to mAChR	Schmeller *et al.* (1997b), Wink *et al.* (1998b)
Scopolamine, hyoscyamine and other tropane alkaloids	Tropane	*Atropa*, *Hyoscyamus*, *Datura*, *Mandragora*, *Scopolia*, *Duboisia*, *Brugmansia* (Solanaceae)	mAChR antagonist (M1-M5)	Kebabian and Neumeyer (1994), Schmeller *et al.* (1995)
Seneciphylline/ senecionine	Pyrrolizidine	*Senecio* spp. and other Asteraceae, *Crotalaria* spp. (Fabaceae)	Binding to mAChR	Schmeller *et al.* (1997a), Wink *et al.* (1998b)
Sparteine and other tetracyclic alkaloids	Quinolizidine	*Lupinus*, *Cytisus*, *Genista* and other Fabaceae	Binding to mAChR	Schmeller *et al.* (1994), Wink *et al.* (1998b)
Tetrandrine	Bis-isoquinoline	*Cocculus*, *Cyclea*, *Stephania* and other Menispermaceae	Binding to mACHR	Hou and Liu (1988)

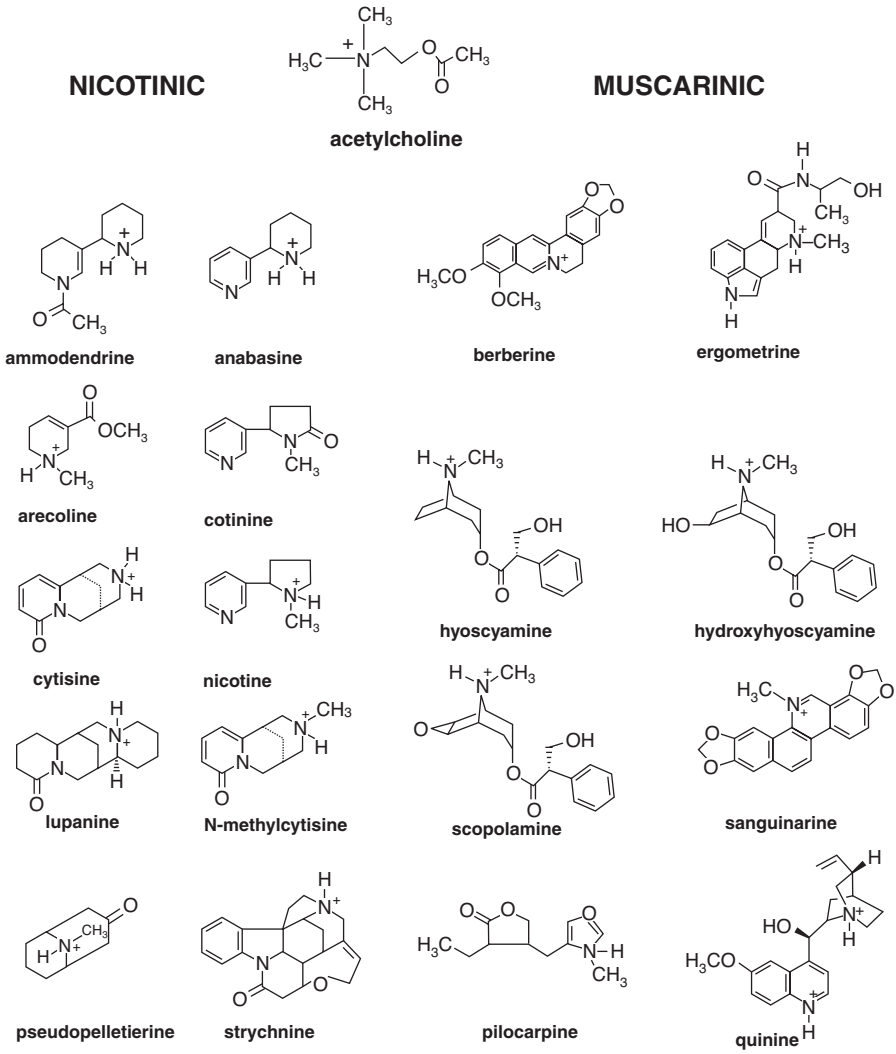

Figure 2.17 Structural similarities between acetylcholine and alkaloids that bind to nicotinic or muscarinic acetylcholine receptors. Under physiological conditions, alkaloids are protonated.

changes usually elicited by ACh. Whether a molecule binds to nicotinic or muscarinic receptors, which exhibit a number of subtypes, depends on the fine structure of the respective bindings sites and their potential interactions with the alkaloid molecules carrying various substituents.

2.2.3.3 Adrenergic receptors

Agonists at α- and β-receptors (Table 2.9) clearly represent analogues of the natural ligands, adrenaline and NA. Ephedrine and its derivative,

Table 2.9 Examples of alkaloids which bind to adrenergic receptors (natural ligand: noradrenaline, adrenaline)

Alkaloid	Type	Occurrence	Activity	Reference
Acetylheliosupine	Pyrrolizidine	Boraginaceae	Binding α_{1+2} receptors	Schmeller *et al.* (1997a), Wink *et al.* (1998b)
Adrenaline	Phenylalkyl amine	Banana (*Musa* spp.) and several other plants, venom of Hymenoptera	α_2, β-agonist	Teuscher and Lindequist (1994), Buckingham (1996)
Ajmalicine	Indole	*Rauwolfia* spp., *Catharanthus roseus* and other Apocynaceae	α blocker, vasodilator	Nagase and Hagihara (1986), Buckingham (1996)
Ammodendrine	Piperidine	*Lupinus*, *Cytisus*, *Genista* and other Fabaceae	α_2 receptor binding	Schmeller *et al.* (1994), Wink *et al.* (1998b)
Anabasine	Pyridine	*Anabasis aphylla* (Chenopodiaceae), *Nicotiana* spp., *Duboisia myoporoides* (Solancaceae)	Binding to α_2 receptor	Buckingham (1996), Wink *et al.* (1998b)
Arecoline	Piperidine	*Areca catechu* (Palmae)	Binding to α_2 receptor	Wink *et al.* (1998b)
Berbamine	Bis-isoquinoline	*Berberis* spp. and other Berberidaceae, Menispermaceae, Ranunculaceae	α_2 blocker	Han and Liu (1988)
Berberine and related alkaloids	Protoberberine	*Berberis* spp., *Mahonia* spp., *Jateorhiza* (Berberidaceae) and other families	α_{1+2} blocker	Liu *et al.* (1989a), Liu *et al.* (1989b), Dong *et al.* (1997), Schmeller *et al.* (1997b)
Boldine	Aporphine	*Peumus boldo* (Monimiaceae) and several Annonaceae and Lauraceae	α_{1A} antagonist; strong binding to α_{1+2} receptors	Ivorra *et al.* (1993a), Madrero *et al.* (1996), Wink *et al.* (1998b)
Brucine/strychnine	Indole	*Strychnos nux-vomica* and many other *Strychnos* spp. (Loganiaceae)	Binding to α_1 receptor	Wink *et al.* (1998b)

Cinchonidine and related alkaloids	Quinoline	*Cinchona* spp. (Rubiaceae)	Strong binding to α_{1+2} receptors	Wink *et al.* (1998b)
Cocaine	Tropane	*Erythroxylum* spp. (Erythroxylaceae)	Adrenergic antagonist	Buckingham (1996)
Colchicine	Tropolone	*Colchicum autumnale* and many other *Colchicum* spp., several *Merendera* spp., *Gloriosa superba* and other Liliaceae	Binding to α_2 receptor	Wink *et al.* (1998b)
Corynanthine	Indole	*Corynanthe*, *Rauwolfia*, *Pausinystalia*, (Rubiaceae, Apocynaceae)	α_1 blocker	Kebabian and Neumeyer (1994)
Crebanine	Aporphine	*Stephania* spp. (Menispermaceae)	α_1 blocker	Han and Liu (1988), Liu *et al.* (1989b)
Daurisoline	Bis-isoquinoline	*Menispermum dauricum* (Menispermaceae)	Binding to α_{1+2} receptors	Waldmeier *et al.* (1995)
Dehydroevodiamine	Isaquinazolinocarboline	*Evodia rutaecarpa* (Rutaceae)	Endothelial α_1 blockade	Chiou *et al.* (1994)
Dispegatrine	Indole	*Rauwolfa verticillata* (Apocynaceae)	α blocker	Buckingham (1996)
Dopamine	Phenylalkylamine	*Musa sapientium* (Musaceae), *Cytisus* spp., (Fabaceae) and other families	Adrenergic agonist	Buckingham (1996)
Emetine	Isoquinoline	*Alangium*, *Psychotria* (*Cephaelis*) sp. (Alangiaceae, Rubiaceae)	Binding to α_1, and strongly to α_2 receptor	Wink *et al.* (1998b)

(*Continued*)

Table 2.9 *(Continued)*

Alkaloid	Type	Occurrence	Activity	Reference
Ephedrine, cathinone and related alkaloids	Phenylalkylamine	*Ephedra* spp. (Ephedraceae) *Aconitum napellus* (Ranunculaceae), *Catha edulis* (Celastraceae), *Taxus baccata* (Taxaceae), *Sida cordifolia* (Malvaceae), *Roemeria refracta* (Papaveraceae)	α, β agonist; binding to α_1 receptor	Buckingham (1996), Wink *et al.* (1998b)
Epiallocorynantheine	Indole	*Uncaria rhynchophylla*	Binding to β receptors	Zhu *et al.* (1997)
Ergometrine (ergonovine)	Ergot	*Claviceps* and several Convolvulaceae (*Argyreia*, *Stictocardia*, *Rivea*, *Ipomoea*)	Binding to α_2, strongly to α_1 receptor	Wink *et al.* (1998b)
Ergosine and related alkaloids	Ergot	*Claviceps purpurea* and several Convolvulaceae	α_1 antagonist	Kazic *et al.* (1989)
Ergotamine	Ergot	*Claviceps purpurea*	Peripheral α_1 antagonist, vasoconstrictor; partial α_2 agonist	Roquebert and Grenie (1986), Buckingham (1996)
Glaucine	Aporphine	Many Monimiaceae, Berberidaceae, Annonaceae, Lauraceae, Ranunculaccae, Papaveraceae	$\alpha_{1A,2}$ antagonist	Orallo *et al.* (1993, 1995), Madrero *et al.* (1996)
Gramine	Indole	Poaceae, Fabaceae, Aceraceae	Binding to α_{1and2} receptors	Wink *et al.* (1998b)
Harmaline and related alkaloids	β-Carboline	*Peganum harmala* (Zygophyllaceae), *Banisteriopsis* spp. (Malpighiaceae)	Binding to α_{1+2} receptors	Wink *et al.* (1998b)

Heliosupine	Pyrrolizidine	*Heliotropium* spp., *Cynoglossum* spp. (Boraginaceae)	Binding to α_1 (weak) and α_2 receptors	Schmeller *et al.* (1997a), Wink *et al.* (1998b)
Higenamine	Isoquinoline	*Aconitum japonicum* (Ranunculaceae), *Gnetum parviorum* (Gnetaceae)	β-receptor agonist	Yun-Choi and Kim (1994)
Hirsutine	Indole	*Uncaria rhynchophylla*	Binding to α_2, β receptor	Zhu *et al.* (1997)
Hyoscyamine (atropine)	Tropane	*Atropa* spp., *Hyoscyamus* spp., *Datura* ssp., *Mandragora* spp., *Scopolia* spp., *Duboisia* spp. (Solanaceae)	Binding to α_{1+2} receptors	Schmeller *et al.* (1995), Wink *et al.* (1998b)
Ibogaine	Indole	*Tabernanthe iboga*, *Voacanga thouarsii*, *Tabernaemontana* spp. (Apocynaceae)	Binding to norepinephrine uptake sites	Sweetnam *et al.* (1995)
Laudanosine	Isoquinoline	*Papaver somniferum*, *Argemone grandiflora* (Papaveraceae)	Cardiovascular α_1 blocker; binding to α_{1and2} receptors	Chulia *et al.* (1994), Wink *et al.* (1998b)
Lysergamid	Ergot	*Claviceps*, *Acremonium* and other fungi, various Convolvulaceae	Vasoconstrictor, adrenergic	Oliver *et al.* (1993)
Martinelline	Pyrroloquinoline	*Martinella iquitosensis* (Bignoniaceae)	Affinity for α_1	Witherup *et al.* (1995)
N-methyldopamine (epinine)	Phenylalkylamine	*Cytisus scoparius*, *Vicia faba* (Fabaceae), *Lophophora williamsii* (Cactaceae)	Adrenergic agonist	Buckingham (1996)

(*Continued*)

Table 2.9 (*Continued*)

Alkaloid	Type	Occurrence	Activity	Reference
Noradrenaline	Phenylalkylamine	Many plants, venom of many Hymenoptera	α agonist	Buckingham (1996)
Octopamine	Phenylalkylamine	*Capsisum frutescens* (Solanaceae), *Citrus* spp. (Rutaceae), *Cyperus* spp. (Cyperaceae), venom of Octobrachia and spiders	α agonist	Kebabian and Neumeyer (1994), Teuscher and Lindequist (1994), Buckingham (1996)
Predicentrine	Aporphine	Many Lauraceae, Magnoliaceae, Fumariaceae, Papaveraceae	α_{1A} antagonist	Madrero *et al.* (1996)
Reserpine	Indole	*Rauwolfia* spp. *Vinca minor*, *Alstonia constrictor*, *Tenduzia longifolia*, *Vallesia dichotoma*, *Excavatia coccinea* and other genera (Apocynaceae)	Adrenergic antagonist	Buckingham (1996)
Salsoline	Isoquinoline	*Salsola* spp. and many other Chenopodiaceae, Fabaceae, Cactaceae, Alangiaceae	Binding to α_2 receptor	Wink *et al.* (1998b)
Sanguinarine	Benzophenanthridine	Several Papaveraceae, Fumariaceae	Binding to α_{1+2} receptors	Schmeller *et al.* (1997b), Wink *et al.* (1998b)
Stephanine and related alkaloids	Aporphine	*Stephania* spp. (Menispermaceae)	Modulation of central α_1 receptor	Han and Liu (1988)
Topsentin B2	bis-Indole	Marine sponges (*Topsentia* spp., *Spongosorites* spp., *Hexadella* spp.)	Binding to α_{1A} and $_{1B}$ receptors	Buckingham (1996)
Xylopine	Aporphine	*Xylopia* spp. (Annonaceae)	α_1 antagonist	Han and Liu (1988)
Yohimbine	Indole	Several Apocynaceae and Rubiaceae	strong α_2 antagonist	Renouard *et al.* (1994), Buckingham (1996)

norephedrine, have an *R*-configurated hydroxyl function at position 1 of the side chain (as do the endogenous ligands; only cathinone has a keto-function instead) and either a free amino group, as in NA, or a methylated amino group, as in adrenaline (Fig. 2.18), both of which are protonated under physiological conditions. If the hydroxyl group at C1 is *S*-configurated, the activity is reduced. The phenolic hydroxy groups at position 3 and 4 of the aromatic ring are apparently not essential for agonistic properties, although ephedrine

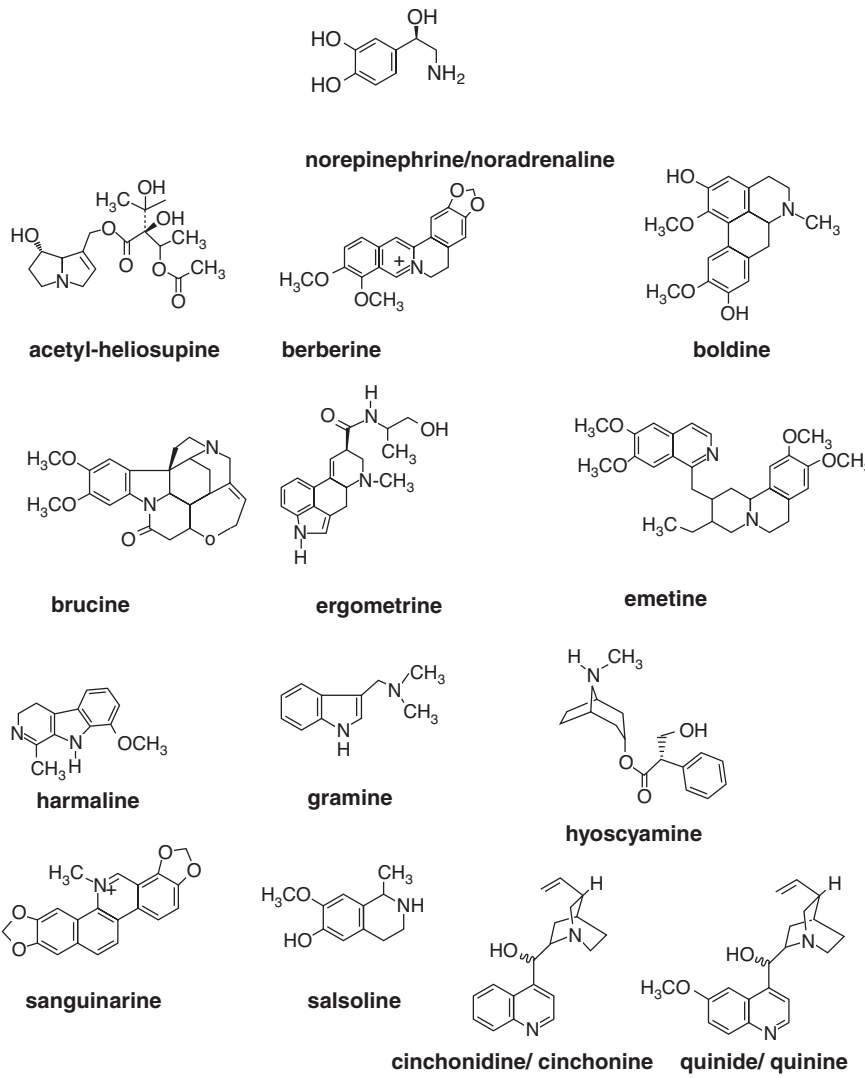

Figure 2.18 Structural similarities between noradrenaline and alkaloids which bind to adrenergic neuroreceptors.

is a weaker agonist than adrenaline. If the hydroxy groups are not present, this provides the advantage, firstly, that the molecules cannot be transformed by COMTs and, secondly, that they can cross biomembranes by simple diffusion. One or two phenolic hydroxyl groups are present in octopamine, dopamine and *N*-methyldopamine; the two latter compounds have no hydroxyl group at C1 of the side chain and are weaker agonists than the endogenous ligands.

Many of the alkaloids shown in Fig. 2.18 are adrenergic antagonists. In alkaloids with an isoquinoline ring, such as aporphines (e.g. boldine, crebanine, glaucine or xylopine), the protoberberines (e.g. berberine, palmatine, govedine, gindarine or stylopine) or the simple or dimeric tetrahydroisoquinolines (e.g. higenamine, laudanosine, berbamine or oxyacanthine), the structure of the biogenic precursor dopamine can easily be detected (Fig. 2.18): The aromatic ring usually contains either two free hydroxyl groups or corresponding methoxy groups, or even a methylenedioxy bridge, as in berberine. The secondary or tertiary amino group is still protonable under physiological conditions. Indole alkaloids, such as ajmalicine, corynanthine, reserpine, rauwolfscine, yohimbine and most ergot alkaloids are adrenergic antagonists. These alkaloids are usually of complex structure (see Fig. 2.18) and carry part of the adrenaline backbone: an aromatic ring (usually unsubstituted as in ephedrine) but a protonable tertiary N that is two carbon atoms adjacent to the aromatic ring. Whereas the protonable N and the aromatic ring A contribute to the binding of these alkaloids at adrenergic receptors, the other functional groups appear to interact with other amino acid residues in the receptor and inhibit a consequent conformational change.

2.2.3.4 Dopaminergic receptors

Table 2.10 lists several D_1 and D_2 agonists. *N*-Methyldopamine and salsolinol can be regarded as sharing most functional groups with dopamine, such as a catechol moiety and a protonable nitrogen. Tyramine, with a single hydroxyl group, is also accepted as an agonist. Surprisingly, the rather large ergot alkaloids, such as agroclavine, ergocornine and ergovaline, also function as dopamine agonists. The structure of dopamine can be superimposed on ergot alkaloids. The aporphine alkaloid, bulbocapnine, is a potent dopamine receptor antagonist. Two molecules of dopamine can be superimposed on this molecule.

2.2.3.5 Serotonergic receptors

Table 2.11 lists several serotonin receptor agonists and antagonists. Agonists include simple structural analogues, such as bufotenine, psilocine, *N*-methyltryptamine and *N*,*N*-dimethyltryptamine, differing in the presence/absence of a hydroxylfunction at C5 or C6 of ring A or whether the still protonable amino group bears one or two methyl groups (Fig. 2.19). In psilocybine, the hydroxyl group is phosphorylated, which does not interfere

Table 2.10 Examples of alkaloids which bind to dopamine receptors (DRs) (natural ligand: dopamine)

Alkaloid	Type	Occurrence	Activity	Reference
Agroclavine	Ergot	*Argyreia*, *Rivea*, *Cuscuta*, *Ipomoea* (Convolvulaceae) and *Penicillium* spp.	Dopamine receptor agonist	Buckingham (1996)
Anisocycline	Protoberberine	*Anisocycla cymosa* (Menispermaceae)	Binding to D_1, D_2	Markstein *et al.* (1992)
Bulbocapnine	Aporphine	*Corydalis cava* (Fumariaceae)	Dopamine antagonist	Kebabian and Neumeyer (1994), Buckingham (1996)
Canadine and related alkaloids	Protoberberine	Several Fumariaceae, Papaveraceae	Binding to D_2 receptor	Chen *et al.* (1987)
3,4-Dihydroxy-phenylalanine	Dopa	Many Fabaceae, Aristolochiaceae	Dopamine precursor	Buckingham (1996)
Dopamine	Phenylalkylamine	Endogenous neurotransmitter, and in several higher plants, Fabaceae, *Musa* spp., in venoms of Hymenoptera	Endogenous ligand	Teuscher and Lindequist (1994), Buckingham (1996)
Epinine	Phenylalkylamine	*Cytisus scoparius*, *Vicia faba* and *Lophophora williamsii* (Fabaceae, Cactaceae), animal skin secretions (Anura)	Dopamine agonist	Kebabian and Neumeyer (1994), Buckingham (1996)
Ergocornine	Ergot	*Claviceps purpurea*	Dopamine receptor agonist	Buckingham (1996)
Ergovaline and related alkaloids	Ergot	*Claviceps purpurea* and other endophytic fungi	D_2 agonist	Strickland *et al.* (1994), Larson *et al.* (1995)
Ioline	Pyrrolizidine	*Lolium* spp., *Festuca* spp. (Poaceae)	D_2 agonist	Strickland *et al.* (1994)

(*Continued*)

Table 2.10 *(Continued)*

Alkaloid	Type	Occurrence	Activity	Reference
Salsolinol	Isoquinoline	*Annona reticulata* (Annonaceae), *Musa* spp. (Musaceae), *Aconitum carmichaeli* (Ranunculaceae)	Dopamine agonist	Nimit *et al.* (1983)
Stephanine	Aporphine	*Stephania* spp. (Menispermaceae)	High affinity for D_2 receptor	Chen *et al.* (1987)
Stylopine	Protoberberine	*Corydalis* spp. (Fumariaceae), *Chelidonium majus* and other Papaveraceae	Binding to D_2 receptor	Chen *et al.* (1987)
Tetrahydropalmatine	Protoberberine	*Corydalis* spp. (Fumariaceae), *Stephania glabra* (Menispermaceae), *Berberis tinctoria* (Berberidaceae) and *Coptis tecta* (Ranunculaceae)	Binding to D_1, D_2 receptor	Chen *et al.* (1987), Chen *et al.* (1992), Markstein *et al.* (1992)
Tyramine	Biogenic phenylalkyl amine	In several plant species, Magnoliaceae, Fabaceae, Poaceae, Cactaceae	Dopamine agonist	Kebabian and Neumeyer (1994)

DOPA, 3,4-dihydroxy-phenylalanine.

Table 2.11 Examples of alkaloids which bind to serotonin receptors (5-HTR) (natural ligand: serotonin)

Alkaloid	Type	Occurrence	Activity	Reference
Akuammine	Indole	*Picralima* spp., *Cabucala* spp. and *Vinca* spp. (Apocynaceae)	Serotonin antagonist	Lebanidze and Gedevanishvili (1985)
Annonaine	Aporphine	Annonaceae, Nelumbonaceae, also from the Lauraceae, Magnoliaceae, Monimiaceae, Papaveraceae, Rhamnaceae and Menispermaceae.	Binding to 5-HT_{1A}	Hasrat *et al.* (1997)
Asimilobine	Aporphine	Several Annonaceae, Nelumbonaceae, Magnoliaceae	Serotonergic antagonist	Shoji *et al.* (1987), Hasrat *et al.* (1997)
Berberine and related alkaloids	Protoberberine	*Berberis* spp., *Mahonia* spp. (Berberidaceae) and other families	Strong binding to 5-HT_2 receptor	Schmeller *et al.* (1997b)
Boldine	Aporphine	*Peumus boldo* (Monimiaceae) and several Annonaceae and Lauraceae	Strong binding to 5-HT_2 receptor	Wink *et al.* (1998b)
Bufotenine and related alkaloids	Indolamine	Poisonous secretion of toads (*Bufo* spp.), of *Piptadenia peregrina*, many genera in the Fabaceae and Poaceae, and in some mushrooms (*Amanita* spp.), also in the gorgonian *Paramuricia chamaeleon*	Agonist at 5-HT receptors, hallucinogenic	Kebabian and Neumeyer (1994), Buckingham (1996)
Cinchonidine and related alkaloids	Quinoline	*Cinchona* spp. (Rubiaceae)	Binding to 5-HT_2 receptor	Wink *et al.* (1998b)
Confusameline	Furoquinoline	*Evodia* spp., *Melicope* spp. (Rutaceae)	5-HT2 antagonist	Cheng *et al.* (1994)
Emetine	Isoquinoline	*Alangium*, *Psychotria* (*Cephaelis*) spp. (Alangiaceae, Rubiaceae)	Binding to 5-HT_2 receptor	Wink *et al.* (1998b)

(*Continued*)

Table 2.11 (*Continued*)

Alkaloid	Type	Occurrence	Activity	Reference
Ephedrine	Phenylalkylamine	*Ephedra* spp. (Ephedraceae) *Aconitum napellus* (Ranunculaceae), *Catha edulis* (Celastraceae), *Taxus baccata* (Taxaceae), *Sida cordifolia* (Malvaceae), *Roemeria refracta* (Papaveraceae)	Weak binding to 5-HT_2 receptor	Kebabian and Neumeyer (1994), Buckingham (1996), Wink *et al.* (1998b)
Ergometrine and related alkaloids	Ergot	*Claviceps* and several Convolvulaceae (*Argyreia*, *Stictocardia*, *Rivea*, *Ipomoea*)	5-HT_2 antagonist	Buckingham (1996), Wink *et al.* (1998b)
Ergotamine	Ergot	*Claviceps purpurea*	5-HT_{1C} antagonist	Brown *et al.* (1992)
Gramine	Indole	Poaceae, Fabaceae, Aceraceae	Binding to 5-HT_2 receptor	Wink *et al.* (1998b)
Harmaline and related alkaloids	β-Carboline	*Peganum harmala* (Zygophyllaceae), *Banisteriopsis* spp. (Malpighiaceae)	5-HT_{1A} agonist, binding to 5-HT_2	Abdel-Fattah *et al.* (1995), Wink *et al.* (1998b)
Hirsutine	Indole	*Uncaria rhynchophylla*	Binding to 5-HT_{1A} and 5-HT_2 receptors	Zhu *et al.* (1997)
Hyoscyamine and related alkaloids	Tropane	*Atropa* spp., *Hyoscyamus* spp., *Datura* spp., *Mandragora* spp., *Scopolia* spp., *Duboisia* spp. (Solanaceae)	Binding to 5-HT_2 receptor	Schmeller *et al.* (1995)
Ibogaine	Indole	*Tabernanthe iboga*, *Voacanga thouarsii*, *Tabernaemontana* spp. (Apocynaceae)	Binding to 5-HT_2 and 5-HT_3 receptors, modulation of serotonin-mediated dopamine release	Sweetnam *et al.* (1995), Sershen *et al.* (1996, 1997)
Kokusaginine	Furoquinoline	*Evodia* spp., *Orixa* spp. (Rutaceae)	5-HT_2 antagonist	Cheng *et al.* (1994)

Liridinine	Aporphine	*Liriodendron tulipifera* (Magnoliaceae)	Serotonergic antagonist	Shoji *et al.* (1987)
Mescaline	Phenylalkylamine	*Lophophora williamsii*, *Trichocercus* spp., *Gymnocalycium gibbosum*, *Opuntia cyclindrica* and other species, Cactaceae.	5-HT agonist	Kebabian and Neumeyer (1994)
Mitragynine	Indole	*Mitragyna speciosa*, *Uncaria* spp. (Rubiaceae)	Narcotic, serotonergic	Matsumoto *et al.* (1996)
N-Methyltryptamine and related alkaloids	Indolamine	*Girgensohnia diptera*, *Acacia maidenii*, *Anadenanthera piptadena*, *Desmodium* spp., *Mimosa hostilis*, *Arthrophytum leptocladum*, *Arundo donax*, *Phalaris* spp., *Banisteriopsis argentea*, *Psychotria* spp., *Virola* spp., *Zanthoxylum* spp. (Myristicaceae, Chenopodiaceae, Fabaceae, Poaceae, Rutaceae)	Binding to 5-HT receptors;	McKenna *et al.* (1984), Buckingham (1996)
Nornuciferine	Aporphine	Nelumbonaceae, Rhamnaceae, Annonaceae, Magnoliaceae, Menispermaceae	Binding to 5-HT_{1A} receptor	Hasrat *et al.* (1997)
Psilocybine/Psilocine	Indole	*Psilocybe* spp., *Conocybe* spp., *Stropharia* spp. and other fungi	Hallucinogen, 5-HT affinity	Teuscher and Lindequist (1994), Buckingham (1996)
Serotonin	Indoleamine	Many plants (stinging hairs), also in venoms of Cnidaria, molluscs, and arthropods and skin secretions of amphibia	Endogenous 5-HT ligand	Teuscher and Lindequist (1994), Buckingham (1996)
Stephanine	Aporphine	*Stephania* spp. (Menispermaceae)	High affinity for 5-HT_1, 5-HT_2	Chen *et al.* (1987)

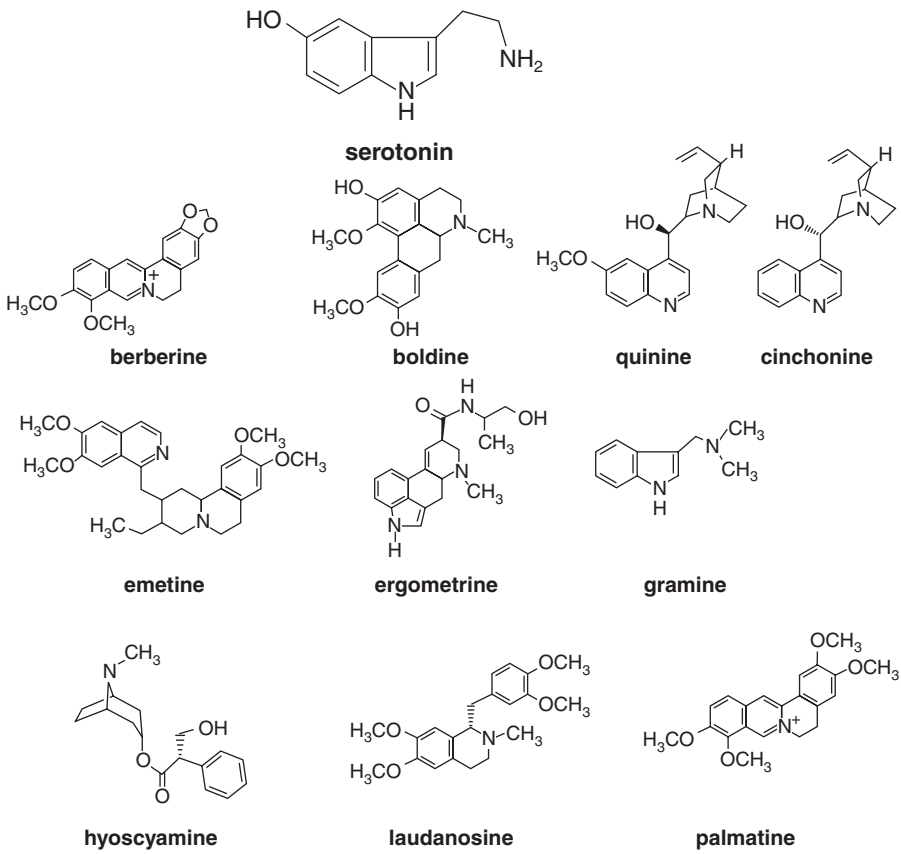

Figure 2.19 Structural similarities between serotonin (5-HT) and alkaloids which bind to 5-HT receptors.

with the agonistic activity. β-Carboline alkaloids can be regarded as compounds that are closely related to serotonin; the structural similarity is close enough for harmaline and harmine to function as agonists. The simple ergot alkaloid lysergamide is also an agonist, in which the serotonin backbone can easily be detected. Mescaline also affects the serotonin receptor, although a structure–function relationship is less visible. Most of these serotonin agonists appear to provoke hallucinations in humans.

A few serotonin receptor antagonists share the serotonin backbone with the endogenous ligand, such as the indole alkaloid, akuammine or the more bulky ergot alkaloids (e.g. ergometrine, ergosine or ergotamine) (Fig. 2.18). Such a structural relationship is not apparent for other antagonists, such as aporphine alkaloids (e.g. asimilobine, liridinine), quinoline alkaloids (e.g. confusameline, kokusaginine) or complex imidazole alkaloids (e.g. hymenine or keramadine).

2.2.3.6 GABAergic receptors

As compared to cholinergic and adrenergic alkaloids, only few GABA receptor agonists or antagonists have been detected; these are listed in Table 2.12. The small fungal metabolite, muscimol, functions as a GABA agonist. Like the endogenous ligand GABA, muscimol contains a protonable amino group. In GABA, there are three methylene groups between the amino group and the bulky carboxyl group. In muscimol, two or four carbon atoms are found between the amino group and the next oxygen function. GABA antagonists include the phthalideisoquinoline alkaloids, bicuculline, corlumine and hydrastine, and the securidan alkaloids, securinine, dihydrosecurinine and virosecurinine. Common to these alkaloids is a tertiary nitrogen, which becomes protonated under physiological conditions, and bulky oxygen substituents, which are three carbon atoms apart, thus mimicking the structure of GABA to some degree. The GABA receptor binding alkaloids with a benzophenanthridine skeleton (e.g. chelerythrine, sanguinarine), the protopine alkaloids (e.g. fagarine I, cryptopine, protopine) and the β-carboline alkaloids also share this structural element. Analogous to the situation at other receptors, the agonist is a small molecule, whereas the antagonist shares a binding core but is usually a larger and bulkier compound that is able to interact with other parts or subunits of the receptor.

2.2.3.7 Glutamate/NMDA receptor

Table 2.13 lists several alkaloids and NPAAs that bind to the glutamate *N*-methyl-D-aspartate (NMDA) receptor. Several NPAAs, such as acromelic acid, domoic acid and kainic acid, are NMDA receptor agonists, which exhibit the following structural similarities with glutamate. Firstly, they contain an alpha nitrogen atom (sometimes in a heterocyclic ring) adjacent to a carboxyl group, common in amino acids. Secondly, they have a second carboxyl group, which is three carbon atoms apart. In the NPAAs, quisqualic acid and willardiine, there are two carbon atoms and one N between the amino group and the next oxygen substituent. In the fungal agonist, ibotenic acid, four carbon atoms are found between the amino group and the next hydroxyl group. It is more difficult to detect a common structural theme in glutamate/NMDA receptor antagonist (e.g. histrionicotoxins, ibogaine, nuciferine or philanthotoxin 433). Only in kynurenin and stizolobic acid are there structural elements similar to those found in the NPAA-type agonists.

2.2.3.8 Other neuroreceptors

Although several more neuroreceptors exist, rather few alkaloids have been identified as agonists or antagonists so far (Table 2.13). The indole alkaloids, brucine, corymine and strychnine, act as glycine receptor antagonists. Morphine, akuammine, daurisoline, 12-hydroxyibogaine, ibogaine and mitragynine affect opiate receptors, which normally bind endorphins and other peptides. The purino receptor is strongly affected by purine alkaloids, such as caffeine. Psycholeine and gelliusine A and B interact with the somatostatin

Table 2.12 Examples of alkaloids which bind to gamma-aminobutyric acid (GABA) receptors (natural ligand: GABA)

Alkaloid	Type	Occurrence	Activity	Reference
Bicuculline	Isoquinoline	*Dicentra* spp., *Corydalis*, *Fumaria* spp. (Fumariaceae)	$GABA_A$ blocker	Kardos *et al.* (1984), Simonyi (1987), Ameri (1997a,b)
Chelerythrine	Benzophenanthridine	Several Papaveraceae, Fumariaceae	Binding to $GABA_A$	Haeberlein *et al.* (1996)
Cryptopine	Isoquinoline	Several Papaveraceae, Fumariaceae, Ranunculaceae	Binding to GABA receptors	Kardos *et al.* (1984)
Corlumine	Isoquinoline	*Corydalis* spp., *Dicentra cucullaria* (Fumariaceae)	GABA antagonist	Buckingham (1996)
Dihydroergosine	Ergot	Sclerotia of *Sphacelia sorghi*	Binding to $GABA_A$ receptor	Pelassy and Aussel (1993)
Harmaline and related alkaloids	β-Carboline	*Peganum harmala* (Zygophyllaceae), *Passiflora* spp. (Passifloraceae), *Banisteriopsis* spp. (Malpighiaceae), *Picrasma quassioides* (Simaroubaceae), *Lolium perenne* and *Festuca arundinacea*	Binding to benzodiazepine receptor ($GABA_A$)	Rommelspacher *et al.* (1980)
β-Hydrastine and related alkaloids	Isoquinoline	*Corydalis fimbrillifera* (Fumariaceae), *Stylomecon heterophylla* (Papaveraceae), *Berberis laurina*, *Hydrastis canadensis* (Berberidaceae)	Competitive $GABA_A$ antagonist	Huang and Johnston (1990)
Muscimol		*Amanita muscaria*	$GABA_A$ agonist	Buckingham (1996)
Protopine	Protoberberine	Several Papaveraceae, Fumariaceae, Berberidaceae	Allosteric modulation of GABA receptors	Kardos *et al.*, (1984), Haeberlein *et al.* (1996)
Sanguinarine	Benzophenanthridine	Several Papaveraceae, Fumariaceae	$GABA_A$ binding	Haeberlein *et al.* (1996)
Securinine and related alkaloids	Indolixidine	*Phyllanthus* spp., *Securinega* spp. (Euphorbiaceae)	Potent centrα_1 antagonist, inhibits GABA stimulated benzodiazepine binding	Beutler *et al.* (1985)

Table 2.13 Examples of alkaloids and NPAAs which bind to other receptors: glutamate (NMDA, AMPA, kainate), opiate, somatostatin, bradykinin and others

Alkaloid	Type	Occurrence	Activity	Reference
Acromelic acid	NPAA	Fungal metabolite, *Clitocybe acromelalga*	Glutamate receptor agonist	Teuscher and Lindequist (1994)
Akuammine	Indole	*Picralima nitida* (*P. klaineana*), *Cabucala erythrocarpa* and *Vinca* spp. (Apocynaceae)	Binding to mu and kappa opiate receptors	Lewin *et al.* (1992)
Baikiain	NPAA	*Baikiaea plurijuga*, *Caesalpinia tinctoria* (Fabaceae), fungus, *Russula subnigricans*	Glutamate receptor antagonist	Teuscher and Lindequist (1994)
Caffeine	Purine	*Coffea arabica*, many other *Coffea* spp., *Theobroma cacao*, *Camellia thea*, *Cola acuminata* and several other *Cola* spp., and several other plants (Rubiaceae, Sterculiaceae, Theaceae)	Binding to adenosine receptor	Buckingham (1996)
Corymine	Indole	*Hunteria* spp. (Apocynaceae)	Inhibition of glycine receptor	Leewanich *et al.* (1997)
Daurisoline	Bis-isoquinoline	*Menispermum dauricum* (Menispermaceae)	Antagonistic binding	Waldmeier *et al.* (1995)
Domoic acid	Pyrrolidine	From blue mussels (*Mytilus edulis*), red algae, *Chondria armata*, *Alsidium corallinum*	Glutamate/kainate receptor agonist	Buckingham (1996)
Ergocryptine and other ergot alkaloids	Ergot	*Claviceps purpurea*	Inhibition of cAMP production stimulated by vasoactive intestinal peptide	Larson *et al.* (1995)
Histrionicotoxin	Piperidine	Skin of *Dendrobates* spp. (Dendrobatidae) *Mantella madagascariensis* (Ranidae, subfamily Mantellinae)	NMDA receptor blocker	Daly *et al.* (1993)

(*Continued*)

Table 2.13 (*Continued*)

Alkaloid	Type	Occurrence	Activity	Reference
Ibogaine and related alkaloids	Indole	*Tabernanthe iboga*, *Voacanga thouarsii*, *Tabernaemontana* spp. (Apocynaceae)	NMDA receptor antagonist, interaction with NMDA associated Na^+-channels, binding to mu, kappa and delta opioid receptors, hallucinogenic	Sweetnam *et al.* (1995), Chen *et al.* (1996)
Ibotenic acid	NPAA	Fungal metabolite, *Amanita* spp.	Glutamate (aspartate) receptor agonist	Buckingham (1996)
Kainic acid		Red algae, *Digenea simplex* and *Centroceras clavulatum*	Glutamate receptor agonist (kainate site)	Buckingham (1996)
Martinellic acid	Pyrroloquinoline	*Martinella iquitosensis* (Bignoniaceae)	Bradykinin receptor antagonist	Witherup *et al.* (1995)
Martinelline	Pyrroloquinoline	*Martinella iquitosensis* (Bignoniaceae)	Affinity for histaminergic receptors	Witherup *et al.* (1995)
Mitragynine	Indole	*Mitragyna speciosa*, *Uncaria* spp. (Rubiaceae)	Narcotic, binding to opioid receptors	Watanabe *et al.* (1997)
Morphine	Morphinane	*Papaver somniferum* (Papaveraceae)	Stimulation of NO release in endothelial cells; binding to μ3 receptors, inhibition of presynaptic dopamine release	Stefano *et al.* (1995, 1997), Liu *et al.* (1996)
Nuciferine	Aporphine	*Nelumbo lutea* (Nelumbonaceae), *Colubrina faralaotra* (Rhamnceae)	Glutamate receptor blocker	Kettenes *et al.* (1981), Buckingham (1996)
Psycholeine	Tris-indole	*Psychotria oleoides* (Rubiaceae)	Somatostatin receptor antagonist	Rasolonjanahary *et al.* (1995)

Quisqualic acid	NPAA	*Quisqualis indica* (Combretaceae)	Glutamate receptor agonist (AMPA)	Buckingham (1996)
Rutaecarpine	Indole	*Evodia rutaecarpa*, *Hortia* spp., *Zanthoxylum* spp. (Rutaceae)	Interaction with endothelial nitric oxide and guanylyl cyclase	Chiou *et al.* (1994)
Stizolobic acid	NPAA	*Amanita pantherina*, *Stizolobium* spp. and *Mucuna irukande* (Fabaceae)	Glutamate receptor antagonist	Teuscher and Lindequist (1994)
Strychnine	Indole	*Strychnos* spp. (Apocynaceae)	Antagonist of glycine-gated Cl^- channels	Perez Leon and Salceda Sacanelles (1996), Leewanich *et al.* (1997)
Willardiine	NPAA	*Acacia willardiana* and other *Acacia* spp. (Fabaceae)	AMPA/kainate receptor agonist	Buckingham (1996)

cAMP, cyclic adenosine monophosphate; NMDA, *N*-methyl-D-aspartate; AMPA, alpha-amino-3-hydroxy-5-methyl-isoxazol-proprionic acid; NPAA, non-protein amino acid.

receptor, and the latter compounds also interact with the receptor of neuropeptide Y. Martinellic acid binds to bradykinin receptors. This list is incomplete and it is certain that many more interactions with alkaloids will be discovered as soon as research directed towards these targets is carried out.

2.2.3.9 Ion channels

Alkaloids which modulate ion channels are tabulated in Table 2.14. Voltage-gated Na^+-channels are a target for several steroidal alkaloids, (e.g. batrachotoxinin, samandarine, veratrine, veratridine and zygadenine), indole alkaloids (e.g. ajmaline), aporphines (e.g. dicentrine, liriodenine), quinoline alkaloids (quinine, quinidine), QAs (e.g. sparteine, lupanine), alkaloids present in animals (e.g. chiriquitoxin, μ-conotoxins, dibromosceptrine, gonyautoxins, histrionicotoxins, pumiliotoxins, saxitoxin and tetrodotoxin). Activation of Na^+ channels inhibits a subsequent repolarization and, thus, leads to a total blockage of neuronal and neuromuscular signalling. This might explain why most Na^+ agonists are highly potent poisons in animals and humans (e.g. aconitine, veratrine, tetrodotoxin) (Wink and van Wyk, 2008).

Ca^{2+}-channels are inhibited by several bis-isoquinoline alkaloids (e.g. berbamine, hernandezine, liensinine, monterine, tetrandrine), aporphines (e.g. glaucine, norushinsunine), complex indole alkaloids (bis-nortoxiferine, hirsutine, mitragynine, paspaline, paspalitrem, paxilline, penitrem) or other bulky alkaloids (agelasine, contotoxins, crambescidin, ryanodine).

An apparent common theme is barely visible in these ion channel blockers but most of these alkaloids have tertiary nitrogen atoms Ns, which are protonated under physiological conditions, and are relatively large and bulky molecules that contain many functional groups, so that interactions are mediated with amino acid residues of the channel proteins.

2.2.3.10 Cell membranes

The integrity of biomembranes and the maintenance of membrane potential are of ultimate importance for the functioning of cells and of all neuronal activities. Compounds which disturb biomembranes, and thus make cells leaky, are usually strong cell poisons and interfere with membrane potential. Natural products that exhibit these properties are either highly lipophilic or amphiphilic. Several SM, such as mono-, sesqui- and diterpenes, or triterpene and steroid saponins, respectively, fall into this category.

Steroidal alkaloids, such as solanine and tomatine, which are present in many members of the Solanaceae, can form complexes with the cholesterol and other lipids present in biomembranes. Important for this interaction is the presence of a lipophilic portion of the molecule (given by the steroidal moiety) and a hydrophilic portion (provided by the sugar side chain). Whereas the lipophilic moiety 'dives' into the lipophilic interior of the membrane and interacts with the structurally similar cholesterol, the hydrophilic side chain remains outside and binds to external sugar receptors. Since phospholipids

Table 2.14 Alkaloids which affect ion channels

Alkaloid	Type	Occurrence	Activity	Reference
Aconitine and related alkaloids	Diterpene	*Aconitum* spp. (Ranunculaceae)	Activation of Na^+-channel (site 2)	Hardick *et al.* (1995), Friese *et al.* (1997)
Ajmaline	Indole	*Rauwolfia* spp., *Melodinus balansae*, *Tonduzia logifolia* (Apocynaceae)	Inhibition of Na^+- and K^+-channel, antiarrhythmic (class Ia)	Koerper *et al.* (1998), Friedrich *et al.* (2007)
Antioquine	Bis-isoquinoline	*Pseudoxandra lucida* (Annonanceae)	Ca^{2+} entry blocker, via benzothiazepine recognition site	Ivorra *et al.* (1993b)
Berbamine	Bis-isoquinoline	*Berberis* spp. and other Berberidaceae, Menispermaceae, Ranunculaceae	Ca^{2+} channel blocker	Pan *et al.* (1989)
Bisnordehydrotoxiferine	Bis-indole	*Strychnos* spp. (Loganiaceae)	Inhibition of voltage-dependent Ca^{2+} channels	da Silva *et al.* (1993)
Boldine	Aporphine	*Peumus boldo* (Monimiaceae), several Annonaceae, Lauraceae	Ca^{2+} blocker via benzothiazepine receptor site	Ivorra *et al.* (1993a)
Caffeine	Purine	*Coffea* spp., *Theobroma cacao*, *Camellia thea*, *Cola* spp. and several other plants (Rubiaceae, Sterculiaceae, Theaceae)	Inhibition of sarcoplasmic Ca^{2+} IP_3 sensitive release channels, activation of ryanodine sensitive SR Ca^{2+} release channels	Teuscher and Lindequist (1994), Berridge and Bootman (1996)
Capsaicin	Amide	*Capsicum* spp. (Solanaceae)	Inhibition of type I K^+ currents	Baker and Ritchie (1994)
Cocaine	Tropane	*Erythroxylum* spp. (Erythroxylaceae)	Inhibition of Ca^{2+} release channel from SR	Gawin (1991)

(*Continued*)

Table 2.14 (*Continued*)

Alkaloid	Type	Occurrence	Activity	Reference
Cordobimine	Bis-isoquinoline	*Crematosperma* spp. (Annonaceae)	Ca^{2+} entry blocker	Ivorra *et al.* (1992a)
Corlumidine	Isoquinoline	*Corydalis* scouleri (Fumariaceae)	Increase of Ca^{2+} currents	Kadota *et al.* (1996)
Daurisoline	Bis-isoquinoline	*Menispermum dauricum* (Menispermaceae)	Inhibition of P-type Ca^{2+}-channels	Waldmeier *et al.* (1995)
Dehydroevodiamine	Isaquinazolino-carboline	*Evodia rutaecarpa* (Rutaceae)	Endothelial K^+-channel activation; Ca^{2+} blockade	Chiou *et al.* (1996)
Dicentrine	Aporphine	Menispermaceae, Lauraceae, Fumariaceae, Papaveraceae	Inhibition of Na^+ -, K^+-channels	Su *et al.* (1994)
Dopamine	Phenylalkyl amine	Many plants	Activation of K^+-channels in snail neurons	Nesic and Pasic (1992)
Egenine	Isoquinoline	*Corydalis* spp., *Fumaria vaillantii* (Fumariaceae)	Inhibition of Ca^{2+} currents	Kadota *et al.* (1996)
Ervatamine	Indole	*Ervatamia* spp. (Apocynaceae)	Na^+-channel blocker	Buckingham (1996)
Glaucine	Aporphine	Many Monimiaceae, Berberidaceae, Annonaceae, Lauraceae, Ranunculaceae, Papaveraceae	Inhibition of voltage-dependent Ca^{2+}-channels	Loza *et al.* (1993), Ivorra *et al.* (1993c)
Gonyautoxins	Purine	*Gonyaulax* and *Protogonyaulax* spp. and isolated from marine animals, mussels, crabs, fish	Inhibition of Na^+-channels	Teuscher and Lindequist (1994), Buckingham (1996)
Granjine	Bis-isoquinoline	*Crematosperma* spp. (Annonaceae)	Ca^{2+} entry blocker	Ivorra *et al.* (1992a)
Hemandezine	Bis-isoquinoline	*Thalictrum* spp. (Ranunculaceae)	Inhibition of Ca^{2+}-channels	Low *et al.* (1996)
Hirsutine	Indole	*Mitragyna* spp., *Uncaria* spp., *Cephalanthus* spp.	Inhibition of voltage-gated Ca^{2+} -channels	Nakazawa *et al.*, (1991), Yano *et al.*, (1991), Horie *et al.* (1992)

Isotetrandrine	Bis-isoquinoline	Several Atherospermataceae, Berberidaceae, Menispermaceae, Ranunculaceae	Ca^{2+}-channel blocker	D'Ocon *et al.* (1992)
Liensinine	Bis-isoquinoline	*Nelumbo nucifera* (Nelumbonaceae)	Ca^{2+}-channel antagonist	Wang *et al.* (1993b)
Liriodenine	Aporphine	Many genera in the Annonaceae, Araceae, Eupomatiaceae, Lauraceae, Magnoiiaceae, Menispermaceae, Nelumbonaceae, Papaveraceae, Rhamnaceae, Rutaceae, Atherospermataceae	Inhibition of Na^{+}-channels, L-type Ca^{2+} currents, and 4-AP sensitive transient outward currents	Chang *et al.* (1996)
Mitragynine	Indole	*Mitragyna speciosa*, *Uncaria* spp. (Rubiaceae)	Neuronal Ca^{2+} -channel blocker	Horie *et al.* (1995)
Monterine	Bis-isoquinoline	*Crematosperma* spp. (Annonaceae)	Ca^{2+} entry blocker	Ivorra *et al.* (1992a)
Norushinsunine	Aporphine	In a wide variety of genera in the Annonaceae, Magnoliaceae Menispermaceae, Eupomatiaceae and Monimiaceae	Blocker of L-type Ca^{2+}-channels	Chulia *et al.* (1995)
Paragracine	Imidazol	*Dentitheca habereri* (coral)	Selective Na^{+}-channel blocker	Buckingham (1996)
Paspalinine	Indole	Fungal toxins, Claviceps paspali, *Aspergillus flavus*	Inhibition of Ca^{2+}-sensitive K^{+}-channels	Knaus *et al.* (1994)

(*Continued*)

Table 2.14 (*Continued*)

Alkaloid	Type	Occurrence	Activity	Reference
Paspalitrem A, C	Indole	Fungus, *Claviceps paspali*	Inhibition of Ca^{2+}-sensitive K^+-channels	DeFarias *et al.* (1996)
Paxilline	Indole	*Penicillium paxilli*, *Acremonium lorii*, *Emericella foveolata*, *Emericella desertorum* and *Emericella striata*	Inhibition of Ca^{2+}-sensitive K^+-channels	DeFarias *et al.* (1996)
Penitrem A	Indole	Fungus, *Penicillium crustosum*	Inhibition of Ca^{2+}-sensitive K^+-channels	Edwards and Weston (1996)
Phalloidin	Cyclic peptide	Fungal metabolite, *Aminata phalloides* and *Amanita* spp.	Voltage-gated K^+-channel blocker	Buckingham (1996)
Quinidine/quinine	Quinoline	*Cinchona* spp. (Rubiaceae)	Inhibition of voltage-gated Na^+-channel, opening of hemigap junctional channel	Malchow *et al.*, (1994), Koerper *et al.* (1998)
Rhynchophylline	Indole	*Uncaria rhynchophylla*, *Mitragyna* spp. (Rubiaceae)	Inhibitor of voltage-gated Ca^{2+}channels	Huang *et al.* (1993)
Ryanodine and derivatives	Pyrrole	*Ryania speciosa* (Flacourtiaceae)	Activation of ER Ca^{2+}-channels at nano-micromolar concn., inhibition of SR Ca^{2+} release channels at higher concn.	Inui (1992), Bidasee *et al.* (1995), Vais and Usherwood (1995), Allouche *et al.* (1996), Schmitt *et al.* (1996), Tinker *et al.* (1996)
Saxitoxin	Purine	*Protogonyaulax tamarensis*, *Gonyaulax cantenella* (dinoflagellates), accumulate in clams (*Saxidomus giganteus*) and mussels (*Mytilus californianus*)	Inhibition of Na^+-channels (I, II, III, h1)	Kebabian and Neumeyer (1994), Buckingham (1996)

Sparteine and related alkaloids	Quinolizidine	*Cytisus*, *Lupinus*, *Genista* and many other Fabaceae	Inhibition of ATP-regulated K^+channel in insulin-secreting betacells, in muscle, stronger inhibition of K^+- than of Na^+-channels	Ashcroft *et al.* (1991), Koerper *et al.* (1998)
Strychnine	Indole	*Strychnos* spp. (Loganiaceae)	Inhibition of muscle Na^+-channels	Koerper *et al.* (1998)
Tetrandrine	Bis-isoquinoline	*Cocculus*, *Cyclea*, *Stephania* and other Menispermaceae	Inhibits voltage-dependent L- and T-type Ca^{2+}-channels in excitable cells	Wang and Lemos (1992), Bickmeyer and Wiegand (1993), Wang *et al.* (1993a), 1994, Bickmeyer *et al.* (1996), Dworetzky *et al.* (1996), Gribkoff *et al.* (1996), Wu *et al.* (1997)
Tetrodotoxin	Guanidinium	Potent neurotoxin isolated from the ovaries and liver of fish, many amphibians and marine organisms, probably a metabolic product of a *Alteromonas* sp.	Sodium channel (I, II, III, mu,1, h1) blocker	Lu and De Clerck, (1993), Teuscher and Lindequist (1994), Buckingham (1996)
Thalidasine	Bis-isoquinoline	*Thalictrum* spp. (Ranunculaceae)	Interaction with voltage- and receptor-dependent Ca^{2+}-channels	Wu *et al.* (1977)

(*Continued*)

Table 2.14 (*Continued*)

Alkaloid	Type	Occurrence	Activity	Reference
Veratrine/veratridine	Steroidal	*Schoenocaulon officinale*, *Veratrum lobelianum* and *Veratrum viride* (Liliaceae), *Helleborus viridis* (Ranuculaceae)	Activation of Na^+ channel, no effect on inward rectifier K^+ current	Van Huizen *et al.* (1988), Nanasi *et al.* (1990), Sheldon *et al.* (1990), Honerjaeger *et al.* (1992), Matsumoto and Shimizu (1995)
Vincamine	Indole	*Vinca* spp., *Tabaernaemontana rigida* (Apocynaceae)	Inhibitor of voltage-gated Na^+-channels	Erdo *et al.* (1996)
Vincanol	Indole	*Kopsia* spp., *Melodinus celastroides*	Inhibitor of voltage-gated Na^+-channels	Erdo *et al.* (1996)
Warifteine	Bis-isoquinoline	*Cissampelos* spp. (Menispermaceae)	Inhibition of voltage-gated Ca^{2+}-channels	De Freitas *et al.* (1996)
Yohimbine	Indole	Several Apocynaceae and Rubiaceae	Inhibition of muscle Na^+-channel	Koerper *et al.* (1998)
Zygadenine	Steroidal	*Zygadenus* spp. (Liliaceae)	Activation of voltage-gated Na^+-channels	Badria *et al.* (1995)

SR, sarcoplasmic reticulum; IP_3, inositol-1,4,5-triphosphate; ER, endoplasmic reticulum; ATP, adenosine triphosphate.

are in a continuous motion (spin around their axis and horizontal movements), a tension easily builds up, which leads to membrane disruption; that is transient 'holes' occur in the biomembrane rendering the cell leaky.

Since particular steroidal alkaloids can specifically interact with receptors, ion channels or transmitter transforming enzymes (see veratrine, solanine; Tables 2.6–2.13), specific effects must be distinguished from more non-specific membrane pertubations. The effects of the steroidal alkaloids of Solanaceae, α-chaconine, α-solanine and tomatine, on the intracellular-free Ca^{2+} concentration were studied in various cell lines (Toyoda *et al.*, 1991). In all cultured cells treated with the alkaloids, the intracellular Ca^{2+} concentrations were raised in a dose-dependent manner. The Ca^{2+} influx evoked by α-chaconine could not be prevented by metal ions or by inhibitors of Ca^{2+} transport across membranes, such as voltage-operated channel antagonists, muscarinic and nicotinic antagonists, or Na^+- and K^+-channel blockers. These findings confirm that the ion flux across biomembranes caused by steroidal alkaloids is due to destabilization of the cell membrane. A similar mechanism is plausible for monodesmosidic saponins, a widely distributed group of natural products, to which the steroidal alkaloids may be assigned according to their physicochemical properties.

The alkaloids, tetrandrine, 3β-hydroxylupanine and cepharanthine, have also been reported to interfere with membrane integrity (Wink, 1993a). Weak haemolytic properties were detected for berbamine, harmin, narcotine, norharman and sanguinarine (Wink *et al.*, 1998b). These membrane perturbances will also affect neuronal and neuromuscular signalling, since both processes require intact membranes. Whereas interactions of alkaloids with neuroreceptors, ion channels and corresponding enzymes of the signal pathways show a high degree of specificity, membrane interactions, as shown here for steroidal alkaloids and saponins, are non-specific but, nevertheless, powerful defence strategies of many plants and animals.

The inhibition of Na^+-, K^+-ATPase (Table 2.16) by cardiac glycosides, anthraquinones, some proanthocyanidins and a few alkaloids might also be discussed in this context, since this inhibition will prevent the generation of ion gradients and, thus, of the membrane potential which are important for neuronal activity and active transport.

2.2.3.11 Other elements of neuronal signalling

Alkaloids which inhibit ACE, MAO and COMT are tabulated in Table 2.15. Potent ACE blockers include indole alkaloids of the physostigmine type (e.g. eseramine, geneserine, physovenine, eserine), protoberberine alkaloids (berberine, columbamine, coptisine, jatrorrhizine, palmatine), steroidal alkaloids (leptine I, solanine, solamargine and tomatidine) and others (e.g. galanthamine). No plausible structure–function relationship is apparent, except that all these alkaloids are quaternary under physiological conditions and that an oxygen can be traced 2–4 carbons adjacent to the N.

MAO inhibitors are in the group of simple indole alkaloids (e.g. β-carbolines, *N*,*N*-dimethyltryptamine, *N*-methyltryptamine), simple

Table 2.15 Examples of alkaloids as inhibitors of neurotransmitter degrading enzymes

Enzyme	Transmitter	Alkaloid	Type	Occurrence	Reference
Acetylcholine esterase (ACE)	Acetylcholine	Anatoxin A	Imidazole	*Anabaena fos-aquae* (Cyanobacterium)	Buckingham (1996)
		Berberine and related alkaloids	Protoberberine	Many *Berberis* spp. and *Mahonia* spp. (Berberidaceae) and in several different families	Buckingham (1996), Schmeller *et al.* (1997b)
		Cimiciphytine and related alkaloids	Indole	*Haplophyton cimicidum* (Apocynaceae)	Buckingham (1996)
		Demissine and related alkaloids	Steroidal	*Solanum* spp., *Lycopersicon* spp. (Solanaceae)	Buckingham (1996)
		Decarbomethoxy tetrahydrosecodine	Indole	*Tabernaemontana cumminsii*, *Haplophyton crooksii* (Apocynaceae)	Buckingham (1996)
		Galanthamine		*Galanthus* spp. and many other Amaryllidaceae	Thomsen and Kewitz (1990)
		Harmaline and related alkaloids	β-Carboline	*Peganum harmala* (Zygophyllaceae), *Passiora* spp. (Passifloraceae), *Banisteriopsis* spp. (Malpighiaceae)	Wink *et al.* (1998b)
		Huperzine and related alkaloids	Lycopodium	*Lycopodium* (Huperzia) *serrata* and other *Lycopodium* spp. (Lycopodiaceae)	Xu and Tang (1987), Kozikowski *et al.* (1996)
		Physostigmine and related alkaloids	Indole	*Physostigma venenosum* (Fabaceae), also metabolite of *Streptomyces pseudogriseolus*	Marta and Pomponi (1987), Yu *et al.* (1988), Buckingham (1996)

		Sanguinarine	Benzophenanthridine	Several Papaveraceae, Fumariaceae	Schmeller *et al.* (1997b)
		Strychnine	Indole	*Strychnos* spp. (Loganiaceae)	Buckingham (1996)
		Thebaine	Morphinane	*Papaver bracteatum*, *P. somniferum* (Papaveraceae)	Buckingham (1996)
		Vasicinol	Quinazoline	*Adhatoda vasica*, *Sida cordifolia* and from other *Sida* spp. (Acanthaceae, Malvaccae).	Buckingham (1996)
Monoamine oxidase (MAO)	NA, dopamine, serotonin, histamine	Alstovenine	Indole	*Alstonia venenata* (Apocynaceae)	Buckingham (1996)
		Carnegine (MAO A/B)	Isoquinoline	*Haloxylon* spp. and other Chenopodiaceae and Cactaceae	Bembenek *et al.* (1990)
		N,*N*-dimethyl-tryptamine and related alkaloids (MAO I)	Phenylalkyl amine	*Mimosa hostilis*, *Acacia* spp., *Arundo donax*, *Desmodium* spp., *Phalaris* spp., *Banisteriopsis* argentea, *Girgensohnia* spp., *Psychotria* spp., *Virola* spp., *Zanthoxylum* spp. and others (Fabaceae, Poaceae, Malphigiaceae, Rubiaceae, Myristicaceae, Rutaceae)	McKenna *et al.* (1984)

(*Continued*)

Table 2.15 (*Continued*)

Enzyme	Transmitter	Alkaloid	Type	Occurrence	Reference
		Harmaline and related alkaloids (MAO A)	β-Carboline	*Peganum harmala* (Zygophyllaceae), *Passiora* spp. (Passifloraceae), *Banisteriopsis* spp. (Malphgiaceae)	McKenna *et al.* (1984)
		O-methylcory-palline (MAO B)	Isoquinoline	Cactaceae, Papaveraceae, Ranunculaceae, Nelumbonaceae	Bembenek *et al.* (1990)
		Quinine and related alkaloids	Quinoline	*Cinchona* spp. (Rubiaceae)	Mitsui *et al.* (1989)
		Saracodine	Steroidal	*Sarcococca pruniformis* (Buxaceae)	Buckingham (1996)
		Salsolidine and related alkaloids (MAO A/B)	Isoquinoline	*Salsola* spp. and many other Chenopodiaceae, Fabaceae, Cactaceae, Annonaceae	Bembenek *et al.* (1990)
		Vinblastine and related alkaloids (MAO B)	Indole	*Cantharanthus roseus* (Apocynaceae)	Son *et al.* (1990)

BSE, butylcholine esterase; ACE, acetylcholine esterase; NA, noradrenaline.

isoquinolines (carnegine, salsolidine, salsolinol), quinoline alkaloids (e.g. quinine) and even complex indole alkaloids (e.g. vinblastine and vincristine). A structural similarity can be seen between the MAO blocker and endogenous substrates: the indole alkaloids and serotonin; simple isoquinoline and dopamine; NA and adrenaline, which implies that these compounds bind to the active site of the enzyme.

Uptake blockers (Table 2.16) display a certain degree of structural relatedness to the endogenous neurotransmitters whose transport is inhibited: dopamine uptake by annonaine, cocaine, ibogaine and salsolinol; serotonin by 12-hydroxyibogaine, ibogaine and norharman; NA and adrenaline by cathinone, ephedrine, salsolinol; GABA by arecaidine and guvacine. Reserpine and deserpidine inhibit an H^+-ATPase at the synaptic vesicle, which builds up a proton gradient, necessary to drive the transport of biogenic amines into the vesicles against a concentration gradient.

A few alkaloids have been recognized as adenylyl cyclase modulators (Table 2.17), as inhibitors of phosphodiesterase, protein kinases and phospholipases. The higher representation of fungal and animal alkaloids in the last two groups might be biased, because the latter targets have been included in screening programmes only during the last two decades, when extensive programmes were directed towards metabolites from fungi and marine animals. At present, no apparent structure–function relationship can be seen between alkaloids that inhibit the same molecular target.

2.2.4 Interaction with multiple targets

In general, the interactions of a particular allelochemical with a molecular target suggest a high degree of specificity. A closer look, however, shows that many substances interfere with more than one target (compare Tables 2.1–2.17). Since the data included in these tables were often produced in studies that did not aim to assess all of the activities of an isolated compound (and also the tables do not indicate when no interaction was recognized), the picture could be misleading or incomplete. In order to have a more solid basis for discussion, the results of a comparative study performed on more than 70 alkaloids (representing most structural types) will be discussed (Wink *et al.*, 1998a,b).

For this purpose, we have determined whether an alkaloid can displace a specifically bound ligand from a neuroreceptor, such as α_1 and α_2 adrenergic receptors, serotonin (5-HT_2) receptor and nicotinic and mAChR (Table 2.18) obtained from porcine brains (Schmeller *et al.*, 1994, 1995, 1997a,1997b). In addition, we have determined whether the same alkaloids inhibit ACE, whether they intercalate DNA, inhibit DNA polymerase 1, RT (reverse transcriptase), protein biosynthesis and membrane stability (Table 2.19) (Wink and Latz-Brüning, 1995; Wink *et al.*, 1998a). The results are summarized below.

Most alkaloids displace radioligands at more than one receptor (Table 2.18); for a number of known alkaloids, specific interactions were discovered

Table 2.16 Examples of alkaloids as inhibitors of neurotransmitter uptake (transport via presynaptic membrane and into vesicles), and of Na^+-, K^+- or Ca^{2+}-ATPases

Alkaloid	Type	Occurrence	Activity	Reference
Annonaine	Aporphine	Apocnaceae, Nelumbonaceae, Lauraceae, Magnoliaceae, Monimiaceae, Papaveraceae, Rhamnaceae and Menispermaceae	Inhibition of dopamine uptake	Bermejo *et al.* (1995), Protais *et al.* (1995)
Arecaidine and related alkaloids	Piperidine	*Areca catechu* (Palmae)	Inhibits GABA reuptake	Teuscher and Lindequist (1994)
Cocaine	Tropane	*Erythroxylum* spp. (Erythroxylaceae)	Inhibition of dopamine transporter	Berger *et al.* (1990), Kilty *et al.* (1991), Lever *et al.* (1993)
Ephedrine and related alkaloids	Phenylalkyl amine	Ephedraceae, *Aconitum napellus* (Ranunculaceae), *Catha edulis* (Celastraceae), *Taxus baccata* (Taxaceae), *Sida cordifolia* (Malvaceae), *Roemeria refracta* (Papaveraceae)	Induces release of NA and inhibits reuptake	Teuscher and Lindequist (1994), Mutschler (2008)
Ibogaine and related alkaloids	Indole	*Tabernanthe iboga*, *Voacanga thouarsii*, *Tabernaemontana* spp. (Apocynaceae)	Binding to vesicular dopamine and monoamine transporter, binding to 5-HT transporter	Staley *et al.* (1996)
Norharman	β-Carboline	*Chrysophyllum lacourtianum*, *Nocardia* spp. and *Streptomyces* spp., *Catharanthus roseus*, *Lolium perenne* and *Festuca arundinacea* (Sapotaceae, Apocynaceae, Poaceae)	Inhibition of dopamine and tryptamine uptake	Rommelspacher *et al.* (1980), Brossi, (1993)

Reserpine and related alkaloids	Indole	*Rauwolfia* spp., *Vinca minor*, *Alstonia constrictor*, *Tenduzia longifolia*, *Vallesia dichotoma*, *Excavatia coccinea* and other genera (Apocynaceae)	Depletes stores of NA and 5-HT in vesicles, H^+-ATPase inhibitor	Eiden *et al.* (1984), Buckingham (1996), Mutschler (1996)
Salsolinol	Isoquinoline	*Annona reticulata* (Annonaceae), *Musa paradisiaca* (Musaceae), *Theobroma cacao* (Sterculiaceae) and *Aconitum carmichaeli* (Ranunculaceae)	Inhibition of uptake of biogenic amines	Melchior and Collins (1982)
Tyramine	Phenylalkyl amine	In several plants of Magnoliaceae, Fabaceae, Poaceae, Cactaceae	Induces release of NA and inhibits reuptake	Mutschler (1996)
Veratramine	Steroidal	*Veratrum* spp. (Liliaceae)	Both releaser and uptake inhibitor of 5-HT	Nagata *et al.* (1991)

GABA, gamma-aminobutyric acid; NA, noradrenaline; 5-HT, 5-hydroxytryptamine (serotonin); ATPase, adenosine triphosphatase.

Table 2.17 Examples of alkaloids modulating enzymes involved in signal transduction

Enzyme	Alkaloid	Type	Occurrence	Activity	Reference
Adenylyl cyclase (AC)	Ergometrine and related alkaloids	Ergot	*Claviceps* and several Convolvulaceae	AC agonist	Rosenfeld *et al.* (1980)
	Nuciferine	Aporphine	*Nelumbo lutea* (Nelumbonaceae), *Colubrina faralaotra* (Rhamnceae)	AC inhibition	Buckingham (1996)
Phospho-diesterase (PDE)	Allocryptopine	Protoberberine	*Glaucium arabicum*	PDE inhibition	Abu-Ghalyun *et al.* (1997)
	Caffeine and related alkaloids	Purine	Rubiaceae, Aquifoliaceae, Sapindaceae, Sterculiaceae, Theaceae	PDE inhibition	Buckingham (1996)
	Chelerythrine	Benzophenanthridine	Several Papaveraceae, Fumariaceae	PDE inhibition	Moriyasu *et al.* (1990)
	Colchicine	Tropolone	*Colchicum autumnale*, many other *Colchicum* spp., several *Merendera* spp., *Gloriosa superba* and other Liliaceae	PDE inhibition	Ewart and Bradford (1988), Teuscher and Lindequist (1994)
	Glaucine	Aporphine	Many Monimiaceae, Berberidaceae, Annonaceae, Lauraceae, Ranunculaceae, Papaveraceae	Inhibition of a Ca^{2+}-independent PDE	Ivorra *et al.* (1992b)
	Griseolic acid	Purine	Fungal metabolite, *Streptomyces*	PDE inhibition	Buckingham (1996)
	Infractine	Indole	Fungus, *Cortinarius infractus*, *Picrasma quassioides* (Simaroubiaceae)	PDE inhibition	Bracher and Hildebrand (1995)

	Laccarin		Fungus, *Laccaria vinaceoavellanea*	PDE inhibition	Matsuda *et al.* (1996)
	Papaverine	Isoquinoline	*Papaver somniferum* (Papaveraceae); *Rauwolfia serpentina* (Apocynaceae)	Unselective PDE inhibition	Ivorra *et al.* (1992b), Buckingham (1996)
	Persicanidine A	Steroidal	*Fritillaria* persica (Liliaceae)	PDE inhibition	Ori *et al.* (1992)
	Pseudodistomin	Piperidine	Tunicate *Pseudodistoma kanoko*	PDE inhibition	Ishibashi *et al.* (1987)
	Sanguinarine	Benzophenanthridine	Several Papaveraceae, Fumariaceae	PDE inhibition	Moriyasu *et al.* (1990)
Protein kinase C (PKC)	Balanol		Fungal metabolite, *Cordyceps ophioglossoides*, *Fusarium merismoides*, *Verticillium balanoides*	PKC inhibition	Buckingham (1996)
	Cepharanthine	Bis-isoquinoline	*Stephania* spp. (Menispermaceae)	PKC inhibition	Edashige *et al.* (1991)
	Chelerythrine	Benzophenanthridine	*Chelidonium majus*	Selective PKC inhibition	Herbert *et al.* (1990)
	Erbstatin	Amine	*Streptomyces* spp.	EGF tyrosine kinase inhibitor	Buckingham (1996)
	Ellipticine	Indole	*Ochrosia elliptica* and several other *Ochrosia* spp., *Aspidosperma subincanum*, *Bleekeria vitiensis* (Apocynaceae)	Selective inhibition of p53 phosphorylation	Ohashi and Matsuoka (1985)
	9-Hydroxyellipticine	Indole	*Strychnos dinklagei* (Loganiaceae)	Selective inhibition of p53 phosphorylation, suppression of CDK2 kinase	Ohashi and Matsuoka (1985)

(*Continued*)

Table 2.17 (*Continued*)

Enzyme	Alkaloid	Type	Occurrence	Activity	Reference
	Lavendustin A		*Streptomyces griseolavendus*	Tyrosine kinase inhibitor	Buckingham (1996)
	Lyngbyatoxin A	Indole	Marine blue-green alga, *Lyngbya majuscula*, also *Streptomyces mediocidicus*	PKC activator	Buckingham (1996)
	Melittin	Peptide	Bee venom (*Apis* spp.)	Inhibitor of PKC and cAMP protein kinases	Buckingham (1996)
	16-Methylpendolmycin	Indole	*Nocardiopsis* spp.	PKC inhibition	Sun *et al.* (1991)
	Michellamine B	Isoquinoline	*Ancistrocladus* spp. (Ancistocladraceae)	PKC inhibition	Upender *et al.* (1996)
	Polymyxin B	Cyclic peptide	*Bacillus polymixa*	PKC inhibition	Buckingham (1996)
	Swainsonine	Indolizidine	*Swainsonia canescens*, *Astragalus* spp. (Fabaceae)	Indirect PKC activation	Breton *et al.* (1990)
Phospholipase	Aristolochic acid	'aporphine'	*Aristolochia* spp. (Aristolochiaceae)	PLA_2 blocker	Teh *et al.* (1990), Moreno (1993)
	Berbamine and related alkaloids	Bis-isoquinoline	*Berberis* spp. and other Berberidaceae, Menispermaceae, Ranunculaceae	Inhibition of cytosolic PLA_2, uncoupling of G-protein from PL	Hashizume *et al.* (1991), Akiba *et al.* (1992, 1995)
	Topsentin B2	Bis-indole	Marine sponges (*Topsentia* spp., *Spongosorites* spp., *Hexadella* spp.)	Inactivation of PLA_2	McConnell *et al.* (1993)

cAMP, cyclic adenosine monophosphate; PLA_2, phospolipase A_2; EGF, epidermal growth factor.

Table 2.18 Interaction of alkaloids with neuroreceptors and acetylcholine-related enzymes

Alkaloids	Adrenergic receptors		Serotonin receptor	Acetylcholine receptors		AChE	BChE	ChAT
	α_1	α_2	(5-HT_2)	mAChR	nAChR			
Imidazole alkaloids								
Pilocarpine	n.a.	n.a.	n.a.	11.0	1656	n.a.	n.a.	n.a.
Indole alkaloids								
Brucine	4.7	347.3	n.a.	51.3	13.6	n.a.	n.a.	n.a.
Ergometrine	4.4	0.9	1.5	2.0	178.2	n.a.	161.3	n.a.
Gramine	26.3	8.7	8.5	677.1	30.7	1019	232.3	n.a.
Harmaline	34.0	7.5	14.6	33.5	n.a.	173.2	90.4	n.a.
Harmalol	56.8	16.9	30.6	60.4	623.7	93.1	37.1	n.a.
Harman	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	72.0	n.d.
Harmine	n.d.	n.d.	n.d.	n.d.	n.d.	1005	175.3	n.d.
Harmol	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	26.1	n.d.
Norharman	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	63.5	n.d.
Physostigmine	n.a.	73.6	394.2	66.2	1992	0.03	16.2	n.a.
Strychnine	25.1	172.3	51.6	32.8	10.2	n.a.	130.8	n.a.
Isoquinoline alkaloids								
Berberine	3.2	0.48	1.9	1.0	35.5	167.4	55.8	n.a.
Boldine	0.53	0.09	0.67	118.1	11.1	n.a.	n.a.	n.a.
Emetine	6.2	0.07	7.6	58.2	n.a.	n.a.	n.a.	n.a.
Laudanosine	18.4	0.82	8.2	67.1	1313	n.a.	415.7	n.a.
Morphine	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Palmatine	5.8	0.96	2.9	4.1	100	124.5	425.6	100
Salsoline	115.1	9.8	146.2	n.a.	n.a.	n.a.	n.a.	n.a.
Sanguinarine	33.6	6.4	91.7	2.4	11.8	10.9	17.4	0.3
Phenylalkylamines								
L-Ephedrine	186.4	14.6	60.0	2649	n.a.	n.a.	n.a.	n.a.

(*Continued*)

Table 2.18 (*Continued*)

Alkaloids	Adrenergic receptors		Serotonin receptor	Acetylcholine receptors				
	α_1	α_2	(5-HT_2)	mAChR	nAChR	AChE	BChE	ChAT
Piperidine/pyridine alkaloids								
Ammodendrine	257.5	11.6	109.0	523.6	9.1	n.a.	275.4	n.a.
Anabasine	2374	47.6	n.a.	n.a.	0.58	n.a.	n.a.	n.a.
Arecoline	n.a.	29.7	n.a.	32.1	5.7	n.a.	n.a.	n.a.
Coniine	n.a.	260.0	492.7	2071	19.0	n.a.	327.5	n.a.
Cotinine	n.a.	n.a.	n.a.	1776	2.5	n.a.	n.a.	n.a.
Nicotine	n.a.	n.a.	n.a.	882.8	0.008	n.a.	n.a.	n.a.
Pseudopelletierine	n.a.	n.a.	n.a.	386.1	0.7	n.a.	n.a.	n.a.
Purine alkaloids								
Caffeine	n.a.	n.a.	n.a.	464.8	n.a.	n.a.	n.a.	n.a.
Pyrrolizidine alkaloids								
Acetylheliosupine	39.1	2.9	23.2	71.3	159.7	n.a.	n.a.	n.a.
Echihumiline	n.a.	358.8	549.0	89.2	n.a.	n.a.	314.4	n.a.
Echihumuline *N*-oxide	n.a.	50	182.0	8.7	n.a.	n.a.	n.a.	n.a.
Echimidine	n.a.	900	257.6	512.5	n.a.	n.a.	n.a.	n.a.
Heliosupine	149.1	15.0	77.1	392.0	n.a.	n.a.	n.a.	n.a.
Heliosupine *N*-oxide	n.a.	n.a.	n.a.	350.0	n.a.	n.a.	n.a.	n.a.
Heliotrine	n.a.	n.a.	535.4	52.2	n.a.	n.a.	n.a.	n.a.
Monocrotaline	n.a.	n.a.	203.4	n.a.	n.a.	n.a.	n.a.	n.a.
Pycnanthine	n.a.	n.a.	407.6	177.2	n.a.	n.a.	462.6	n.a.
Retronecine	ma.	n.a.	n.a.	127.9	n.a.	n.a.	n.a.	n.a.
Riddelline	n.a.	n.a.	n.a.	208.7	n.a.	n.a.	n.a.	n.a.
Senecionine	ma.	n.a.	249.4	43.0	n.a.	n.a.	n.a.	n.a.
Seneciphylline	n.a.	341.4	608.6	52.6	n.a.	n.a.	n.a.	n.a.

Quinoline alkaloids								
Cinchonine	5.2	1.4	5.5	19.2	n.a.	n.a.	22.9	n.a.
Cinchonidine	1.1	1.3	10.7	19.7	n.a.	n.a.	n.a.	n.a.
Quinine	5.7	2.5	6.4	4.5	n.a.	n.a.	109.0	n.a.
Quinidine	29.7	1.3	14.4	18.4	n.a.	n.a.	n.a.	n.a.
Quinolizidine alkaloids								
Albine	n.a.	n.a.	n.a.	32.9	237.7	n.a.	n.a.	n.a.
Anagyrine	n.a.	n.a.	n.a.	132.1	2096	n.a.	n.a.	n.a.
Angustifoline	n.a.	n.a.	n.a.	25.3	n.a.	n.a.	n.a.	n.a.
Annotinine	n.a.	n.a.	n.a.	260.5	n.a.	n.a.	n.a.	n.a.
Cytisine	n.a.	n.a.	n.a.	398.2	0.14	n.a.	n.a.	n.a.
13-Hydroxylupanine	n.a.	n.a.	n.a.	139.7	467.2	n.a.	n.a.	n.a.
13-Tigloyloxylupanine	n.a.	n.a.	508	11.1	99.8	n.a.	648.3	n.a.
Lupanine	n.a.	n.a.	n.a.	118.0	5.3	n.a.	n.a.	n.a.
Lupinine	n.a.	207.8	n.a.	189.9	n.a.	n.a.	n.a.	n.a.
N-Methylcytisine	n.a.	n.a.	n.a.	416.7	0.05	n.a.	n.a.	n.a.
17-Oxosparteine	n.a.	180.7	n.a.	117.9	155.0	n.a.	n.a.	n.a.
3β-Hydroxylupanine	n.a.	n.a.	n.a.	74.1	192.4	n.a.	n.a.	n.a.
Multiflorine	n.a.	n.a.	n.a.	49.4	n.a.	n.a.	n.a.	n.a.
Sparteine	n.a.	127.7	n.a.	21.3	330.8	n.a.	165.5	n.a.
Tetrahydrorhombifoline	n.a.	n.a.	n.a.	128.8	347.6	n.a.	n.a.	n.a.

(*Continued*)

Table 2.18 (*Continued*)

Alkaloids	Adrenergic receptors		Serotonin receptor	Acetylcholine receptors		AChE	BChE	ChAT
	α_1	α_2	(5-HT_2)	mAChR	nAChR			
Terpene alkaloids								
Aconitine	n.a.	331.6	n.a.	1.3	n.a.	n.a.	n.a.	n.a.
Tropane alkaloids								
Atropine	6.1	10.1	6.0	0.005	284.4	n.a.	n.a.	n.a.
Cocaine	n.a.	506.7	317.3	56.7	371.4	n.a.	274.3	n.a.
6β-Hydroxyhyoscyamine	12.6	42.0	n.d.	0.039	n.a.	n.a.	n.a.	n.a.
7β-Hydroxyhyoscyamine	n.d.	37.6	n.d.	0.008	n.a.	n.a.	n.a.	n.a.
Littorine	66.3	72.3	68.9	0.003	909.8	n.a.	n.a.	n.a.
Noratropine	16.4	30.4	22.7	0.2	494.4	n.a.	n.a.	n.a.
Scopolamine	113.0	359.7	168.0	0.002	928.4	n.a.	n.a.	n.a.
Tropine	n.a.	n.a.	n.a.	2631	n.a.	n.a.	n.a.	n.a.
Tropolone alkaloids								
Colchicine	n.a.	23.8	133.2	347.3	30.0	n.a.	n.a.	n.a.

Note: The data presented are the concentrations (in μM) which replace 50% of the specifically bound ligand or which inhibit the enzymes by 50% (IC_{50}). 5-HT_2, serotonin receptor, mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; AChE, acetylcholine esterase; BChE, butylcholine esterase; ChAT, choline acetyltransferase; n.a., not active at 500 μM; n.d., not determined.
Source: After Wink *et al.* (1998a,b).

Table 2.19 Interaction of alkaloids with basic molecular targets

	DNA melting temperature increase[a] °C	DNA methylgreen release IC_{50}[b]	DNA DNA Pol I inhibition IC_{50}[c]	RNA RT inhibition IC_{50}[c]	Protein biosynthesis inhibition %[d]	Membrane permeability haemolysis %[e]
Imidazole alkaloids						
Pilocarpine	0	n.a.	n.a.	n.a.	n.a.	n.a.
Indole alkaloids						
Ajmalicine	1.0[f]	> 1 mM	n.a. (0.5 mM)	n.d.	n.d.	n.a.
Ajmaline	2.3[f]	> 5 mM	> 10 mM	7 mM	46 (4 mM)	n.a. (1 mM)
Brucine	n.d.	> 5 mM	n.a.	5 mM	36	n.a.
Ergometrine	n.d.	> 5 mM	n.a.	> 10 mM	n.a.	n.a.
Ergotamine	13.7[f]	n.d.	n.d.	n.d.	20 (0.25 mM)	n.a.
Gramine	n.d.	n.a.	n.a.	> 10 mM	n.a.	n.a.
Harmaline	8.6[f]	0.3 mM	3.2 mM	2.4 mM	70	n.a.
Harmine	16.l[f]	0.4 mM	0.9 mM	0.5 mM	95	2.3
Norharman	6.2[f]	< 1 mM	8 mM	< 0.2 mM	50 (0.2 mM)	3.3
Physostigmine	n.d.	n.a.	n.a.	> 10 mM	15	n.a.
Strychnine	n.d.	n.a.	n.a.	7 mM	20	n.a.
Vincamine	n.d.	n.d.	n.a.	n.a.	54	2 (1 mM)
Yohimbine	n.d.	n.a.	n.a.	> 10 mM	62	n.a.
Isoquinoline alkaloids						
Berbamine	13.2[f]	0.5 mM	0.7 mM	< 0.25 mM	n.d.	2.3 (2 mM)
Berberine	15[f]	0.1 mM	0.4 mM	0.2 mM	100	n.a.
Boldine	6[f]	< 0.7 mM	5 mM	< 1.5 mM	30	n.a.
Canadine	1.8[f]	n.d.	n.d.	n.d.	15 (0.1 mM)	1.5 (0.2 mM)
Chelidonine	1.5[f]	(> 2 mM)	(> 2 mM)	(> 2 mM)	28 (0.14 mM)	n.a.
Emetine	7.4[f]	0.7 mM	5 mM	< 0.5 mM	100 (0.01 mM)	n.a.

(*Continued*)

Table 2.19 (*Continued*)

	DNA melting temperature increase[a] °C	DNA methylgreen release IC_{50}[b]	DNA DNA Pol I inhibition IC_{50}[c]	RNA RT inhibition IC_{50}[c]	Protein biosynthesis inhibition %[d]	Membrane permeability haemolysis %[e]
Laudanosine	n.d.	n.a.	n.a.	4 mM	15	n.a.
Narcotine	0	n.a.	1 mM	n.a. (0.5 mM)	n.a. (0.1 mM)	4.5 (0.5 mM)
Papaverine	0	n.a.	n.a.	n.d.	55 (0.8 mM)	1 (0.5 mM)
Salsoline	n.d.	n.a.	n.a.	n.a.	30	n.a.
Sanguinarine	24[f]	0.02 mM	< 0.02 mM	0.03 mM	n.d.	5.2 (0.1 mM)
Phenylalkylamines						
L-Ephedrine	n.d.	n.a.	n.a.	n.a.	n.a.	n.a.
Piperidine/pyridine alkaloids						
Anabasine	n.d.	n.a.	n.a.	n.a.	n.a.	n.a.
Arecoline	n.d.	n.a.	n.a.	> 10 mM	n.a.	n.a.
Cycloheximide	n.d.	n.a.	n.a.	n.a.	90 (0.01 mM)	n.a.
Lobeline	1.5′	> 5mM	> 10 mM	< 2 mM	45	n.a.
Nicotine	n.d.	n.a.	n.a.	n.a.	20	n.a.
Piperine	n.d.	n.a.	n.a.	n.d.	35 (0.3 mM)	n.a.
Pseudopelletierine	n.d.	n.a.	n.a.	n.a.	n.a.	n.a.
Purine alkaloids						
Caffeine	n.d.	n.a.	n.a.	n.a.	n.a.	n.a.
Pyrrolizidine alkaloids						
Heliotrine	n.d.	n.a.	n.a.	n.a.	20	1
Monocrotaline	n.d.	n.a.	n.a.	n.a.	25	n.a.
Retronecine	n.d.	n.a.	n.a.	n.a.	20	n.a.
Riddelline	n.d.	n.a.	n.a.	n.a.	20	n.a.
Senecionine	n.d.	n.a.	n.a.	n.a.	50 (5 mM)	1.2

Quinoline alkaloids						
Cinchonine	5′	n.a.	8 mM	6 mM	58 (5mM)	n.a.
Cinchonidine	6′	< 5mM	10 mM	< 1 mM	90 (5 mM)	n.a.
Quinidine	8′	1 mM	2.4mM	< 1 rum	63	0.6 (1 mM)
Quinine	6{	2 mM	3.2 mM	< 2 mM	80	n.a.
Quinolizidine alkaloids						
Anagyrine	n.d.	n.a.	n.a.	n.a.	n.a.	n.a.
Angustifoline	n.d.	n.a.	n.a.	n.a.	15 (5 mM)	n.a.
Cytisine	n.d.	n.a.	n.a.	n.a.	n.a.	n.a.
3β-Hydroxylupanine	n.a.	n.a.	n.a.	n.a.	n.a.	91
13-Hydroxylupanine	n.a.	n.a.	n.a.	n.a.	38	3.5
Lupanine	n.a.	n.a.	n.a.	n.a.	15 (5 mM)	0.8
Lupinine	n.d.	n.a.	n.a.	n.a.	31 (5mM)	n.a.
17-Oxosparteine	n.a.	n.a.	n.a.	n.a.	37	n.a.
Sparteine	n.d.	n.a.	n.a.	n.a.	n.a.	n.a.
Tetrahydrorhombifoline	n.d.	n.a.	n.a.	n.a.	n.a.	0.9
13-Tigloyloxylupanine	n.a.	n.a.	n.a.	> 10 mM	20	n.a.
Terpene alkaloids						
Aconitine	n.d.	n.a.	n.a.	n.a.	n.a.	n.a.
Protoveratrine B	0	n.a.	n.a.	n.a.	30	n.a.
Solanine	3′	n.a. (0.5 mM)	n.a. (0.5 mM)	<0.1mM	50 (0.025 mM)	100 (0.2 mM)

(*Continued*)

Table 2.19 (*Continued*)

	DNA melting temperature increase[a] °C	DNA methylgreen release IC_{50}[b]	DNA DNA Pol I inhibition IC_{50}[c]	RNA RT inhibition IC_{50}[c]	Protein biosynthesis inhibition %[d]	Membrane permeability haemolysis %[e]
Tropane alkaloids						
Hyoscyamine	n.d.	n.a.	n.a.	n.a.	n.a.	n.a.
Scopolamine	0.3	n.a.	n.a.	n.a.	n.a.	n.a.
Scopine	n.d.	n.a.	n.a.	n.a.	n.a.	n.a.
Tropine	n.d.	n.a.	n.a.	n.a.	15	n.a.
Tropolone alkaloids						
Colchicine	n.d,	n.a,	n.a.	< 10 mM	n.a.	0.7 (1 mM)

[a] 70 pM alkaloid solution and 70 μM Sinapis DNA.
[b] Alkaloids were tested up to 5 mM (n.a. = release <25% at 5 mM).
[c] Alkaloids were preincubated with DNA or RNA (n.a. = inhibition <25% at 10 mM).
[d] Alkaloids were tested at 1 mM (n.a. = inhibition <10%). If strong activities were discovered then lower concentrations were also assayed.
[e] Alkaloids were tested at 5 mM (n.a. = inhibition <0.6%). If strong activities were discovered then lower concentrations were also assayed.
[f] Alkaloid and DNA coelute when separated by gel chromatography on PD10 columns.
DNA, deoxyribonucleic acid; Pol 1, polymerase 1; RNA, ribonucleic acid; RT, reverse transcriptase; ICSO, concentration required to cause 50% maximum inhibition; n.d., not determined; n.a., not active.
Source: After Wink and Latz-Brüning (1995), Wink *et al.* (1998a,b).

that (to our knowledge) had not been reported in the literature. Affinities to a particular receptor are often significantly correlated with effects at other neuroreceptors. For example, alkaloids that affect α_1 receptors also bind to α_2 and serotonin receptors, and those that affect α_2 are active at serotonin receptors. Activities between muscarinic and nAChRs and between serotonin and adrenergic receptors are much weaker or lack a clear correlation.

For a few alkaloid groups, the hybrid character is well known and well documented, e.g. ergot and QAs. Ergot alkaloids, such as ergotamine, ergometrine or ergoclavine, are produced by fungi of the genus *Claviceps* and other endophytic fungi, which live in close contact with many grasses (family Poaceae), such as the cereal, *Hordeum vulgare*. These alkaloids can modulate several receptors of neurotransmitters, such as dopamine, serotonin and NA. As a consequence, the pharmacological action of ergot alkaloids is rather broad, ranging from vasoconstriction and uterine contraction to hallucinations. These activities can be explained through structural similarities between the alkaloid and the different neurotransmitters (Fig. 2.17). It has been suggested that the interactions between *Claviceps* and its host plant are of a symbiotic nature, i.e. infected plants exploit the chemistry of the fungus for their own protection against herbivores (otherwise it would be difficult to explain why a fungal metabolite should interfere with targets that are only present in animals); grasses with endophytes had better protection against herbivores than fungus-free grasses.

QAs, such as lupanine, sparteine or cytisine, are produced by many members of the Fabaceae. QAs are bitter for many animals (and plants producing them are, therefore, avoided as food). QAs, like many other alkaloids, occur as complex mixtures in plants. We have recently shown (Schmeller *et al.*, 1994), that some QAs preferentially bind to the nAChR, whereas others tend to bind to the muscarinic AChR (Table 2.18). Some QAs exhibit a prominent cross-reactivity. Furthermore, QAs such as lupanine and sparteine inhibit Na^+-channels, thus blocking the signal transduction in nerve cells at a second critical point (Koerper *et al.*, 1998). In addition, QAs slightly interfere with protein biosynthesis. A few particular QAs, such as anagyrine, cytisine and the bipiperidine alkaloid, ammodendrine (which cooccurs with QAs in many plants), are mutagenic and lead to malformations. These results suggest that QAs are indeed defence chemicals with a broad range of targets, which might be affected simultaneously (reviewed in Wink, 1992, 1993a,b,c).

In other instances, such apparent hybrid activities could be missing or less prominent, as shown by the example of epibatidine, a powerful alkaloid from the skin of several arrow poison frogs. Synthetic (+)- and natural (−)-epibatidine have potent agonistic activity at ganglionic-type nicotinic receptors. The epibatidines have little or no activity at a variety of other central receptors, including opioid, muscarinic, adrenergic, dopamine, serotonin and GABA receptors (Badio and Daly, 1994).

Ajmaline, berbamine, berberine, boldine, cinchonine, cinchonidine, ergometrine, harmaline, harmine, lobeline, norharman, papaverine, quinidine, quinine, sanguinarine and solanine affect more than one of the basic molecular targets. In addition, the same compounds can bind to one or several neuroreceptors that are only present in animals (Tables 2.15, 2.16). It is likely that these interactions are responsible (at least in part) for the allelopathic, antibacterial effects and animal toxicity, which are exhibited by these alkaloids (Wink and Twardowski, 1992; Wink and Latz-Brüning, 1995). According to Tables 2.18 and 2.19, many alkaloids are compounds with a very broad activity spectrum. Because of their wide activity, we can consider them 'multipurpose' defence substances. Since plants cannot predict or choose their competitors, infesting microorganisms, insects and other herbivores, such preformed 'multipurpose' compounds are certainly a means of being prepared for most situations.

But, why do neurotransmitters have mimics that also intercalate DNA? Plant–herbivore interactions are mutual processes. If the plant produces an nAChR inhibitor, it is likely that some insects develop a resistance, in such a way that they modify the binding site of their nAChR, so that the inhibitor can no longer bind. It was recently shown that *Danaus plexippus*, which sequesters cardiac glycosides from its host plant, has a modified binding domain for its Na^+, K^+-ATPase, which no longer interacts with cardiac glycosides, thus providing an insensitivity at this target (Holzinger *et al.*, 1992; Holzinger and Wink, 1996). However, if the same compound affected other molecular targets at the same time, such as DNA, an adaptation through target site modification would be much more difficult and unlikely, since it would require several concomitant mutations. According to this hypothesis, the evolution of allelochemicals affecting more than one target would be a strategy, firstly, to counteract adaptations by specialists; and secondly, to help in fighting off different groups of enemies. In conclusion, it can be said that nature has obviously tried 'to catch as many flies with one clap as possible' in the selection of allelochemicals during evolution.

2.3 Accumulation of defence and signal compounds in plants

2.3.1 The importance of high concentrations

In order to deter the feeding of herbivores, allelochemicals need to be accumulated in sufficient quantities. Storage of water-soluble compounds in large concentrations (up to 200–500 mM) in the vacuole is an important requisite for chemical defence in most plants; therefore, we have termed these vacuoles 'defence and signal compartment' (Wink, 1993a). Many vacuolar allelochemicals are positioned at a favourable site for defence because they

are stored in substantial amounts in epidermal and subepidermal cells (Wink *et al.*, 1984; Wink, 1992, 1997). The sequestration of lipophilic metabolites in ducts, laticifers, trichomes, glandular hairs or in the cuticle (reviewed in Wink, 1993a,b,c, 1997) is also important as a defence measure. If a small herbivore or microbe attacks such a plant, it will encounter a high concentration immediately at the periphery upon wounding or entering the tissue, which might deter further feeding (Wink, 1999a).

A further question is whether the inhibitory concentrations determined in in vitro experiments (Tables 2.18 and 2.19) relate in any way to the in vivo situation. A simple calculation may help to assess this problem: alkaloids are usually stored in high concentrations at sites that are important for growth and reproduction and can reach 1–2% of the dry weight (Wink, 1993a). Assuming an alkaloid concentration of 100 mg per 100 g fresh weight (which is approximately equivalent to 1% dry weight) and a small herbivore with a body weight of 1000 g; if this animal ingested 100 g of an alkaloid-producing plant, it would take up 100 mg of alkaloids. Supposing that the alkaloids are completely resorbed and equally distributed in the body, a concentration of 100 mg alkaloids per kg body weight would be obtained. Taking a mean molecular weight of 200 kDa, the alkaloid concentration in this herbivore would be 500 μM, which would be high enough to partially or completely block the binding of ACh, serotonin or noradrenalin at their receptors (compare the values for the concentration required to cause 50% maximum inhibition (IC_{50}) in Table 2.18) or to interfere with DNA and related processes. In reality, incomplete resorption and degradation will prevent such high internal toxin concentrations but even a five to ten times lower internal concentration can produce severe intoxication and disturbance in most instances. Under these conditions, an interaction with multiple targets appears likely. This might explain why alkaloid-producing plants are usually avoided by most herbivores, with the exception of a few adapted organisms that have evolved tolerance or insensitivity towards these toxins.

2.3.2 Variability and timing of accumulation of defence chemicals in vulnerable tissues

In most plants, synthesis and accumulation of SM is regulated in space and time. Some plants make and store a particular compound in one organ only; in other instances, a compound is made in the roots or leaves but exported to other plant parts via xylem, phloem or apoplastically. In general, vulnerable tissues and organs are defended more than old senescing ones; many seeds, seedlings, buds and young tissues either sequester or synthesize large amounts of defence chemicals. Plant parts that are important for survival and multiplication, such as flowers, fruits and seeds, are nearly always heavily defended. It is difficult to summarize this topic, as the relationships differ from system to system and need to be elucidated for each group of plants.

Variation is an important strategy against herbivores, since if all plants followed the same strategy it would be easy for herbivores to adapt.

The profile of SMs in plants usually consists of a series of related compounds; generally, a few major metabolites and several minor components are present that differ in the position of their substituents or their stereochemistry. The differences may appear small from a chemical perspective but they can be important for pharmacological activity; the addition of a simple substituent may open another molecular target (see Tables 2.18 and 2.19) and can thus contribute to an improved fitness. The profile usually varies between plant organs, within developmental stages and sometimes even diurnally (e.g. in lupin alkaloids) (Wink, 1992). Moreover, marked differences can usually be seen between individual plants of a single population, and even more so between members of different populations. In some plants, the population differences appear to be genetically fixed and can be distinguished as particular 'chemotypes'; examples are monoterpene profiles in *Thymus vulgaris*, which lead to differential herbivory. This variation, which is part of the apparent evolutionary 'arms race' between plants and herbivores, makes adaptation by herbivores more difficult.

In most examples discussed so far, variation in patterns and concentrations was found for most secondary compounds but, nevertheless, we consider this defence constitutive; prefabricated metabolites are included that are only activated in case of immediate emergency (e.g. cyanogenic glucosides, coumaroylglycosides, bidesmosidic saponins, ranunculin, alliin, glucosinolates). If a plant is wounded by a herbivore or infected by a pathogen, the defence chemistry often becomes activated (Harborne, 1993; Baldwin, 1994). Two strategies can be distinguished. Either the concentration of the compounds already present is increased by a de novo synthesis, as has been observed for several defence chemicals, such as nicotine in *Nicotiana tabacum* (Baldwin, 1994), lupin alkaloids in *Lupinus polyphyllus* (Wink, 1992) and several others. Alternatively, the de novo synthesis of compounds (usually termed 'phytoalexins') that were hitherto absent is induced. These compounds include several isoflavones, pterocarpans, furanocoumarins, chalcones, stilbenes and others. Many of these metabolites have antifungal properties, so that they are sometimes considered to be part of a specific antimicrobial defence system in plants (see Chapter 4 in this book). However, since most of these compounds also affect herbivores, the plant defence induced appears to be a more general phenomenon. The biochemical mechanisms, starting with binding of elicitors to membrane receptors, via intercellular signalling, to gene activation and protein biosynthesis, have been studied in these induced systems, so that a considerable body of information is already available on the underlying mechanisms. During the last decade, it has been shown that a first important step in the signal pathway is the hydrolysis of unsaturated fatty acids from the cell membrane lipids by phospholipase. The fatty acids are converted into jasmonic acid, which appears as a major signal compound in wounded and infected plants. It

will be a challenging task to elucidate all the steps in these induced signal pathways.

In conclusion, variation in chemical structures, in organ-specific profiles of allelochemicals within and between different groups of plant and in defence strategies can be interpreted as a means of avoiding the adaptation of specialized herbivores and microorganisms.

2.3.3 Costs of chemical defence

The pathways leading to most groups of SM have been elucidated (reviewed in Ashour *et al.*, 2010; Kreis and Müller-Uri, 2010; Petersen *et al.*, 2010; Roberts *et al.*, 2010; Selmar, 2010; Wink *et al.*, 2010) and several of the specific enzymes involved have been purified and studied in detail (Luckner, 1990). Because the enzymes of biosynthesis also show a regular turnover, mRNA must be transcribed and translated into proteins. Both transcription and translation require substantial amounts of ATP, as does the biosynthesis itself: a prerequisite for defence or signal compounds to be present in relatively high amounts at the right place and time. Storage in the vacuole usually requires energy as well; energy for the uphill transport and often for trapping the metabolite in the vacuole is provided by an H^+-ATPase. In addition, some SM are transported into the vacuole with help of ABC transporters which depend on ATP. As a consequence, both biosynthesis and sequestration (and the corresponding transcription and translation) must be costly processes for the plants producing SM. Sequestration in resin ducts, oil cells or trichomes demands the formation of new structures, which can figure as costs for a plant expressing them (Wink, 1999a).

Is there any evidence that these costs matter? Plants are autotrophic and usually not limited by energy or carbon dioxide; theoretically, it should be rather easy for them to provide the ATP and carbon necessary for the synthesis of defence substances. But circumstantial evidence suggests that chemical defence is, nevertheless, carried out parsimoniously and not luxuriously. Legumes, for example, produce QAs as effective defence chemicals. In brooms, we find several species with spikes and thorns which provide mechanical defence (Wink, 1992). In all these species, alkaloid contents are very low, suggesting that chemical protection has been substituted by mechanical defence. A similar feature has been reported for cacti; spiny species usually contain small amounts of alkaloids, whereas soft-bodied and spineless taxa are alkaloid rich (e.g. *Lophophora williamsii* producing mescaline). Species which have colonized volcanic islands often encounter a niche free from herbivores and sometimes they have apparently reduced their alkaloid content. For example, whereas *Echium* species from continental Europe produce PAs in substantial amounts, *Echium* species from the Canary Islands usually sequester only very low amounts of PAs. These few examples, which could be augmented by several population genetics studies, imply that chemical defence can be costly even for autotrophic plants.

2.4 Animal responses: detoxification mechanisms and adaptations

If a herbivore feeds on a plant rich in SM, the following scenarios can be postulated (Rosenthal and Berenbaum, 1991, 1992; Bernays and Chapman, 1994):

1. A herbivore does not taste the allelochemicals, ingests them and is killed immediately, e.g. in the case of an alkaloid-rich plant.
2. A herbivore eats only a small part of the plant and suffers from unpleasant symptoms afterwards. Most vertebrate herbivores can associate both events and will subsequently avoid this plant for feeding.
3. A herbivore does not feed on a single plant species but samples from a wide range of plants. Using this strategy, it will ingest a wide variety of allelochemicals but will avoid an acute intoxication.
4. A herbivore is adapted on a particular plant species (as a food specialist) and can apparently feed on it, although it contains active allelochemicals. Herbivores have evolved degrading enzymes, such as cytochrome p450 enzymes (which make lipophilic xenobiotics more hydrophilic so that they can be eliminated via urine after conjugation with a hydrophilic moiety, such as glucoronic acid). Intenstinal cells but also other tissues (blood endothelia, kidneys) express ABC proteins, which are ATP-driven transporters that can export lipophilic xenobiotics that have entered a cell by free diffusion. A selection of alkaloids which affect CYP and ABC transporters is given in Table 2.20.

In nature, we usually find situations 3 and 4, which provide some protection against herbivores. A plant has a few adapted herbivores, which are often attracted to the noxious chemistry of their host plants, or is afflicted by browsers, which might take a part but not destroy the complete plant. This situation is obviously better than being attacked by a multitude of herbivores, which might devour the whole plant. A number of plants (e.g. with alkaloids), whose constituents interact with important molecular targets, are often avoided even by the casual browser.

On the other hand, a number of adapted herbivores, especially arthropods, are known. This argument is sometimes used to negate the defence function of SM. An example from medicine easily explains why this argument does not count. Our immune system apparently works against the majority of bacteria, fungi and viruses and we are usually unaware of its existence. A few viruses, microbes and parasites have overcome the defence barrier by 'clever' biochemical strategies (e.g. by antigenic variation), so that they escape the immune system and make us sick. Nobody would call the immune system and the antibodies useless because of these few adapted specialists. It is one goal of modern medicine to elucidate the underlying intricate adaptations.

Table 2.20 Detoxification mechanisms of alkaloids mediated by cytochrome p450 and ABC transporters

Alkaloid	Source	Effect
Alkaloids derived from tryptophan		
Ajmalicine, serpentine	*Rauvolfia serpentina* (Apocynaceae)	Substrate of CYP2D6 but not of CYP3A4
Brucine	*Strychnos* sp. (Loganiaceae)	Inducer of CYP2B3
Camptothecin	*Camptotheca acuminata* (Cornaceae)	Substrate for ABC2 transporter in *Botrytis cinerea*; for PMR5 from *Penicillium digitatum*, AtrBp from *Aspergillus nidulans*; induction of MDR overexpression
Cinchondine	*Cinchona pubescens* (Rubiaceae)	P-gp substrate
Cinchonine	*Cinchona pubescens* (Rubiaceae)	Reversal of P-gp mediated drug resistance
Conoduramine	*Peschiera laeta* (Apocynaceae)	P-gp binding
Coronaridine, heyneanine	*Tabernanthe iboga* (Apocynaceae)	Reversal of P-gp mediated drug resistance in vincristine-resistant KB cells
Harmine	*Banisteriopsis caapi* (Malpighiaceae)	Inhibition of CYP2D6; *O*-demethylation by CYP1A1 and CYP2D6
Ibogaine	*Tabernanthe iboga* (Apocynaceae)	*O*-methylation by CYP2D6
Indole-3-carbinol	many Brassicaceae	Downregulation of upregulated P-gp; dietary adjuvant in treatment of cancer with MDR activates CYP1A1
Kopsamine, pleiocarpine Kopsiflorine	*Kopsia dasyrachis* (Apocynaceae)	Reversal of P-gp mediated drug resistance
Quinidine	*Cinchona pubescens* (Rubiaceae)	P-gp substrate
Quinine	*Cinchona pubescens* (Rubiaceae)	Reversal of P-gp mediated drug resistance, P-gp substrate of CYP2D6
Rescinnamine	*Rauvolfia serpentina* (Apocynaceae)	P-gp substrate
Reserpine	*Rauvolfia serpentina* (Apocynaceae)	Substrate for bacterial ABC transporters; reversal of multidrug resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) strains (NorA MDR pump); P-gp substrate
Rutaecarpine	*Evodia rutaecarpa* (Rutaceae)	Reversal of P-gp mediated drug resistance substrate of CYP1A and CYP2B; induction of CYP1A1, CYP1A2, CYP2B, CYP2E1

(*Continued*)

Table 2.20 (*Continued*)

Alkaloid	Source	Effect
Strychnine	*Strychnos* sp. (Loganiaceae)	Inducer of CYP2B1, CYT2B2
Vincristine, vinblastine	*Catharanthus roseus* (Apocynaceae)	Substrate of P-gp; P-gp substrate in BBB; induction of MDR overexpression substrate of CYP3A4
Voacamine	*Peschiera fuchsiaefolia* (Apocynaceae)	Reversal of P-gp mediated multidrug resistance; enhancement of doxorubicin cytotoxicity P-gp substrate in BBB
Alkaloids derived from phenylalanine/tyrosine		
Berbamine	*Berberis* sp. (Berberidaceae)	P-gp substrate in BBB, and in Caco2 cells
Berberine	*Hydrastis canadensis* (Ranunculaceae)	Substrate of P-gp and bacterial ABC transporters; P-gp substrate in BBB, inhibition of CYP1A1; substrate of CYP2C9, CYP2D6, CYP3A4
Boldine	*Peumus boldo* (Monimiaceae)	P-gp substrate
Californine	*Eschscholtzia californica* (Papaveraceae)	*N*-demethylation by CYP3A2; demethylenation by CYP2D1 and CYP2C11
Cephaeline	*Psychotria ipecacuanha* (Rubicaeae)	*O*-demethylation by CYP3A4, CYP2D6
Cepharanthine	*Stephania cepharantha* (Menispermaceae)	Inhibition of P-gp; enhancement of sensitivity of K562 cells towards doxorubicin and vincristine; apoptotic
Chelerythrine	*Zanthoxylum clava-herculis* (Rutaceae)	Reversal of multidrug resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) strains inhibition of CYP1A1; detoxification by CYP1A
Colcemide	*Colchicum autumnale* (Colchicaceae)	Spindle poison; P-gp substrate
Colchicine	*Colchicum autumnale* (Colchicaceae)	Spindle poison; apoptotic; P-gp substrate
Emetine	*Psychotria ipecacuanha* (Rubicaeae)	P-gp substrate; induction of MDR overexpression *O*-demethylation by CYP3A4, CYP2D6
Fangchinoline	*Stephania tetrandra* (Menispermaceae)	Reversal of P-gp mediated drug resistance; P-gp inhibition
Galanthamine	*Galanthus nivalis* (Amaryllidaceae)	Metabolization by CYP2D6 and CYP3A4
Glaucine	*Papaver* sp. (Papaveraceae)	P-gp substrate
Homoharringtonine	*Cephalotaxus harringtonia* (Cephalotaxaceae)	Induction of MDR overexpression, P-gp substrate
Hydrastine	*Hydrastis canadensis* (Ranunculaceae)	Substrate of CYP2C9, CYP2D6, CYP3A4

Table 2.20 (*Continued*)

Alkaloid	Source	Effect
5-Methoxyhydnocarpine	*Hydnocarpus kurzii* (Flacourtiaceae)	Inhibitor of NorA MDR pump in *Staphylococcus aureus*
Palmatine	Ranunculaceae; Berberidaceae	Substrate of bacterial ABC transporters
Protopine	*Eschscholtzia californica* (Papaveraceae)	Demethylenation by CYP2D1 and CYP2C11
Roemerine	*Annona senegalensis* (Annonaceae)	P-gp substrate; reversal of P-gp mediated drug resistance
Sanguinarine	*Sanguinaria canadensis* (Papaveraceae)	Apoptotic; intercalator; reversal of P-gp mediated drug resistance inhibition of CYP1A1, detoxification by CYP1A
Tetrandrine	*Stephania tetrandra* (Menispermaceae)	Reversal of P-gp mediated drug resistance; enhancement of sensitivity of KBv200 cells towards paclitaxel, docetaxel, vinblastine, doxorubicin; P-gp substrate in BBB
Thaliblastine	*Thalictrum* sp. (Ranunculaceae)	Apoptotic, reversal of MDR phenotype
Alkaloids derived from ornithine/arginine		
Clivorine	*Ligularia hodgsonii* (Asteraceae)	Metabolic activation by CYP3A1 and CYP3A2
Monocrotaline	*Crotalaria* sp. (Fabaceae)	CYP3A substrate; CYP oxidation to alkylating dehydromonocrotaline
Nicotine, cotinine	*Nicotiana tabacum* (Solanaceae)	Metabolization by CYP2A6; inhibition of CYP2E1; CYP1A1 inducer
Pervilleine B, C, F	*Erythroxylum pervillei* (Erythroxylaceae)	Reversal of P-gp mediated drug resistance in KB-V1 cells and in NCr n/nu mice
Retrorsine	*Senecio* sp. (Asteraceae)	Induction of CYP1A1, 1°2, 2EI, 2B1/2
Senecionine	*Senecio* sp. (Asteraceae)	CYP2B and CYP3A4 oxidation to alkylating dehydroderivatives
Alkaloids derived from lysine		
Lobeline	*Lobelia* sp. (Campanulaceae)	Pg-p substrate
Tetrahydrorhombifoline	*Lupinus* sp. (Fabaceae)	Pg-p substrate
Sparteine	*Cytisus scoparius* (Fabaceae)	Metabolization by CYP
Alkaloids with a terpenoid backbone		
Aconitine	*Aconitum napellus* (Ranunculaceae)	P-gp substrate
Cyclopamine	*Veratrum album* (Liliaceae)	Apoptotic; inhibitors of P-gp; reversal multidrug resistance in NCI AdrR cells to adriamycin and vinblastine

(*Continued*)

Table 2.20 (*Continued*)

Alkaloid	Source	Effect
Lycaconitine	*Aconitum pseudo-laeve* (Ranunculaceae)	MDR inhibitor in nKBV20c cells
Paclitaxel	*Taxus* sp. (Taxaceae)	P-gp substrate; induction of MDR overexpression, CYP3A substrate
Tomatidine	*Lycopersicon esculentum* (Solanaceae)	Inhibitor of P-gp; reversal multidrug resistance in NCI AdrR cells to adriamycin and vinblastine
Miscellaneous alkaloids		
Caffeine	*Coffea arabica* (Rubiaceae)	Demethylation by CYP1A
Capsaicin	*Capsicum frutescens* (Solanaceae)	P-gp substrate
Dipiperamide A, B, C, D, E	*Piper nigrum* (Piperaceae)	Inhibition of CYP3A4
Pilocarpine	*Pilocarpus jaborandi* (Rutaceae)	Substrate of CYP2A6; CYP2B
Piperine	*Piper nigrum* (Piperaceae)	Inhibition of CYP activity and CYP induction

Source: After Wink (2007a).

A similar argument also applies for plant SM. The question is therefore: What are the mechanisms which enable a herbivore to feed on chemically defended plants? Because there are so many herbivores, generalizations are difficult and dangerous but a few general strategies can be observed (Harborne, 1993; Bernays and Chapman, 1994). Many insects and vertebrates have evolved highly potent degrading enzymes, which can hydrolyze or hydroxylate (among them inducible cytochrome P_{450} hydroxylases) a compound taken up from the gut (Table 2.20). In a second step, these compounds are conjugated with glucuronic acid, glutathione or sulphate, so that they become more water soluble. In vertebrates, these conjugates are then excreted via the bile duct or more commonly via the kidneys. A detoxification of defence chemicals by rumen or gut microbes has also been discussed. Experiments with QAs suggest that they are not degraded by rumen microorganisms (Aguiar and Wink, 2005b). A short intestinal transition time or the ingestion of clay, which can adsorb dietary toxins (geophagy), can be additional mechanisms that have been observed in some herbivores. The invention of ABC transporters which can export lipophilic toxins from cells (Pg-p, MDR) is another line of defence mechanisms common in herbivores (Möller *et al.*, 2006; Ma and Wink, 2008).

Quite a number of insect herbivores but only few vertebrates have adapted to the defence chemistry of a given host plant in a particularly close way (for overviews, see Blum, 1981; Meinwald, 1990; Harborne, 1993; Bernays and Chapman, 1994; Brown and Trigo, 1995; Hartmann and Witte, 1995;

Braekman *et al.*, 1998; Roberts and Wink, 1998; Eisner *et al.*, 2005). As a first step in adaptation, these organisms must develop an insensitivity or tolerance against the dietary toxin. The underlying mechanisms are understood for only a few species. In the case of *Manduca sexta* and other Sphingidae that can feed on alkaloid rich plants, a very active degradation and excretion system appears to be the main mechanism. In the case of the monarch butterfly (*D. plexippus*), whose larvae feed on *Asclepias* plants that are rich in cardenolides, it was discovered that its Na^+, K^+-ATPase (the molecular target of cardenolides) is insensitive to cardiac glycosides. Molecular analysis revealed that an amino acid of the ouabain binding site is exchanged, so that cardiac glycosides can no longer bind (Holzinger and Wink, 1996). This insect can now tolerate cardiac glycosides; however, adaptations are even more advanced, since *Danaus* can actively sequester cardenolides and store them in its integument as acquired defence compounds against predators. A similar strategy has been observed for the sequestration of cardiac glycosides in aphids, bugs and Lepidoptera, for PAs in beetles and Lepidoptera (for reviews, see Meinwald, 1990; Brown and Trigo, 1995; Hartmann and Witte, 1995) and for QAs in aphids and moths (Wink, 1992). In the case of some arctiid moth, the acquired PAs serve as a precursor for pheromones, which are dissipated from coremata that are only developed if the larvae feed on PAs (Schneider *et al.*, 1982). While females transport PAs to the eggs, the male can also contribute to the chemical defence of its clutch by transferring PAs with its spermatophore (as a 'nuptial gift').

Insects which defend themselves chemically, often bear warning colouration (aposematism) to advertise their inpalatability to potential predators. A fascinating coevolution has been observed in several groups of insects, which show the same colouration but do not sequester the corresponding toxins. These mimics, nevertheless, gain protection by this strategy. The few examples given show the intricate and complex interactions that can be encountered in insects that have adapted to the defence chemistry of their host plants (Wink, 1999a; for further discussions, see also Chapter 3 in this book).

2.5 Concluding remarks

Although relatively few of the several tens of thousands of known SM have been analyzed biochemically or ecologically in any detail, we can nevertheless generalize that many of them are allelochemicals that serve as defence compounds against herbivores or microorganisms. Many others are signal compounds and a few may be without obvious function.

If studied in detail, it is usually possible to define the mode of action of each allelochemical and, in particular, the molecular target with which it interferes. Knowing the targets and the functional groups of the active molecule, the toxic or pharmacological effects observed can generally be explained. This knowledge will help in understanding the role of these compounds in

nature but is also useful for the exploitation of these active components in medicine or agriculture. Thus, chemical ecology and biotechnology share a common evolutionary base. Given the richness of structures and potential targets which might have been selected during evolution, 'bioprospection' in combination with chemical ecology, pharmacology and medicine will remain an interesting topic for science in the future.

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Chapter 3

CHEMICAL DEFENCE IN MARINE ECOSYSTEMS

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Abstract: Nature has provided a broad arsenal of structurally diverse and pharmacologically active compounds that serve as highly effective drugs or lead structures for the development of novel drugs to combat a multitude of diseases. Traditionally, terrestrial organisms represent the richest source of natural drugs. Considering the fact that over 70% of the surface of the earth is covered by oceans that harbour a rich biodiversity, aspiration in marine bioprospecting as a viable counterpart for the discovery of bioactive compounds from the terrestrial environment seems justified. Interestingly, the majority of marine natural products involved in clinical or preclinical trials is produced by invertebrates, which is in contrast to compounds derived from the terrestrial environment where plants by far exceed animals with respect to the production of bioactive metabolites. The fact that bioactive metabolites are predominantly found in sessile or slow-moving marine organisms that lack physical defence structures thus appears to reflect the ecological importance of these compounds with respect to inter- as well as intraspecific interactions, for example predation, competition for space and fouling. Numerous natural products from marine invertebrates show striking structural similarities to known metabolites of microbial origin. This fact suggests that microorganisms such as bacteria and microalgae that often live associated with marine invertebrates are at least involved in the biosynthesis or are in fact the true sources of these respective metabolites in many cases.

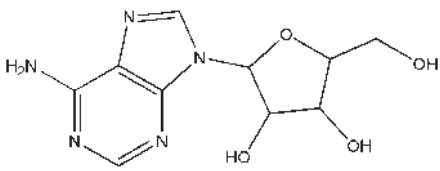
Keywords: marine natural products, allelopathy, fouling, endosymbionts

3.1 Introduction

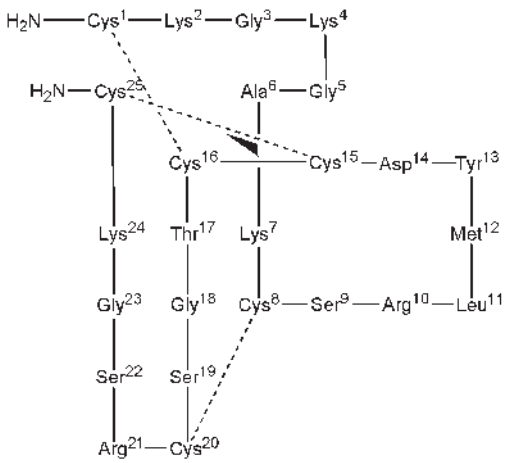
Even though close to 21 000 different marine natural products have been isolated until now (MarinLit, 2009), this scientific field is still young when compared to the long tradition of natural product research in the terrestrial habitat. The isolation of sizeable amounts of prostaglandin derivatives from the Caribbean gorgonian, *Plexaura homomalla*, by Weinheimer and Spraggins

in 1969 is usually considered as the starting point of marine chemistry even though the initial spark dates back as early as the 1950s with the discovery of unusual nucleoside derivatives from the sponge *Tethya crypta* (Bergmann and Feeney, 1951). These compounds later on served as a model for the development of modern nucleoside drugs such as Ara-A (**1**) for the chemotherapy of viral infections. Interestingly, the synthetic product Ara-A was later isolated in 1995 by De Rosa *et al*. from the gorgonian *Eunicella clavolini*. Since these early times, interest in marine natural products has constantly grown fuelled mainly by the search for 'drugs from the sea' as the early discoveries of bioactive compounds from the sea had aroused considerable hope in marine bioprospecting as a counterpart for such endeavours on land. Given the fact that over 70% of the surface of the earth is covered by oceans and considering furthermore the rich biodiversity of the sea (several phyla of the animal kingdom such as the Porifera and Bryozoa are exclusively aquatic) such hopes seem indeed justified. In fact, marine organisms have in recent decades yielded a plethora of structurally unprecedented secondary metabolites that are unknown from the terrestrial habitat and hence constitute an important library of new natural products for drug discovery. Presently, close to 20 different marine natural products or natural product derivatives are in clinical studies, most of them in the field of cancer but also in other areas such as inflammation (Proksch *et al*., 2006). Recently, the peptide ziconotide (**2**) which represents a toxin from marine *Conus* snails was introduced into

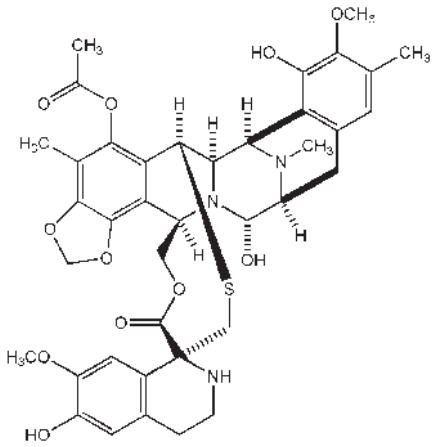
1 Ara-A



2 Ziconotide



3 ET-743



the drug market as a new analgesic under the trade name 'Prialt'. Other compounds such as the potential new anticancer drug ET-743 (**3**) from the marine tunicate *Ecteinascidia turbinata* are in late stages of clinical trials and could enter the drug market already in the near future (Proksch *et al.*, 2006).

The high incidence of strongly bioactive compounds especially in those marine organisms that are sessile or slow moving and that lack physical defences (such as algae and most marine invertebrates) has furthermore served as a stimulus for studies on the ecological functions of these metabolites which commenced in the 1970s at a time when the general perception of the 'raison d'ètre' of secondary products changed from the traditional view as 'waste products' to the current appreciation of their ecological functions (it should not be forgotten, however, that 'chemical ecology' is no invention of the late twentieth century but reaches back more than 100 years to the pioneering work of Stahl (1904) that was sadly ignored by his successors in the field). Since then the vital roles that natural products may play in inter- as well as intraspecific interactions in the marine as well as in the terrestrial environment such as intraspecific signalling (pheromones), deterrence of herbivores and predators, suppression of competing neighbours, inhibition of bacterial and fungal infections, protection against harmful ultraviolet radiation and others have been documented by numerous studies and the ecological importance of secondary metabolites is now generally accepted.

Whereas the major threats against which organisms have to defend themselves are very similar regardless of the physical differences of their environment (marine or terrestrial), there is nevertheless one clear difference: in the terrestrial habitat, higher plants have been most ingenious with regard to the biosynthesis and accumulation of secondary metabolites, whereas in the marine environment this role is taken over by the invertebrates such as sponges, tunicates, molluscs and others that elaborate a more complex chemical diversity of metabolites than encountered in algae. Hence, this chapter focuses

on the chemical ecology of marine invertebrates even though examples from algae are also covered. Given the complexity of ecological functions of marine natural products that have been reported in recent years (especially since the first version of this chapter [Proksch, 1998] went into print), any treatise on marine chemical ecology is bound to be selective. This is also true for the present chapter which focuses on the roles of natural products for allelopathy, defence against fouling and consumers as well as on the contribution of marine microorganisms for the biosynthesis of secondary metabolites recovered from invertebrates. Readers who wish to engulf more deeply into this field are referred to other recent reviews in this field (Paul and Puglisi, 2004; Paul *et al*., 2006; Proksch *et al*., 2006).

3.2 Marine natural products in allelopathic interactions

Competition for space is generally intense on marine hard bottom substrates but appears to be most pronounced on tropical coral reefs, which are characterized by an exceedingly high species diversity and remarkable population density, unmatched in any other marine ecosystem (Jackson and Buss, 1975; Jackson, 1977; Branch, 1984; Porter and Targett, 1988; Sale, 1991). Given the high incidence of toxic natural products that have been isolated from marine algae (e.g. Cembella, 2003 and references therein) and especially from marine invertebrates, such as sponges (e.g. Sarma *et al*., 1993; Kelmann *et al*., 2001; Martí *et al*., 2003; Selvin and Lipton, 2004), as well as the bare zones observed around some sponges in their natural habitat (Porter and Targett, 1988; Turon *et al*., 1996a), allelopathic effects through biosynthesis and exudation of toxic secondary metabolites appear, indeed, to play a crucial role in structuring benthic marine ecosystems.

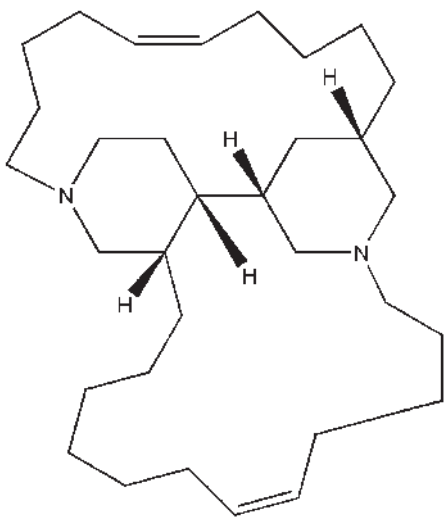
Aquatic photoautotrophs often face severe competition for resources, namely for light, space and nutrients (Gross, 2003). Marine macrolagae are known to prevent epiphyte growth by allelopathic mechanisms (Harlin, 1987; Young Cho *et al*., 2001), exhibit antifouling activity against bacteria and fungi (Hellio *et al*., 2000) and can inhibit germination and development of algae and invertebrates (Nelson *et al*., 2003; Jin *et al*., 2005). Several bloom-forming microalgae may dominate phytoplankton not only because they produce feeding deterrents, but also because of allelopathic interactions with other plankton species (Arzul *et al*., 1999; Legrand *et al*., 2003; Kubanek *et al*., 2005) including planktonic invertebrates (Ianora *et al*., 2004; Pohnert, 2005). Despite the vast number of bioactive secondary metabolites isolated from cyanobacteria (Pietra, 1997; Singh *et al*., 2005), little is known about allelopathic interactions (Gross, 2003).

Proof of allelopathy is one of the great challenges of marine chemical ecology (Cembella, 2003). In the case of marine invertebrates, most of the experiments conducted in order to prove the significance of allelochemicals in competition for space have employed crude extracts (Porter and Targett, 1988; Turon *et al*., 1996b; Engel and Pawlik, 2000; De Voogd *et al*., 2004; Lages

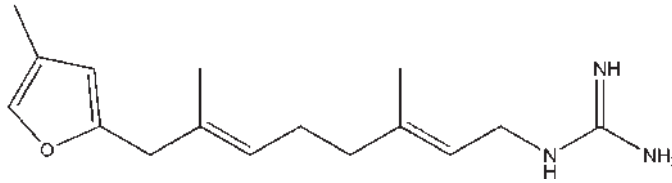
et al., 2006). As an example, De Voogd and co-workers (2004) demonstrated specificity of allelochemically mediated interactions of four species of Indonesian sponges causing tissue necrosis in corals. It was demonstrated that the same sponge individual could cause tissue necrosis in particular neighbouring corals, but not in others (De Voogd *et al*., 2004). The disproportionately frequent association of the Great Barrier reef sponge *Haliclona* sp. 628 which bears nematocysts and zooxanthellae with dead patches of the coral *Acropora nobilis* was explained by sponge larvae settling on and then killing coral tissue, since necrosis of live corals has been observed within a 1 cm radius of this sponge (Garson *et al*., 1999; Russell *et al*., 2003).

The compounds responsible for the claimed allelopathic effects, however, have rarely been isolated and characterized. A notable exception is a recent study by Green and co-workers (2002) showing that haliclonacyclamine A (**4**) isolated from the above-mentioned nematocyst-bearing sponge *Haliclona* sp. 628 induced settlement of larvae of the ascidian *Herdmania curvata*, but subsequently inhibited completion of metamorphosis, resulting in the death of post-larvae. Another example is that of burrowing sponges from the genus *Siphonodictyon*, which burrow into the heads of living corals. Sullivan and coworkers (1981, 1983) demonstrated in elegant experiments that overgrowth and thus killing of *Siphonodictyon coralliphagum* by corals (e.g. *Acropora formosa*) is prevented by exudation of the toxic secondary metabolite, siphonodictidin (**5**), which suppresses photosynthesis of the coral's zooxanthellae and, thereby, coral growth, even at concentrations as low as 10 ppm. Interestingly, the related sponge, *S. mucosa*, which burrows into dead corals (in contrast to the above-mentioned *S. coralliphagum*), lacks the toxic siphonodictidin, thereby corroborating the proposed ecological significance of the respective secondary metabolite.

4 Haliclonacyclamine A

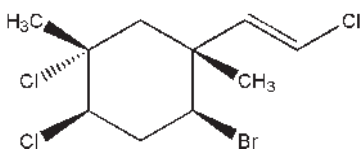


5 Siphonodictidin



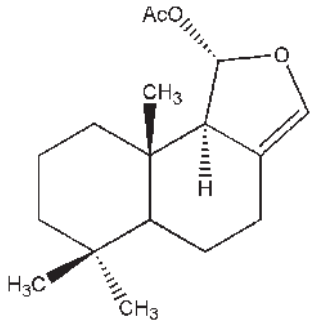
Allelopathic effects are not restricted to competition of marine algae or invertebrates with each other but are apparently also of importance in interactions between these two classes of organisms. The red alga, *Plocamium hamatum* present on reefs of North Queensland, was found to cause tissue necrosis of several marine invertebrates when in physical contact with the alga (De Nys *et al*., 1991). Since allelochemicals, especially the algal secondary metabolite chloromertensene (**6**), were suspected of involvement in this interaction, a series of field experiments was conducted that included *P. hamatum* and the soft coral, *Sinularia cruciata*. Healthy algae and soft corals were relocated on mesh grids. In one set of experiments, individuals of both taxa were brought into physical contact, whereas in the second set of experiments, algae and soft corals were kept in a non-contact situation. Only those soft corals that had physical contact with red algae developed tissue necrosis, indicating that the suspected allelochemicals are not waterborne but act upon physical contact. In further experiments, the algal metabolite chloromertensene was coated onto 'artificial algae', which were again brought into physical contact with the soft corals. In all cases where coated 'algae' were in contact with *S. cruciata*, the soft corals exhibited tissue necrosis, whereas contact with uncoated 'algae' merely caused abrasion but not necrosis (De Nys *et al*., 1991). In addition to its allelopathic effects, coating with chloromertensene inhibited fouling of the artificial 'algae' as well as predation, suggesting multiple ecological roles for this natural product.

6 Chloromertensene



Another example of allelopathic interactions between marine sponges includes two hitherto undescribed sponges of the genera *Dysidea* and *Cacospongia*, that co-occur on reefs of the tropical island, Guam. *Dysidea* sp. was frequently observed to overgrow adjacent specimens of *Cacospongia* sp. and to cause necrosis of the latter sponge (Thacker *et al*., 1998) When crude extracts of *Dysidea* sp. or its major secondary metabolite, 7-deacetoxyolepupuane (**7**), were incorporated into agar strips and placed in contact with *Cacospongia* sp. in the field, typical tissue necrosis of the latter was observed. This suggests that natural products are involved in this allelopathic interaction and are probably the major reason for the success of *Dysidea* sp. over *Cacospongia* sp.

7 Deacetoxyolepupuane



The study of *Dysidea* sp. and *Cacospongia* sp., as well as most other studies of marine allelopathy, have not unequivocally established whether the compounds suspected to be involved in allelopathy are present on the surface of the aggressor or are exuded upon contact with other species. However, the tissue-specific localization of any natural product suspected to be involved in allelopathy is of critical importance, since only those compounds that come into contact with other organisms can realistically be expected to be of ecological significance in any scenario involving competition for space.

3.3 Chemical defence against fouling

Fouling is a biological phenomenon ubiquitously observed in the marine environment. In 1952, Woods Hole Oceanographic Institution referred to biofouling as growth of animals and plants on artificial submerged surfaces. The process of fouling is, however, not restricted to artificial man-made substrates but extends also to all kinds of natural surfaces such as rocks, reefs, driftwood as well as the surfaces of marine animals and algae. Fouling organisms include microorganisms (e.g. bacteria, diatoms and protozoa) that constitute the so-called 'primary film' as well as macroorganisms like macroalgae and invertebrates (Bakus *et al*., 1986, 1990; Melton and Bodnar, 1988; Davis *et al*., 1989; Clare, 1996a,b; Ortlepp *et al*., 2007). Of the latter, mussels (*Mytilus* spp.) as well as barnacles (*Balanus* sp.) are especially important, also in an economic sense since they settle and grow on hulls of ships, cooling systems and similar man-made substrates thereby causing large annual losses reaching billions of dollars due to example to higher fuel consumption of ships that are slowed down by fouling organisms growing on their hulls (Rascio, 2000). Also for filter-feeding invertebrates, fouling may be disadvantageous or even dangerous since inhalant canals may be blocked by epibionts resulting in a reduced feeding capacity. Furthermore, epibionts may compete with their hosts for food.

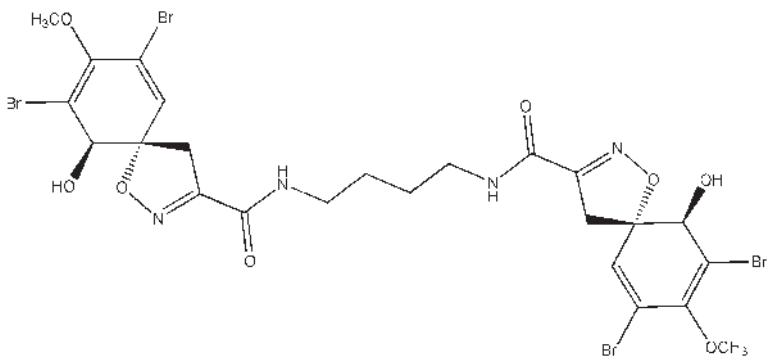
In spite of the omnipresence of fouling organisms in the marine environment, the surfaces of many marine invertebrates such as sponges are often remarkably free at least of macrofouling organisms giving rise to the hypothesis that these invertebrates produce and secrete antifouling compounds that protect them from being overgrown. Given the high incidence of antibiotic,

cytotoxic or otherwise biologically active compounds that are known from sponges and other marine invertebrates as well as algae (Blunt *et al*., 2006, 2007; Altmann and Gertsch, 2007) this assumption seems indeed plausible. In recent years, numerous marine natural products with inhibitory activity towards epibionts have been identified from marine microorganisms, algae and invertebrates alike (Fusetani, 2004). These natural compounds provide valuable leads for the development of new antifouling ingredients for marine paints and coatings as there is an urgent need to replace the commonly used organo tin (TBTO), copper oxide and herbicide coatings that are still used today even though they are toxic to the aquatic environment (Alzieu *et al*., 1986, 1989; Ortlepp *et al*., 2007). These toxic ingredients were recently banned by the International Maritime Organization (IMO) in a resolution that called for a stepwise reduction of the use of organotin compounds by 2003 and complete prohibition by 2008 (IMO, 2001; Ortlepp *et al*., 2007). Thus it is hoped that natural products may provide environmentally friendly alternatives to these toxic ingredients.

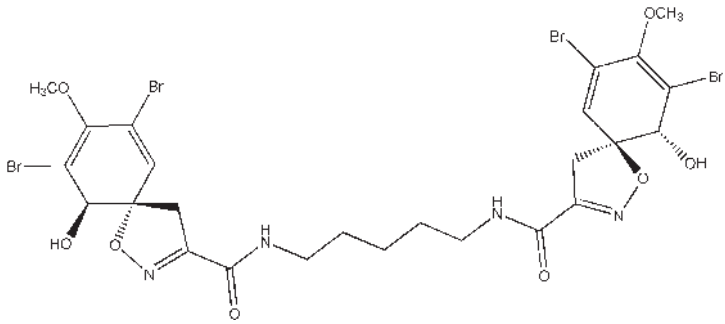
Whereas it is clear that marine organisms hold a plethora of new and biologically active compounds with antifouling activity (Fusetani, 2004), unequivocal proof of their ecological significance with regard to the suppression of fouling is missing in most cases. The mere presence of such compounds in marine organisms is not a hint per se in favour of their ecological function as it is unclear where these compounds are stored and whether at all they get in contact with epibionts. For this purpose, surface allocation of the respective compounds and/or exudation would have to be demonstrated in addition to antifouling activity. In most publications on this topic, this evidence is lacking.

One of the first studies proving an ecological function of antifouling compounds demonstrated that the sponge *Aplysina fistularis* exudes the brominated isoxazoline alkaloids aerothionin (**8**) and homaerothionin (**9**) into the surrounding sea water at rates of 8.9×10^{-3} to 7.7×10^{-4} μg/min/g (Walker *et al*., 1985). Physiologically relevant concentrations of both compounds had earlier been shown to prevent settlement of fouling organisms on *A. fistularis* (Thompson *et al*., 1985). Following mechanical injury of the sponge tissue which mimics attack by predators, a strong acceleration of the rate of exudation by the factor of 10–100 was observed (Walker *et al*., 1985).

8 Aerothionin

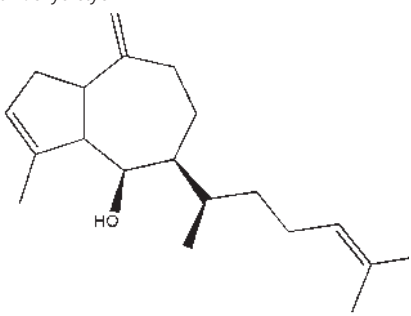


9 Homoaerothionin

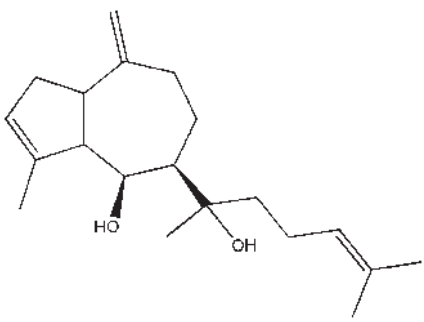


Antifouling constituents are also known from algae such as the brown alga *Dictyota menstrualis*, which appears to be less frequently covered by fouling organisms than co-occurring algae from other taxa (Schmitt *et al*., 1995). Laboratory assays indicated that the bryozoan, *Bugula neritina*, which was used as a model for epibionts, did not settle on surfaces of *D. menstrualis*. It was found that rejection of the alga by the bryozoan occurred after physical contact with the algal surface and was not mediated through waterborne signals. Analysis of a crude lipophilic extract obtained after rubbing the surface of *D. menstrualis* yielded the known diterpenes, pachydictyol A (**10**) and dictyol E (**11**). When exposed to these compounds, larval mortality and abnormal larval development increased significantly, suggesting that both compounds are the causative agents for the observed antifouling effects.

10 Pachydictyol A



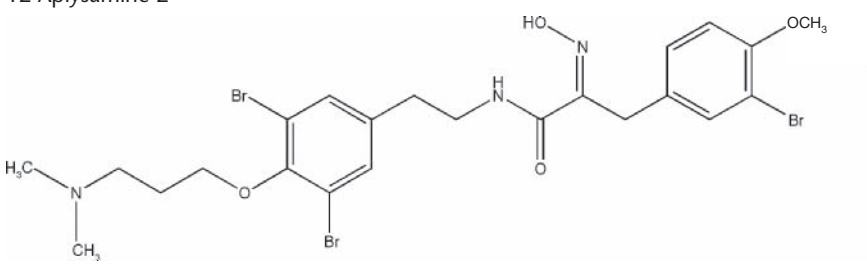
11 Dictyol E



There is growing evidence that bacterial communities that live on the surfaces of marine sponges are involved in the inhibition of other bacteria that are not members of sponge-specific bacterial community. Surface-sterilized colonies of the marine sponge *Mycale adhaerens* were colonized by taxonomically different bacteria than sterilized polystyrene dishes over a 7-day period (Lee and Qian, 2004). Whereas 50% of the bacterial strains attached to the polystyrene dishes proved to be susceptible to an *M. adhaerens* extract, none of the bacteria that had settled on the sponge surface were affected. Moreover, extracts from surface bacteria of the sponge were inhibitory to bacteria settling on the polystyrene dishes suggesting that sponge-associated bacteria are possibly involved in the defence of their hosts against other non-adapted bacteria. A further study by Dobretsov *et al*. (2004) likewise indicates that bacteria which are naturally associated with the surfaces of sponges are not affected by natural products secreted by their sponge hosts, whereas other potential fouling organisms such as the hydroid *Hydroides elegans* or the diatom *Nitzschia paleacea* are.

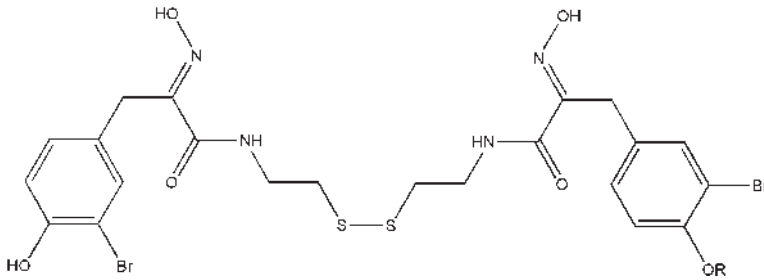
The sponge *Ianthella basta* is the source of a series of structurally unique compounds, the bastadins, which may be either linear or cyclic and consist of several brominated tyrosine and tyramine units that are linked to each other by peptide and diphenyl ether bonds. Moreover, the bastadins exhibit oxime substituents that are rarely found in natural products. Several bastadin derivatives proved to inhibit settlement of *Balanus improvisus* larvae at concentrations of 1–10 μM in laboratory assays (Ortlepp *et al.*, 2007). Similar activities were observed for other oxime-bearing sponge metabolites such as aplysamine-2 (**12**) isolated from *Pseudoceratina purpurea* and for psammaplin A (**13**) from *Aplysinella rhax*, whereas other brominated sponge-derived natural products that lacked oxime groups and included several bromopyrrol alkaloids and isoxazoline derivatives showed no inhibition of barnacle settling (Ortlepp *et al.*, 2007). Hemibastadin-1 (**14**) due to its simplified structure when compared to the larger bastadins was selected for preparation of synthetic analogues and subsequent structure activity studies. Three synthetic congeners including debromohemibastadin-1 (**15**), 2,2′-dibromohemibastadin-1 (**16**) and tyrosinyl-tyramide (**17**) were prepared. Hemibastadin-1 and its 2,2′-dibromoderivative were essentially comparable with regard to the suppression of barnacle settlement. Debromohemibastadin-1 was less active and tyrosinyl-tyramide was completely inactive (Ortlepp *et al.*, 2007). This

12 Aplysamine-2



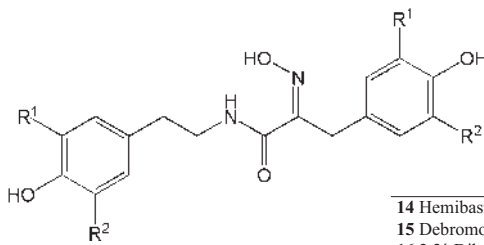
comparative study demonstrates that presence of an oxime group is an essential prerequisite, whereas bromine atoms enhance antifouling activity. Interestingly, ianthelline (**18**) which is another oxime bearing bromotyrosine derivative isolated from the Caribbean sponge *Ailochroia crassa* was recently shown to inhibit bacterial attachment (Kelly *et al*., 2005; Ortlepp *et al*., 2007).

13, 48



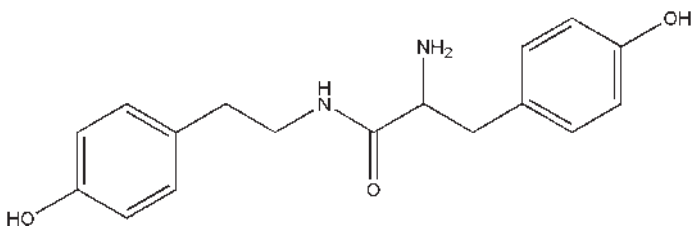
	R
13 Psammaplin A	H
48 Psammaplin A sulphate	SO_3^-

14, 15, 16

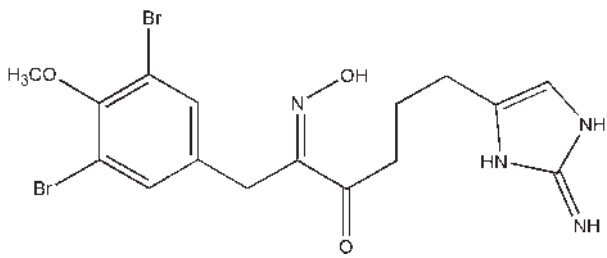


	R^1	R^2
14 Hemibastadin-1	Br	Br
15 Debromo-hemibastadin-1	H	H
16 2,2′-Dibromochemibastadin-1	Br	Br

17 Tyrosinyl-Tyramide



18 Ianthelline



One of the best documented and understood examples of the ecological significance of antifouling marine natural products involves the red alga *Delisea pulchra.* The algae produce halogenated furanones that interfere with bacterial colonization of the algal surface (Maximilien *et al.*, 1998; Paul *et al.*, 2006). The algal metabolites which accumulate in cells at the surface of *D. pulchra* are structurally similar to bacterial acylated homoserine lactones which act as chemical signals, bind to LuxR receptor proteins in gram-negative bacteria and regulate swarming and biofilm formation (Manefield *et al.*, 2002; Steinberg *et al.*, 2002; Steinberg and De Nys, 2002). By competing with the physiological acylated homoserine lactone ligands at the bacterial receptors, the furanones prevent biofilm formation at the algal surface which is a prerequisite for settlement of fouling macroorganisms (Melton and Bodnar, 1988). Bacterial biofilm formation does not only occur in the marine environment as part of biofouling but causes also considerable problems for human health by plugging catheters or colonizing the surfaces of artificial joints (Morales *et al.*, 2004; Ramage *et al.*, 2006). It is of interest to note that halogenated furanones and similar metabolites that are able to interfere with bacterial quorum sensing are explored as a possible mechanism to control biofilm formation in humans (Hentzer *et al.*, 2003; Paul *et al.*, 2006).

3.4 Chemical defences of marine invertebrates and algae against consumers

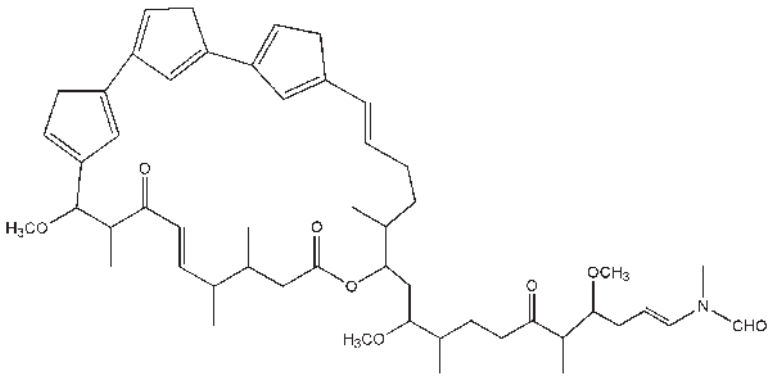
Numerous studies of marine natural products derived from various invertebrates as well as from algae have indicated the significance of these metabolites in chemical defence against predatory or herbivorous fish. Consumer pressure is high in marine ecosystems but especially pronounced on tropical coral reefs, where fish have been estimated to bite the bottom in excess of 150 000 times/m^2/day (Carpenter, 1986). Mainly, the intense predation and herbivory by fishes in tropical regions is thought to have resulted in high selection for noxious and toxic chemical compounds in tropical marine organisms (Bakus and Green, 1974; Green, 1977; Hay and Fenical, 1988). Based on early investigations of toxicity of marine invertebrates, the latitudinal hypothesis was suggested, predicting an inverse correlation between the incidence of chemical defence and latitude (Bakus and Green, 1974). However, this simplistic hypothesis has come into question since pronounced within-region variance has been found (Bolser and Hay, 1996). Moreover, several studies proved toxicity of Antarctic marine invertebrates to be comparable to those of tropic invertebrates (McClintock, 1987; Avila *et al.*, 2000; McClintock *et al.*, 2003, 2004). In northern high latitudes, however, the incidence of feeding-deterrent compounds present in marine invertebrates

appears to be lower than in tropical and Antarctic species (Lippert *et al.*, 2004).

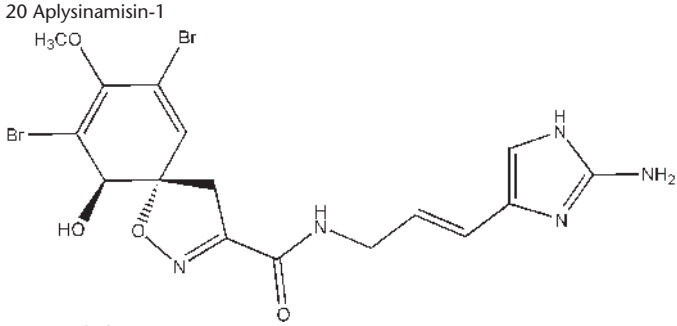
Early investigators in the field primarily used laboratory assays in order to assess feeding-deterrent or toxic properties of marine natural products (e.g. Braekman and Daloze, 1986). In some of these studies, freshwater rather than marine fish were employed as test organisms. Whereas these studies yielded interesting insights into the general toxicity of marine natural products and sometimes also into their mode of action (Groweiss *et al.*, 1983), the ecological significance of the data obtained is often questionable. More recent studies on chemical defence of invertebrates or algae against fish focus, therefore, on field rather than laboratory assays and employ naturally occurring assemblages of consumers (e.g. Meyer and Paul, 1995; Pawlik *et al.*, 1995; Wulff, 1997).

In some cases, the origin of fish-deterrent natural products in marine invertebrates can be traced through the food chain. For example, sponges belonging to the genus *Halichondria* were traced as the dietary source of macrolide oxazole alkaloids detected in the nudibranch mollusc, *Hexabranchus sanguineus* (Kernan *et al.*, 1988), also known as 'Spanish dancer' due to its bright colouration. Even though lacking a protective shell, the soft-bodied nudibranchs are rejected by Indo-Pacific reef fish as well as by other potential predators, such as the hermit crab *Dardanus negistos* (Pawlik *et al.*, 1988). Macrocyclic oxazole alkaloids, such as halichondramide (**19**), were traced as the major feeding-deterrent compounds present in the nudibranch, where they are concentrated in the most vulnerable parts, such as the dorsal mantle or the conspicuous egg ribbons (Pawlik *et al.*, 1988). Sponges of the genus *Halichondria* could ultimately be traced as the true sources of the defensive metabolites of *H. sanguineus*. Like other nudibranchs that are morphologically defenceless, the 'Spanish dancer' has become specialized to feed on chemically protected marine invertebrates, such as sponges, that are largely unpalatable to other generalist consumers. In this trophic interaction, nudibranchs frequently sequester the defence compounds of their prey (Proksch, 1994). Another example for this kind of trophic interaction is provided by the Mediterranean slug *Tylodina perversa* that feeds almost exclusively on the sponge *Aplysina aerophoba* and selectively sequesters its deterrent secondary metabolites (Thoms *et al*., 2003a). The alkaloid spectrum of hepatopancreas tissue resembles that of the sponge prey *A. aerophoba*, with aplysinamisin-1 (**20**) being the major component, whereas in egg masses and mantle tissue, aerophobin-2 (**21**) is the dominant alkaloid, demonstrating that *T. perversa* sequesters these alkaloids in an organ-specific manner (Thoms *et al*., 2003a). Since chemical defences of sea slugs have been investigated extensively, there is a vast body of literature on specialized trophic relationships between sea slugs and invertebrates as well as algae (e.g. review by Cimino and Ghiselin, 1998, 1999; Cimino and Gavagnin, 2006; Thoms *et al.*, 2006b).

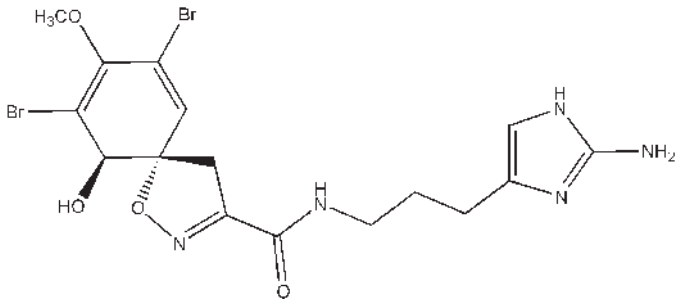
19 Halichondramide



20 Aplysinamisin-1



21 Aerophobin-2

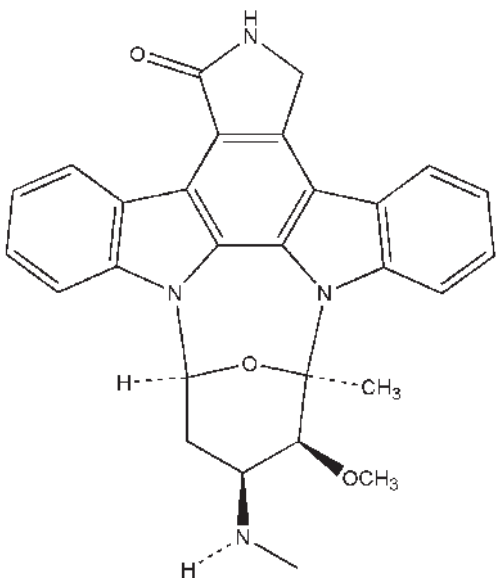


Numerous studies have demonstrated that chemical defences protect potential prey organisms from predation by repelling predators. However, few of these investigated the mechanisms by which predators are affected. A recent study demonstrated that chemical defences may act in a more complex way than palatability assays of prey-derived compounds may suggest. Kicklighter and co-workers (2005) examined the chemical defence of sea hares (*Aplysia californica*) against spiny lobsters (*Panulirus interruptus*). When attacked, individuals of *A. californica* release defensive secretions from ink and opaline glands, facilitating the escape of sea hares by acting through a combination of mechanisms. Ink stimulates appetitive and ingestive

behaviour, opaline can elicit appetitive behaviour but can also inhibit ingestion and evoke escape responses, and both stimulate grooming (Kicklighter *et al.*, 2005). The authors suggest that these secretions function by 'phagomimicry', in which ink–opaline stimulates the feeding pathway to deceive spiny lobsters into attending a false food stimulus, and by sensory disruption, in which the sticky and potent secretions cause high-amplitude, long-lasting chemo–mechano-sensory stimulation. In addition, opaline contains a chemical deterrent opposing appetitive effects. Thus, this study demonstrates a complex interplay of different antipredatory mechanisms.

Sequestration of defensive compounds is not restricted to sponge-feeding nudibranchs but also extends to other marine invertebrates. In the mangrove area of the Micronesian island Truk, the marine flatworm, *Pseudoceros concineus* was observed feeding on the ascidian *Eudistoma toealensis*. Despite the lack of morphological defence mechanisms, neither the ascidians nor the flatworms were attacked by fish, suggesting that both organisms were chemically defended. By incorporating crude extracts from the ascidians or from the flatworms into artificial food at physiological concentrations and offering these treated food pellets to a natural fish assemblage, this hypothesis was corroborated. The treated food pellets were largely avoided, whereas control food was readily consumed (Schupp *et al.*, 1999a). Bioassay-guided fractionation of the respective extracts yielded several staurosporine derivatives as the active constituents responsible for the fish-deterrent effects observed. Staurosporine (**22**) is originally known as a bacterial metabolite produced by actinomycetes such as *Saccharothrix aerocolonigenes* subsp. *staurosporea* (formerly known as *Streptomyces staurosporeus*) or by other microorganisms.

22 Staurosporine



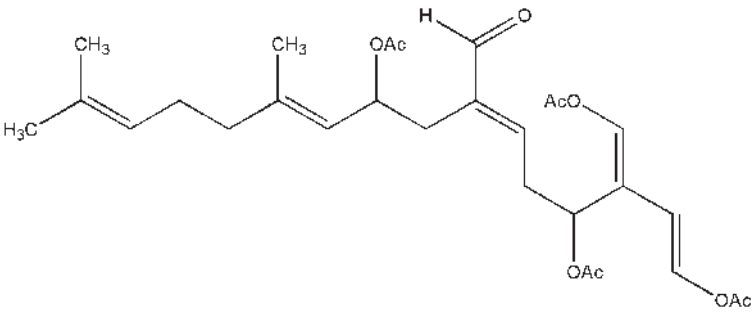
The fact that chemical defence can have a significant impact on the structure of marine habitats has been demonstrated by a recent study. Lages and co-workers (2006) showed that defensive chemicals can facilitate the invasion of alien species in a marine environment. Their experiments revealed that the Indo-Pacific alien soft coral *Stereonephthya* aff. *curvata* possesses an efficient chemical defence against fishes and causes tissue necrosis of the Brazilian endemic gorgonian *Phyllogorgia dilatata*, thus facilitating the expansion of the invasive species. The authors conclude that defensive chemicals can be used to predict the potential invasiveness of introduced species (Lages *et al*., 2006).

As in marine invertebrates, natural products play an important role in protecting marine algae from herbivores, although marine plants have apparently been less 'ingenious' in diversifying their chemical armoury than marine invertebrates (Blunt *et al*., 2006, 2007 and preceding reviews). The majority of natural products isolated from marine macroalgae are terpenoids, polyketides or aromates (Paul, 1992b; Paul *et al*., 2006). Nitrogenous compounds, which are frequently encountered in marine invertebrates, such as sponges or tunicates, are comparatively rare in macroalgae.

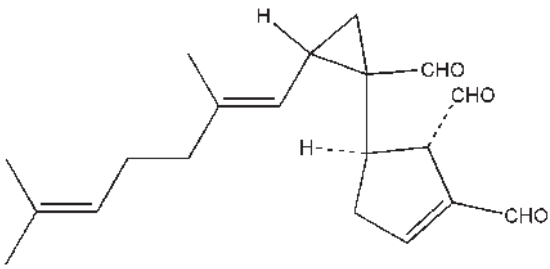
Nevertheless, many of the terpenoids found in green, red or brown algae act as defensive allomones against consumers, such as fish or sea urchins. A vivid example of the ecological significance of terpenoid allomones is provided by the defensive metabolites of green algae from the genera *Halimeda* and *Udotea*. Several species of the latter genera are able to convert biologically weakly active sesquiterpenoids into highly deterrent defence metabolites following tissue damage caused either by the attack of herbivores or by mechanical injury (Paul, 1992b). *Halimeda* species, for example, have been shown to convert the less active sesquiterpenoid halimedatetraacetate (**23**) into the defensive compound halimedatrial (**24**) following breakdown of cellular compartmentation (Paul and Van Alstyne, 1988), whereas under similar conditions, *Udotea flabellum* converts udoteal (**25**) to the more active metabolite, petodial (**26**) (Paul, 1992b). In a more recent study, wound-activated transformation of caulerpenyne (**27**) to oxytoxins 1 (**28**) and 2 (**29**) and related acetoxy aldehydes has been described for the Mediterranean alga *Caulerpa taxifolia* (Jung and Pohnert, 2001). Jung and co-workers (2002) suggest that, in wounded algae, esterases act on caulerpenyne by removing the three acetate residues to rapidly yield the reactive aldehydes. For three species of Mediterranean *Caulerpa*, it was shown that more than 50% of stored caulerpenyne was converted to aldehydes within 1 min (Jung *et al*., 2002). Recently, the biotransformation of dimethylsulfoniopropionate (DMSP) (**30**) to acrylic acid (**31**) and dimethylsulfide (DMS) (**32**) by the enzyme DMSPlyase has been described to occur after tissue damage in temperate macroalgae (Van Alstyne *et al*., 2001; Van Alstyne and Houser, 2003). Both products of the cleavage reaction function as feeding deterrents towards sea urchins, while the precursor of DMSP is a feeding attractant

(Van Alstyne *et al*., 2001; Van Alstyne and Houser, 2003). This biotransformation is especially prevalent in many green and red macroalgae, but also occurs in unicellular phytoplankton (Wolfe and Steinke, 1996; Wolfe *et al*., 1997).

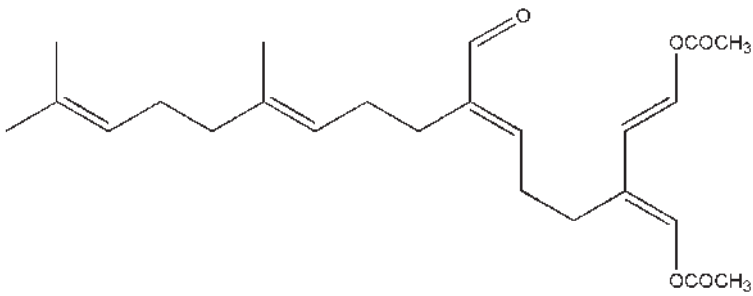
23 Halimedatetraacetate



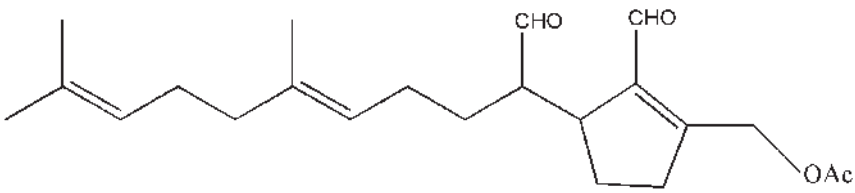
24 Halimedatrial



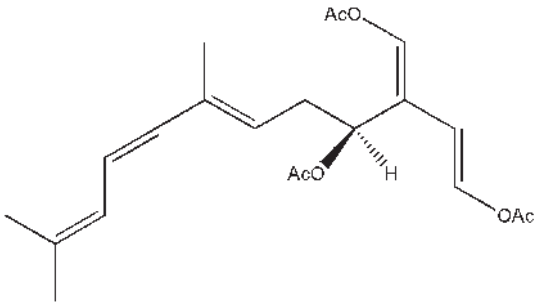
25 Udoteal



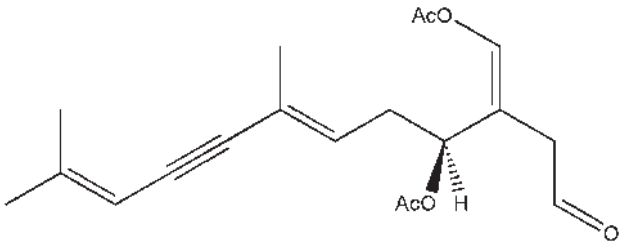
26 Petodial



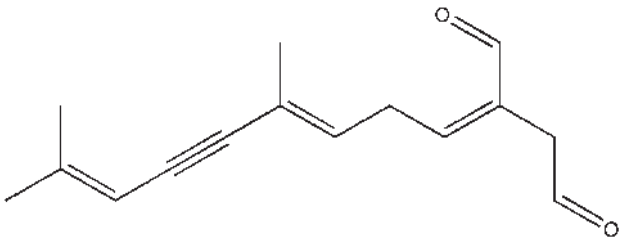
27 Caulerpenyne



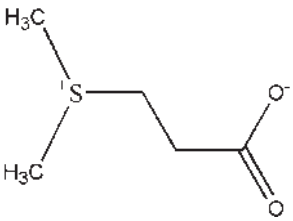
28 Oxytoxin 1



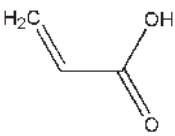
29 Oxytoxin 2



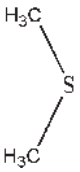
30 Dimethylsulfoniopropionate (DMSP)



31 Acrylic acid

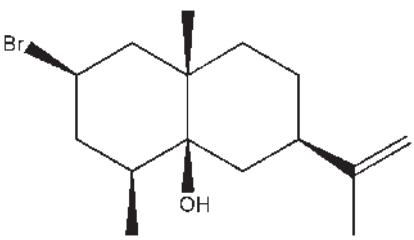


32 Dimethylsulfide



Most chemical defence mechanisms studied in marine algae, however, seem to be constitutive, as reported for example for the tropical green alga *Neomeris annulata*, which is widely distributed on tropical reefs in the Caribbean and Pacific. Specimens of *N. annulata* growing at Guam accumulate brominated sesquiterpenes (**33**) as prominent secondary metabolites. Tips of the thalli, which are most vulnerable to attack by herbivores, usually contain higher concentrations of brominated compounds than the tougher middle or basal parts of the algae. When incorporated into artificial diet and tested at natural concentrations such as present in thallus tips, the major brominated sesquiterpene deterred feeding by three reef herbivores, including the parrot fishes, *Scarus sordidus* and *Scarus schlegeli*, as well as by the sea urchin, *Diadema savignyi* (Lumbang and Paul, 1996). No synergistic effects were observed when the natural mixture of brominated sesquiterpenes rather than individual compounds was tested for deterrent activity (Lumbang and Paul, 1996). Among the unusual chemical defences present in marine algae, some brown algae were found to contain high concentrations of sulphuric acid within cell vacuoles (reviewed by Amsler and Fairhead, 2006). Further examples for the various chemical defence mechanisms of marine algae against consumers are described in the recent review by Paul and co-workers (2006).

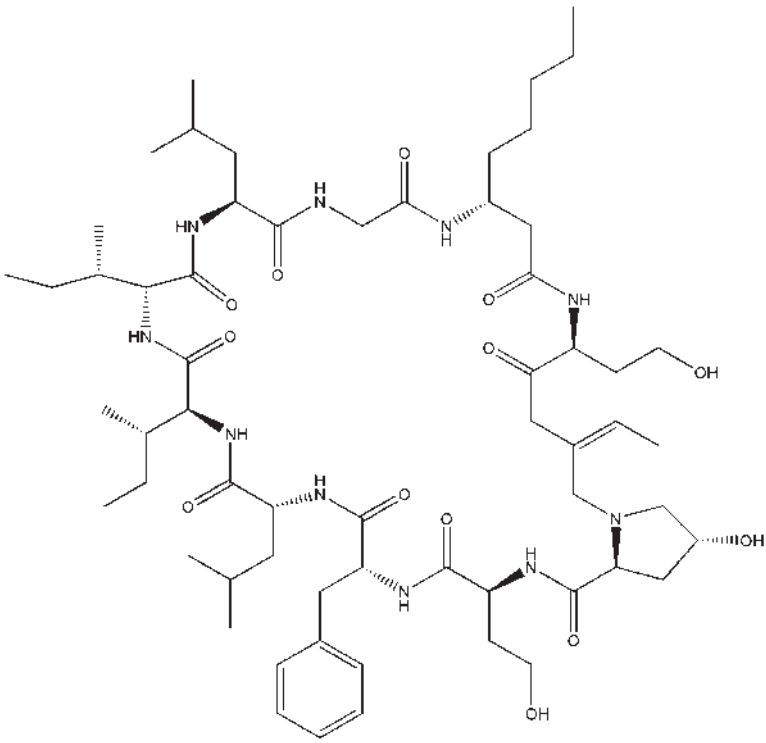
33 Brominated sesquiterpene



Several studies have evaluated the effects of microalgal toxins to consumers. Defence by means of deterrent natural products is employed by some benthic marine cyanobacteria, as reported for the bluegreen alga, *Hormothamnion enteromorphoides* (Pennings *et al.*, 1997). *H. enteromorphoides* periodically dominates shallow reef habitats at Guam, forming erect tufts comparable in size to the thalli of macroalgae. Although at certain times very common,

H. enteromorphoides is avoided by most herbivores, suggesting that the cyanobacterium is chemically defended. This hypothesis was corroborated by incorporating a crude extract of the cyanobacterium into an artificial diet and offering the spiked diet to a natural assemblage of reef fish, including the parrot fish, *S. schlegeli*, as well as to invertebrate grazers, such as the sea urchin, *D. savignyi*, or the crab, *Leptodius* sp. (Pennings *et al.*, 1997). Fractionation of the extract suggested that a mixture of cyclic peptides (which occur frequently in cyanobacteria) including the major constituent laxaphycin A (**34**), were responsible for the deterrency of *H. enteromorphoides*.

34 Laxaphycin A



Some species of marine diatoms produce polyunsaturated aldehydes that have negative effects on copepod reproduction (Ianora *et al.*, 2004; Pohnert, 2005). When diatom cells are damaged, these aldehydes are cleaved from fatty acid precursors and cause developmental arrest and deformed larvae when mothers and larvae are fed diatom diets containing these compounds (Ianora *et al.*, 2004). Relatively few studies have addressed the fate and effects of natural products of unicellular algae through marine food webs. The transfer of microalgal toxins to upper trophic levels can have various consequences. For example, exposure to brevetoxins after a *Karenia brevis* bloom

caused mass mortalities of marine mammals (Flewelling *et al.*, 2005), and microalgal toxins accumulated in prey organisms influence feeding behaviour of predators such as shorebirds and sea otters (Kvitek and Bretz, 2004, 2005).

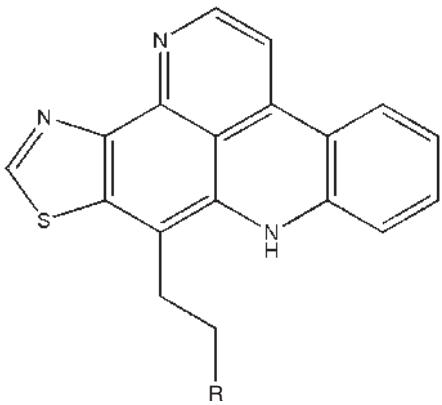
3.5 Favoured allocation of defensive metabolites in vulnerable and valuable parts of marine invertebrates and algae

Several models have been proposed to describe intraspecific patterns of secondary metabolite allocation in marine organisms, among these are the plant apparency model, the optimal defence theory, and the growth differentiation balance hypothesis. The 'plant apparency model', originally developed for higher terrestrial plants, predicts a correlation between chemical defence and the risk of being discovered by herbivores (Feeny, 1976; Rhoades and Cates, 1976). The 'optimal defence theory', also originally formulated for higher terrestrial plants, suggests that metabolically costly chemical defences should be preferentially invested in the most valuable parts of a plant (McKey, 1979; Rhoades, 1979), such as young developing leaves or seeds. The 'growth differentiation balance hypothesis' assumes that acquired resources are allocated between growth processes and differentiation (including cellular specialization and production of defensive chemicals; Herms and Mattson, 1992). The latter hypothesis therefore predicts that actively growing parts of a thallus should be less defended than older, differentiated parts, thus predicting a pattern contrary to the optimal defence theory. However, the predictions of the growth differentiation balance hypothesis so far only seem to hold for particular brown algae that allocate terpenes and phlorotannins to older regions of their thalli (Poore, 1994; Cronin and Hay, 1996). In contrast, there are numerous examples that prove the predictions of the optimal defence theory and the plant apparency model in case of marine algae as well as invertebrates.

A study on the chemical defence of the Indo-Pacific sponge *Oceanapia* sp. demonstrated that both the optimal defence theory and the plant apparency model apply to marine invertebrates. The conspicuously red-coloured Indo-Pacific sponge *Oceanapia* sp. occurs in shallow sandy areas around the Micronesian island Truk. Part of the sponge, the so-called base, is immersed into the substrate, whereas the fistule and an apical round-shaped structure, the so-called capitum (probably an asexual propagation unit), are exposed in the sea water. Underwater observations have indicated that the exposed parts of the sponge, even though easily accessible to potential predators, are not consumed by the frequently occurring reef fish (Schupp *et al.*, 1999b), suggesting chemical defence of the sponge. This hypothesis was corroborated by incorporating a crude extract derived from fistulae of *Oceanapia* sp. into an artificial diet at the respective natural concentration, and offering treated

versus non-treated diet cubes to a natural assemblage of reef fish in a field bioassay. Whereas the fish readily consumed the control diet, the treated diet was clearly avoided. The pyridoacridine alkaloids, kuanoniamine C (**35**) and D (**36**), proved to be responsible for the deterrent properties of the crude extract from *Oceanapia* sp., as shown in a subsequent field feeding experiment. Interestingly, the defensive alkaloids occur at largely different concentrations in the various parts of the sponge analyzed. Whereas total alkaloid concentration amounts to only 0.8% (relative to dry mass) in the base, which is physically protected in the substrate, alkaloid concentrations equal almost 2% in the exposed fistule and are close to 5% in the asexual propagation unit capitum (Schupp *et al.*, 1999b), thus providing an example for both the above-mentioned 'apparency model' and for the 'optimal defence theory' in the marine habitat.

35, 36

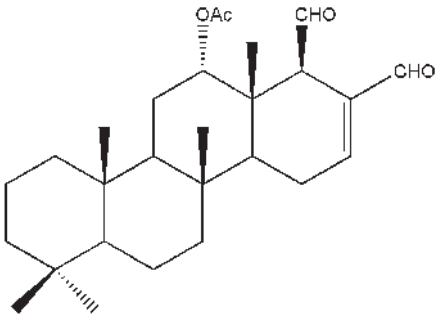


	R
35 Kuanoniamine C	$NHCOC_2H_5$
36 Kuanoniamine D	$NHCOCH_3$

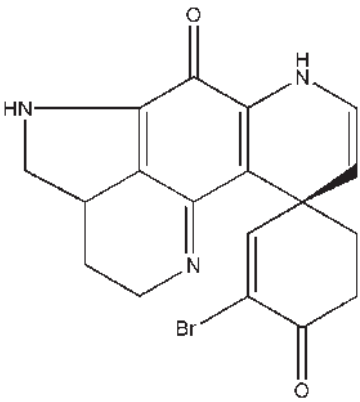
Several further studies investigating the distribution of chemical defences within individual sponges support the predictions of the optimal defence theory for marine sponges as well. Turon and co-workers (1996b) and Uriz and co-workers (1996) detected higher average toxicity values in the periphery than in the centre of the encrusting Mediterranean sponge *Crambe crambe*, proving that this sponge optimizes the deployment of defensive metabolites by concentrating predator deterrents in the surface tissue. Becerro and co-workers (1998) found significantly more scalaradial and desacetyl-scalaradial (**37**) in the tips rather than in the bases of branches of the Micronesian sponge *Cacospongia* sp. from Guam which is preyed upon by the specialist feeding nudibranch *Glossodoris pallida*. The Antarctic sponge *Latrunculia apicalis* contains discorhabdin alkaloids of which discorhabdin G (**38**) serves as defensive agent against the spongivorous sea star *Perknaster fuscus* (Furrow *et al.*, 2003). When they determined discorhabdin G concentrations in discrete sponge layers, Furrow and co-workers (2003) found a gradient of discorhabdin G that falls off rapidly towards the centre of the sponge. On average, 52% of total

discorhabdin G in a given sponge was detected within 2 mm of the sponge surface (Furrow *et al*., 2003).

37 Desacetyl-scalaradial



38 Discorhabdin G



Mahon *et al*. (2003) demonstrated that among several isolated tissues of the Antarctic brachiopod *Liothyrella uva* offered to the abundant, omnivorous sea star *Odontaster validus* and the omnivorous fish *Notothenia coriiceps*, only the pedicle was unpalatable. Since the pedicle affixes individuals to the substrata, it is, in contrast to all other brachiopod tissues that are protected by a shell, constantly exposed and highly vulnerable. In the context of the 'optimal defence theory', an accumulation of defensive metabolites in the pedicle not only protects an exposed and vulnerable body part, but also maximizes fitness by preventing the dislodgement of individuals from the substrata and subsequent probable death (Mahon *et al*., 2003).

Another example from marine invertebrates that fits both the 'apparency model' as well as the 'optimal defence theory' concerns chemical defence of marine invertebrate larvae. Whereas some species of marine invertebrates produce large numbers of small larvae that feed and develop in the plankton and are usually dispersed over large distances ('planktotrophic' larvae), other marine invertebrates produce a smaller number of larger non-feeding larvae

that use yolk for nutrition ('lecitotrophic' larvae) (Lindquist and Hay, 1996). Some marine invertebrates even brood lecitotrophic larvae to an advanced stage of development (Lindquist and Hay, 1996). Lecitotrophic larvae are usually large and conspicuous and should, therefore, be prone to predation by fish or other consumers, especially since the yolk on which these larvae depend is highly nutritious. This latter type of larvae can, therefore, almost certainly be expected to be defended in some way (e.g. by natural products) according to the above-cited 'apparency model' and to the 'optimal defence theory'. In this context, Tarjuelo *et al*. (2002) could show that among six species of colonial Mediterranean ascidians, the larvae of species with high fecundity and small larvae were most palatable to common sympatric predators (fishes and crustaceans) when compared to the larvae of species with lower fecundity and larger larvae that were unpalatable to at least two species of predators tested (Tarjuelo *et al*., 2002). Another example of the chemical defence of lecitotrophic larvae is provided by the ascidian *Ecteinascidia turbinata*. *E. turbinata* releases conspicuously coloured larvae that are deterrent to potential fish predators (Young and Bingham, 1987). After mouthing larvae of *E. turbinata*, the pinfish *Lagodon rhomboides* was reported to avoid the usually palatable larvae of *Clavelina oblonga* when the latter were coloured to resemble larvae of *E. turbinata* (Young and Bingham, 1987), thereby suggesting the importance of colour in fish–invertebrate associations. The list of examples of chemically defended lecitotrophic larvae has been extended by the sea star *Diplasterias bruceri* and the brooding sponge *Isodictya setifera* (McClintock and Baker, 1997), the Antarctic sea stars *Neosmilaster georgianus* and *Lysasterias perrieri* and the common giant isopod *Glyptonotus antarcticus* (McClintock *et al*., 2003).

In a broader survey on the chemical defence of tropical and temperate marine invertebrate larvae, Lindquist and Hay (1996) showed that brooded larvae were significantly more likely to be unpalatable (86% of all species tested) than planktotrophic larvae (33%). Whereas most unpalatable larvae were released during the day (89%), the majority of palatable larvae spawned at night (77%). Furthermore, there was a high incidence of conspicuous colouration in unpalatable larvae (60%), whereas palatable larvae were devoid of aposematic colouration (0%) (Lindquist and Hay, 1996).

There is evidence from several recent studies that the predictions of the optimal defence theory are not restricted to invertebrates in the marine environment, but also applied to macroalgae. Fairhead and co-workers (2005) examined within-thallus variation of both chemical and physical defences in two ecologically dominant Antarctic brown macrolagae of the genus *Desmarestia.* Lipophilic and hydrophilic extracts were obtained from the holdfast, the primary stem, and the lateral branches of the thallus and incorporated into artificial alginate foods offered to the sympatric, herbivorous amphipod *Gondogeneia antarctica*. Consistent with the optimal defence theory, the most valuable part, the primary stem, was most strongly physically and chemically protected in the highly differentiated species *Desmarestia anceps*, followed by the physically strongly defended holdfast. In contrast, the replaceable lateral

branches showed only moderate chemical protection (Fairhead *et al*., 2005). In the congener *Desmarestia menziesii*, no differences were detected between thallus parts, which the authors explained by the lower level of thallus differentiation in this species when compared to *D. anceps* (Fairhead *et al*., 2005). The optimal defence theory is further supported by studies showing that seaweeds preferentially defend younger or more vulnerable portions of the thallus, reproductive structures, or the more exposed parts (reviewed by Paul and Puglisi, 2004).

3.6 The flexible response: stress-induced accumulation of defence metabolites and activation of protoxins

Most chemical defence strategies reported so far for marine invertebrates or algae are constitutive and rely on preformed toxic or deterrent compounds that are stored in tissues and either liberated upon wounding or continuously exuded (Walker *et al*., 1985). Examples for these rather static defence mechanisms from the marine environment have been discussed above. However, chemical ecological studies in the terrestrial environment, especially those conducted on the interaction of higher plants with herbivores or with pathogenic microorganisms (e.g. fungi) have repeatedly shown that the defence response of plants may be highly dynamic rather than static. Plants are, for example, known to react to an attack by pathogens or tissue damage with a significantly increased accumulation of constitutively present natural products (e.g. Sahm *et al*., 1995; Marak *et al*., 2002; Bezemer *et al*., 2004; Alves *et al*., 2007), by a de novo biosynthesis of phytoalexins especially in response to invading fungi (e.g. Bailey and Mansfield, 1982; Pedras *et al*., 2000; Heil and Bostock, 2002), or by an usually enzymatically catalyzed biotransformation of preformed compounds (protoxins). An example for the latter defence strategy is the liberation of the highly toxic hydrocyanic acid (HCN) from non-toxic cyanogenic glycosides that are used as storage compounds (protoxins) for the defence metabolite HCN (Jones, 1988; Seigler, 1991). Further examples for the stress-induced biotransformation of protoxins include the herbivore-induced conversion of phenolic glycosides in *Populus balsamifera* (Clausen *et al*., 1989) or the conversion of sesquiterpenes in the mushroom *Lactarius vellereus* (Sterner *et al*., 1985).

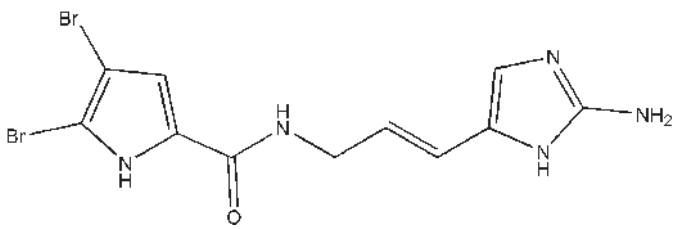
Stress-induced changes in the composition (by de novo biosynthesis of phytoalexins) or amounts of natural products (by an accelerated biosynthesis) are referred to as 'induced defence' in order to distinguish them from the biotransformation of protoxins which is defined as 'activated defence' (Paul and Van Alstyne, 1992; Thoms and Schupp, 2007). Activated defence mechanisms are fast and mostly occur within seconds after cell compartmentalization has been disturbed and protoxins get in contact with liberating enzymes. In the case of cyanogenic plants, β-glucosidases catalyze the hydrolysis of cyanogenic glycosides that are liberated from vacuoles upon cellular disruption to form

chemically unstable cyanohydrins that in turn are transformed either enzymatically or spontaneously to the actual defence metabolite HCN and an aldehyde or ketone (Conn, 1979; Wajant and Effenberger, 1996). The liberated defence compounds like HCN or thiocyanates, isothiocyanates and isonitriles which are formed by cleavage of glucosinolates (Stoewsand, 1995; Fahey *et al*., 2001) are usually toxic to both the invader and the attacked organism and may result in partial tissue necrosis of the latter. Since plants, just like many marine organisms, have the capability to quickly regenerate tissue loss they are, however, able to compensate such self-inflicted damage.

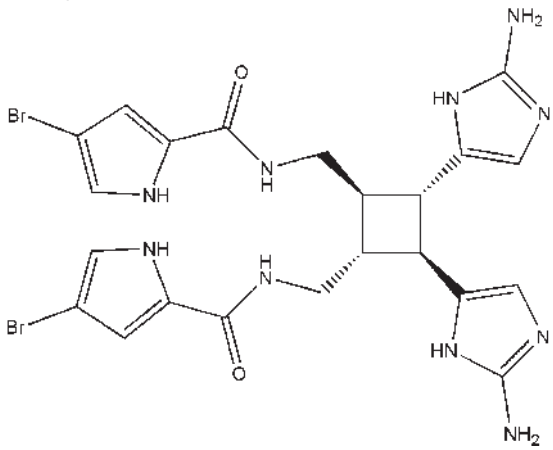
Induced defence mechanisms are much slower and usually take days or even weeks before they become apparent (Taylor *et al*., 2002; Thoms and Schupp, 2008). This makes their detection especially under field conditions difficult as non-stressed 'controls' are virtually not existing in a natural environment that is characterized by a plethora of biotic and abiotic stress factors. Nevertheless, cases of induced defences have been documented for the marine environment. Grazing by the amphipod *Ampithoe longimana* was, for example, reported to induce an increased accumulation of defensive compounds in the marine brown alga *D. menstrualis* (Cronin and Hay, 1996). *A. longimana* preferentially feeds on thalli of *D. menstrualis*, which contain dictyol-type diterpenes. Field observations as well as controlled feeding experiments indicated that tissue damage by the amphipod causes an enhanced accumulation of diterpenes, which in turn decrease the palatability of *D. menstrualis* (Cronin and Hay, 1996). Compared to undamaged control plants, thalli damaged by *D. menstrualis* contained 19–34% more dictyol derivatives and were 50% less palatable to the amphipods. Grazing did not affect protein content or toughness of the thalli, indicating a specific elicitation of diterpenoid accumulation.

A further example is provided by the Caribbean sponge *Agelas conifera* that was shown to react to experimental simulation of predator bites by a marked increase of the bromopyrrol alkaloids oroidin (**39**) and sceptrin (**40**) (Richelle-Maurer *et al*., 2003). Both compounds that were induced by artificial wounding proved to be feeding deterrents against the reef fish *Stegastis partitus*. Both alkaloids were also active against the coral *Madracis mirabilis* which is a potential competitor of *A. conifera* for space, thereby indicating multiple ecological functions of these stress-induced metabolites (Richelle-Maurer *et al*., 2003).

39 Oroidin



40 Sceptrin

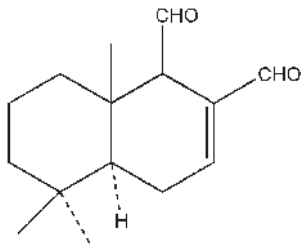


Marine invertebrates like sponges, however, do not only react by induced accumulation of natural products towards predators or competitors but also respond towards pathogenic microorganisms. The Mediterranean sponge *Suberites domuncula*, for example, when exposed to bacterial lipopolysaccharide endotoxin reacted with an increased biosynthesis of two alkyllipid derivatives that show antibacterial activity (Müller *et al*., 2004). The lipopolysaccharide receptor which is expressed at the sponge surface was discovered in a follow-up study (Wiens *et al*., 2005). The same group was also able to show that *S. domuncula* responds with activation of endocytosis and release of the antibacterial enzyme lysozyme when exposed to bacterial peptidoglycans (Thakur *et al*., 2005).

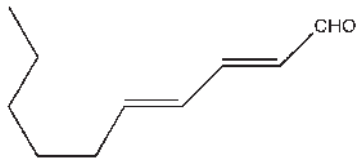
The first example for an activated chemical defence in the marine habitat was described for tropical green algae of the genus *Halimeda* that react towards tissue damage by a fast and presumably enzymatically catalyzed conversion of halimedatetraacetate (**23**) to the chemically highly reactive halimedatrial (**24**) (Paul and Van Alstyne, 1992) that causes an increase of feeding deterrency of the algae towards herbivorous fishes (Paul and Van Alstyne, 1988). Interestingly, the chemically similar and likewise highly reactive compound polygodial (**41**) first isolated from the sprout of *Polygonum hydropiper* known as 'tade' and used as a food spice in Japan but also present in the African trees *Warburgia stuhlmannii* and *Warburgia ugandensis* is a strong feeding deterrent against herbivorous insects (Kubo and Taniguchi, 1988; Kubo and Himejima, 1992; Kubo *et al*., 2001). It is unknown so far if polygodial like halimedatrial arises from a precursor and is formed only upon tissue injury. The wound-induced deacetylation of caulerpenyne (**27**) to the reactive aldehydes oxytoxin 1 (**28**) and 2 (**29**) by green algae of the genus *Caulerpa* (Jung and Pohnert, 2001; Jung *et al*., 2002) is a further example for activated chemical defences in marine macroalgae. Similar chemical reactions culminating in the liberation of highly reactive aldehydes, however, occur also in microalgae. In diatoms, polyunsaturated fatty acids are enzymatically cleaved from

phospholipids and converted within seconds to unsaturated aldehydes like 2,4-decadienal (**42**) or 2,4,7-decatrienal (**43**) (Miralto *et al.*, 1999). It has been shown that the reactive aldehydes diminish the reproductive success of their copepod herbivores (Miralto *et al.*, 1999).

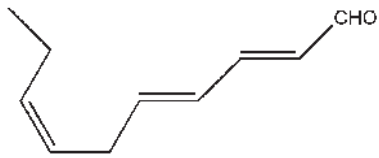
41 Polygodial



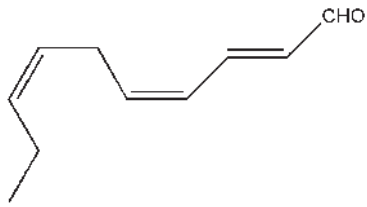
42 2,4-Decadienal



43
2-trans-4-trans-7-cis-decatrienal



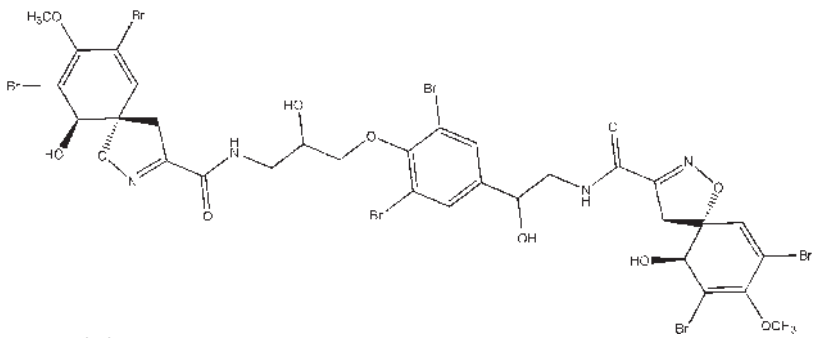
2-trans-4-cis-7-cis-decatrienal



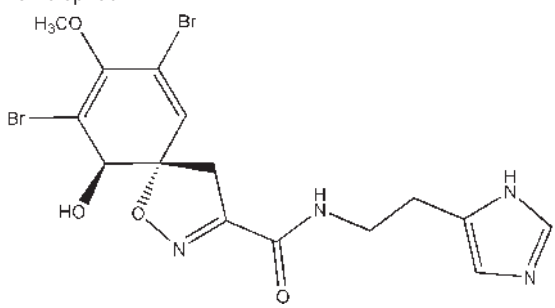
The first example for an activated chemical defence in marine invertebrates was reported for the Mediterranean sponge *A. aerophoba* (Teeyapant and Proksch, 1993). Sponges of the genus *Aplysina* as well as sponges from other genera of the family Aplysinidae sequester structurally unusual brominated isoxazoline alkaloids such as isofistularin-3 (**44**) or aerophobin-1 (**45**) and -2 (**21**) that in total may account for more than 10% of the sponge dry weight (Teeyapant *et al.*, 1993; Ciminiello *et al.*, 1996, 1999; Thoms *et al.*, 2003b). Within the sponges, these compounds are primarily stored in spherolous cells in the mesohyl (Thompson *et al.*, 1983; Turon *et al.*, 2000). Disruption of the

cellular compartmentation, for example by artificial wounding (e.g. grinding of fresh sponge tissue in a mortar for a few seconds) or by immersing freshly collected specimens of *A. aerophoba* in methanol (due to the high water content in the sponges, the resulting methanol concentration is considerably diluted in the extraction medium) a rapid conversion of the alkaloids is observed. The isoxazoline alkaloids are cleaved between the isoxazoline moiety and the adjacent carbonyl group. Opening of the isoxazoline ring gives rise to the nitrile aeroplysinin-1 (**46**) that is transformed in a second reaction step via enolether hydrolysis and partial hydrolysis of the nitrile group to a dienone (**47**). When isofistularin-3 is used as a substrate for the reaction, a chemically stable bisoxazolidinone derivative is recovered as a further product formed, whereas in the case of aerothionin the amine putrescine is detected as a second reaction product (Ebel *et al*., 1997; Thoms *et al*., 2006a). Time course series that involved grinding fresh sponge tissue followed by shock freezing, lyophilization and extraction with methanol proved that these biotransformation reactions occur within seconds (Ebel *et al*., 1997; Thoms *et al*., 2006a). Since these reactions also proceeded in a cell-free extract of *A. aerophoba* but could be inhibited either by boiling of the enzyme extract prior to addition of isoxazoline precursors or by acid treatment, it was concluded that the biotransformations are enzymatically catalyzed. Addition of isoxazoline alkaloids to tissue or cell-free extracts of non-verongid sponges such as *C. crambe* or the sponge-feeding opisthobranch *T. perversa* that is a specialized predator on *Aplysina* sponges (Ebel *et al*., 1999; Thoms *et al*., 2003b) caused no biotransformation of sponge alkaloids suggesting that the enzyme(s) involved are specific for *Aplysina* sponges.

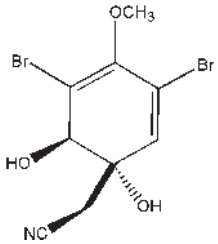
44 Isofistularin-3



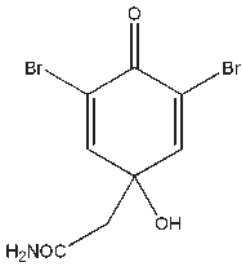
45 Aerophobin-1



46 Aeroplysinin-1



47 Dienone



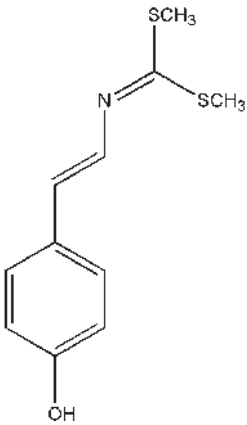
The isoxazoline alkaloids are involved in the chemical defence of *Aplysina* sponges against fishes such as *Blennius sphinx* at concentrations below those encountered in the sponges (Thoms *et al*., 2004). The biotransformation products aeroplysinin-1 and the dienone lack the deterrent activity of their precursors but are strongly antibiotically active against marine as well as terrestrial bacteria (Teeyapant *et al*., 1993; Weiss *et al*., 1996; Debitus *et al*., 1998). It has been proposed that the wound-induced biotransformation of isoxazoline alkaloids generates a protection against invasion of pathogenic bacteria at the site of tissue damage (Thoms *et al*., 2006a).

The wound-induced biotransformation of *Aplysina* alkaloids was the first example proposed as an activated defence strategy in sponges (Teeyapant and Proksch, 1993). Recently, a similar case has been described for the sponge *A. rhax* that occurs in the Pacific Ocean and is systematically related to *Aplysina* sponges as both are placed in the order Verongida (Hooper and Van Soest, 2002). *A. rhax* accumulates large amounts of the nitrogenous metabolite psammaplin A sulphate (**48**) (Pham *et al*., 2000; Shin *et al*., 2000). Psammaplin A sulphate is biogenetically related to the *Aplysina* alkaloids as both are obviously derived from dibromotyrosine. In contrast to the *Aplysina* alkaloids, it lacks an isoxazoline ring but features an oxime group instead as well as a sulphate ester moiety bound to the aromatic ring. A recent study by Thoms and Schupp (2008) demonstrated that upon artificial wounding of the sponges, a rapid biotransformation reaction is observed which yields psammaplin A (**13**) by hydrolysis of the sulphate group from the precursor psammaplin A sulphate. Like described for *A. aerophoba*, the biotransformation of psammaplin A sulphate proceeds within seconds after artificial wounding and appears to be enzymatically catalyzed (Thoms and Schupp, 2008). Unlike the

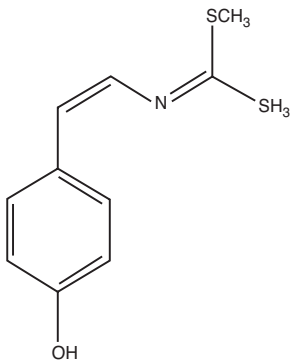
biotransformation of isoxazoline alkaloids in *Aplysina* sponges, however, the conversion of natural products in *A. rhax* is not paralleled by drastic changes in the biological activities as both the precursor and its product show feeding deterrency against fishes (the latter being somewhat more active than the former) as well as antibiotic activity against marine bacteria (Thoms and Schupp, 2008).

A further example for an activated chemical defence in marine invertebrates was reported for the marine hydroid *Tridentata marginata* (Lindquist, 2002). The hydroids accumulate tyrosine derived dithiocarbamates called tridentalols (**49**) (Lindquist *et al*., 1996; Stachowicz and Lindquist, 2000). When the hydroids are attacked (simulated by crushing of the tissue), a rapid transformation of biologically inactive tridentalols to fish repelling derivatives is observed (Lindquist, 2002). Like the biotransformations of sponge metabolites in *A. aerophoba* and in *A. rhax*, the activation of tridentalols appears to be enzymatically catalyzed (Lindquist, 2002).

49
Tridentalol A



Tridentalol B

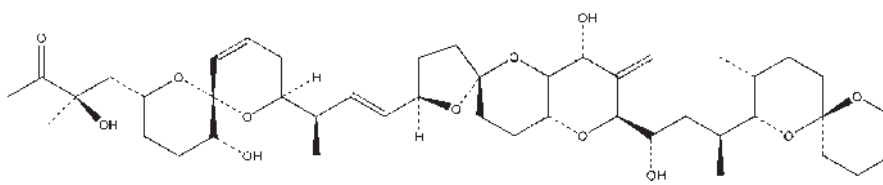


These examples of activated defence mechanisms which are present in marine diatoms, macroalgae and invertebrates suggest that like in terrestrial plants the stress-induced activation of protoxins is no singular event but appears to be more widespread than initially thought. It may thus be expected that further examples will be reported for marine organisms in the near future.

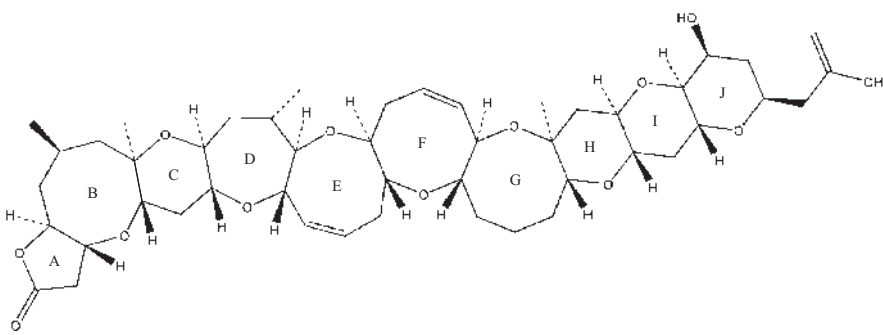
3.7 Endosymbionts as sources for allelochemicals found in marine invertebrates

Marine natural product chemists have for a long time pondered about the obvious chemical similarity or even identity of natural products recovered from invertebrates such as sponges or tunicates and compounds known from microorganisms such as bacteria and microalgae. In some cases, the origin of bioactive compounds found, for example, in sponges as being derived by filter feeding, could be unequivocally proven. This holds true for the highly toxic protein phosphatase inhibitor okadaic acid (**50**) that had originally been isolated from sponges of the genus *Halichondria* such as *H. okadai* and *H. melanodica* (Tachibana *et al*., 1981). Later, it was shown that okadaic acid is in fact produced by dinoflagellates of the genus *Prorocentrum* (Murakami *et al*., 1982) which are now considered to be the true source of this polycylic ether derivative that shares important structural characteristics with other well-known dinoflagellate toxins like brevetoxin A (**51**) or zooxanthellatoxin A (**52**) (Shimizu and Li, 2006).

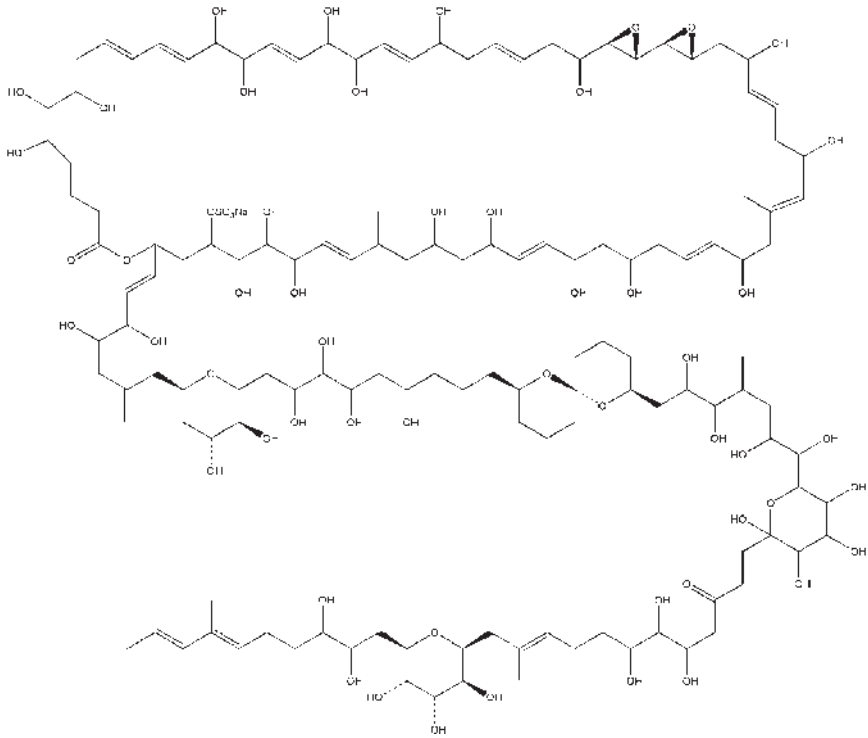
50 Okadaic acid



51 Brevetoxin A

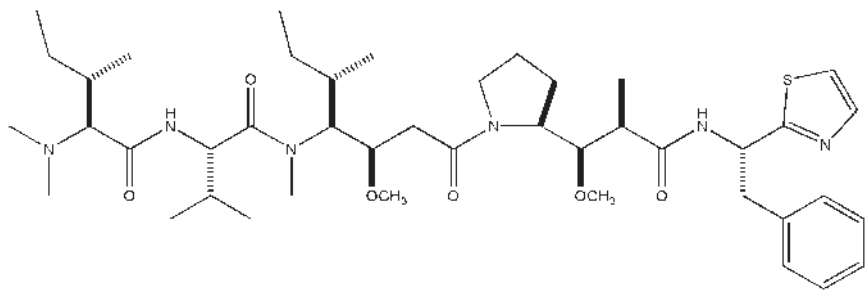


52 Zooxanthellatoxin A

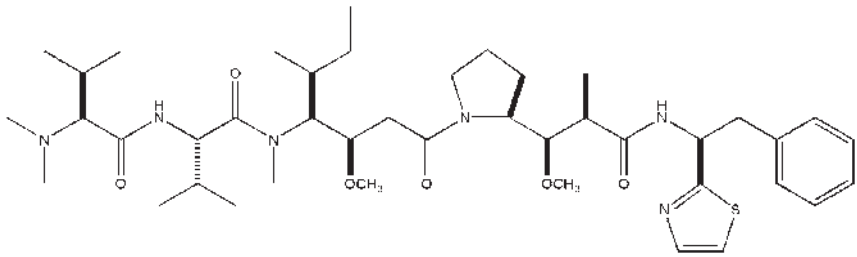


The example of okadaic acid appears to be no isolated case. Closer inspection of other important invertebrate-derived natural products reveals likewise striking similarities to microbial metabolites. Symplostatin 1 (**53**), for example, is known as a metabolite of the blue-green alga *Symploca hydnoides* (Harrigan *et al*., 1998) and bears obvious chemical similarity to dolastatin 10 (**54**) isolated from the marine algivorous mollusc *Dolabella auricularia* which raised suspicion about the origin of the latter. Evidence in support of a dietary origin of dolastatin 10 in the molluscs was provided when the same dolastatin derivatives as found in *D. auricularia* were identified in free-living cyanobacteria such as in the marine cyanobacterium *Symploca* species VP642 (Luesch *et al*., 2001).

53 Symplostatin 1

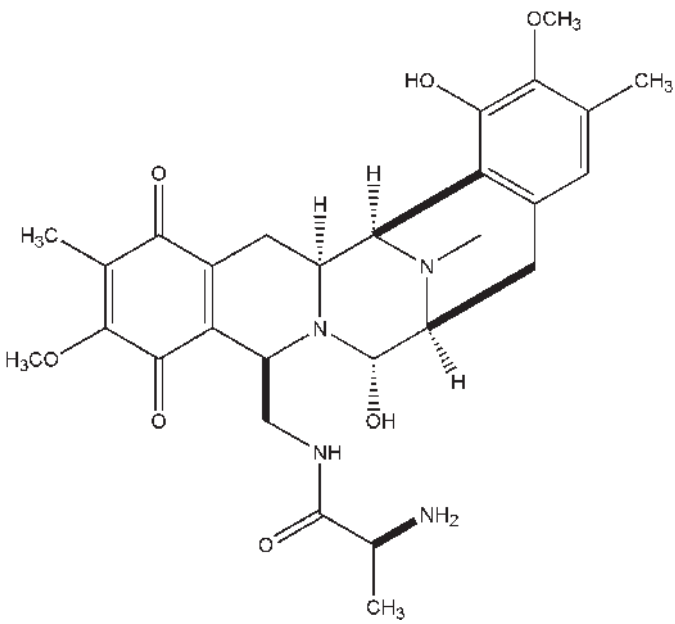


54 Dolastatin 10



743 (ET-743) (**3**) is a tunicate-derived alkaloid isolated from *cteinascidia turbinata* that is presently in clinical studies as a new anticancer drug (Proksch *et al*., 2002, 2006). In the past, insufficient supply of ET-743 from the tunicates had been an obstacle for the clinical investigation of this promising compound. This bottleneck, however, has been overcome by developing a synthetic strategy that makes use of the striking chemical similarity of ET-743 to the bacterial compound safracin B (**55**) which is a metabolite of *Pseudomonas fluorescens* (Ikeda *et al*., 1983). Using safracin B, which can be obtained through fermentation of *P. fluorescens* as a synthetic precursor, ET-743 can be obtained in satisfactory yields by partial synthesis (Cuevas *et al*., 2000).

55 Safracin B

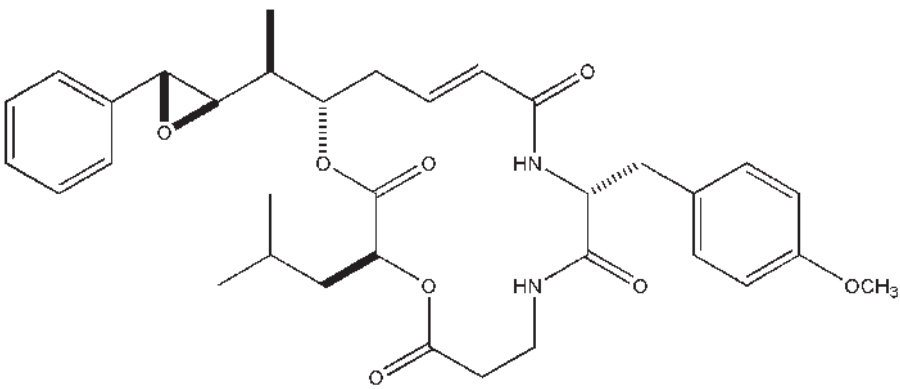


Based on the discussed chemical similarity or even identity of bioactive metabolites from marine invertebrates, it is tempting to assume that like in the case of okadaic acid they are introduced (and perhaps structurally modified)

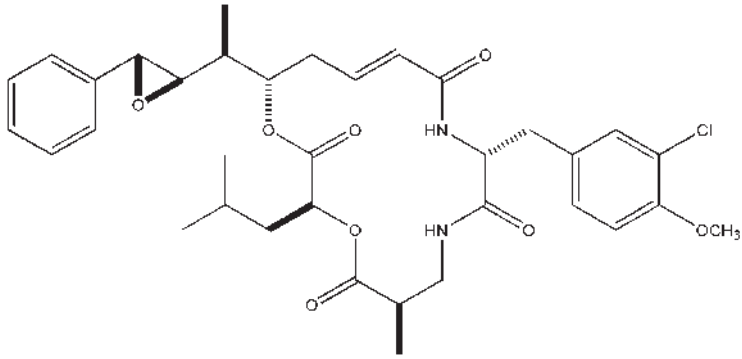
into the respective invertebrates either through the food chain or originate from microbial symbionts that live in close association with their hosts.

Strong evidence for the latter hypothesis (production of 'invertebrate' metabolites by symbiotic microorganisms) has been put forward by a number of studies on sponges of the genus *Dysidea* that harbour symbiotic cyanobacteria, predominantly *Oscillatoria spongeliae* (Thacker and Starnes, 2003). *Dysidea* sponges are well known for their natural products that resemble cyanobacterial metabolites. For example, *D. arenaria* from Okinawa yielded the cytotoxic metabolite arenastatin A (**56**) that shows striking similarity to cryptophycin A (**57**), a cyclic peptide that had been isolated from the terrestrial cyanobacterium *Nostoc* sp. (Smith *et al*., 1994; Trimurtulu *et al*., 1994; Kobayashi and Kitagawa, 1999). Certain populations of *D. herbacea* produce further unusual chlorinated peptides including dysidenin (**58**) and 13-demethylisodysidenin (**59**) (Unson and Faulkner, 1993; Dumdei *et al*., 1997; MacMillan *et al*., 2000; Harrigan *et al*., 2001). The highly unusual trichlormethyl group of dysidenin and of demethylisodysidenin is also found in cyanobacterial metabolites such as barbamide (**60**), pseudodysidenin (**61**), dysidenamide (**62**), nordysidenin (**63**) and herbamide B (**64**) (Orjala and Gerwick, 1996; Jiménez and Scheuer, 2001) suggesting an origin of the trichlorinated sponge metabolites from cyanobacteria. Experimental support for his assumption was provided by flow cytometric studies involving specimens of *D. herbacea* from the Great Barrier Reef, Australia. The unusual chlorinated metabolites were clearly identified in the cyanobacterial fraction (*O. spongeliae*), whereas the fraction consisting mainly of sponge cells was devoid of these natural products (Unson and Faulkner, 1993). Recent molecular analysis of *D. herbacea* and its symbiont *O. spongeliae* succeeded in the isolation of biosynthetic genes showing high homology to the already known barbamide biosynthetic gene cluster (*bar*). Using fluorescence in situ hybridization, it was possible to localize *bar*-like halogenase genes in the cyanobacterial sponge symbiont *O. spongeliae*, whereas other specimens of *D. herbacea* that likewise contain *O. spongeliae* but fail to produce chlorinated compounds lack *bar* homologues (Flatt *et al*., 2005).

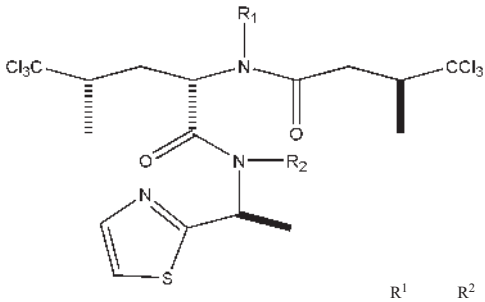
56 Arenastatin A



57 Cryptophycin A

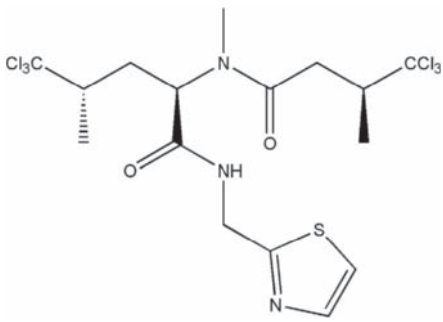


58, 61, 63

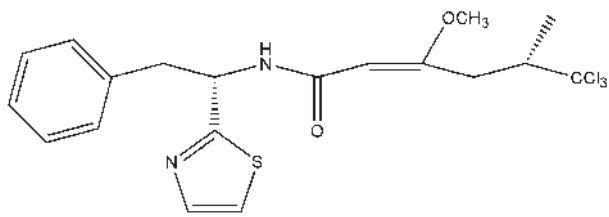


		R^1	R^2
58	Dysidenin	CH_3	H
61	Pseudodysidenin	CH_3	CH_3
62	Nordysidenin	H	H

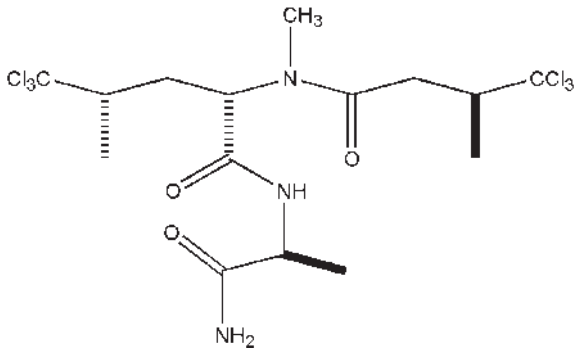
59 Demethylisodysidenin



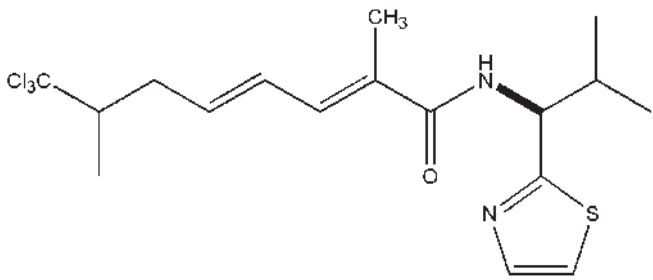
60 Barbamide



62 Dysidenamide

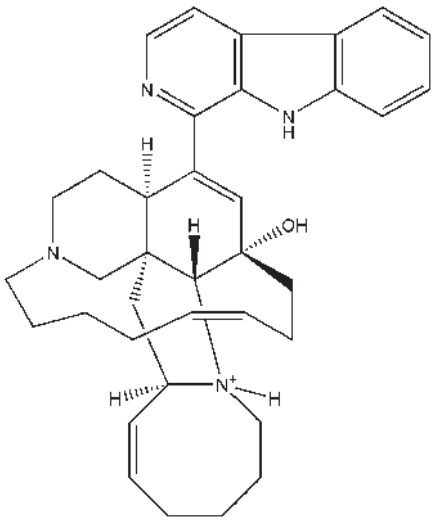


64 Herbamide

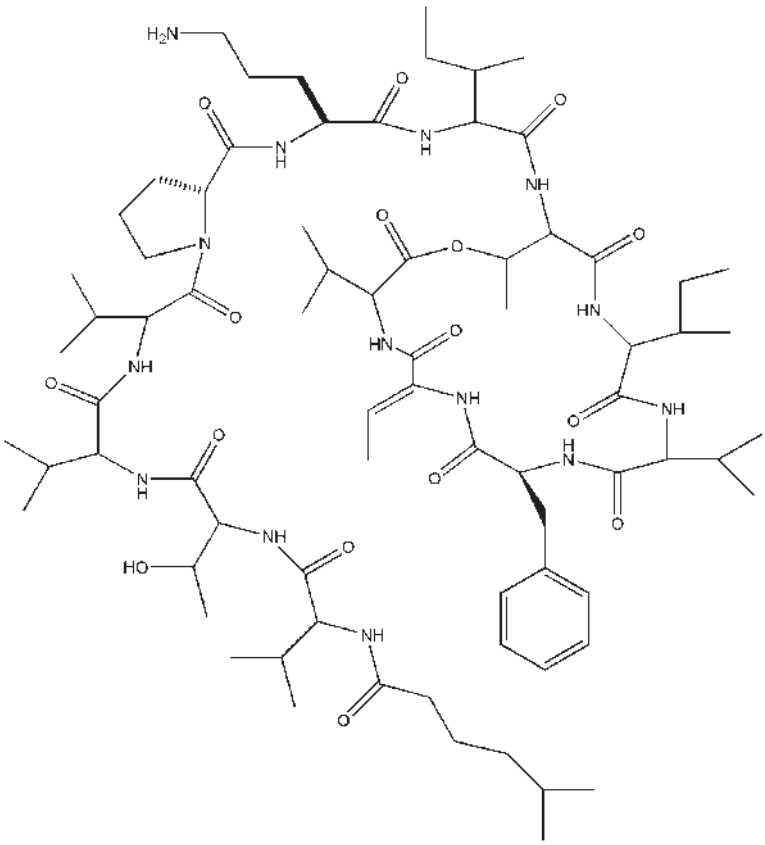


Direct proof for a microbial origin of bioactive invertebrate metabolites by isolation and cultivation of the respective symbionts has been attempted in many cases but has mostly failed as the requirements for special media and cultivation parameters that resemble conditions, for example, in sponges are apparently hard to simulate. Thus, attempts to isolate and cultivate bacteria associated for example with the Mediterranean sponge *A. aerophoba* were only successful for less than 1% of the true bacterial diversity that is present in the sponges and can be assessed culture independently using 16 S rRNA gene sequencing (Friedrich *et al*., 2001). Sponges apparently even harbour bacteria that are unknown from other biota such as the recently discovered candidate phylum 'Poribacteria' (Fieseler *et al*., 2004; Scheuermayer *et al*., 2006) which indicates a long co-evolutionary history of sponge bacterial symbiosis. It is thus not surprising that cultivation of these highly specialized symbiotic bacteria which are probably the most interesting ones with regard to their involvement in the production of unusual bioactive metabolites is hard to achieve, if not impossible. Nevertheless, in a few cases these attempts have been successful. One example is the isolation of a *Micromonospora* strain from *Xestospongia* sponges that produces structurally complex alkaloids of the manzamine (**65**) type that are also found in the sponge host (Hill *et al*., 2004). The second example is from the same group of researchers and involves a group of depsipeptides called kahalalides. Kahalalide F (**66**) has originally been obtained from the Hawaiian mollusc *Elysia rufescens* (Hamann *et al*., 1996) and is presently in

65 Manzamine A



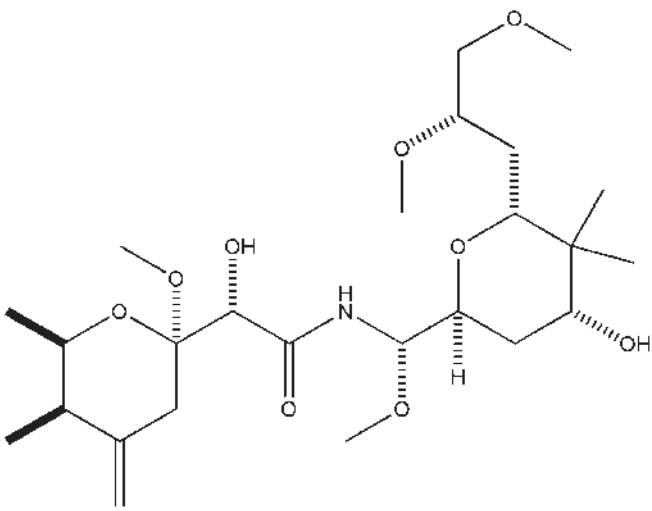
66 Kahalalide F



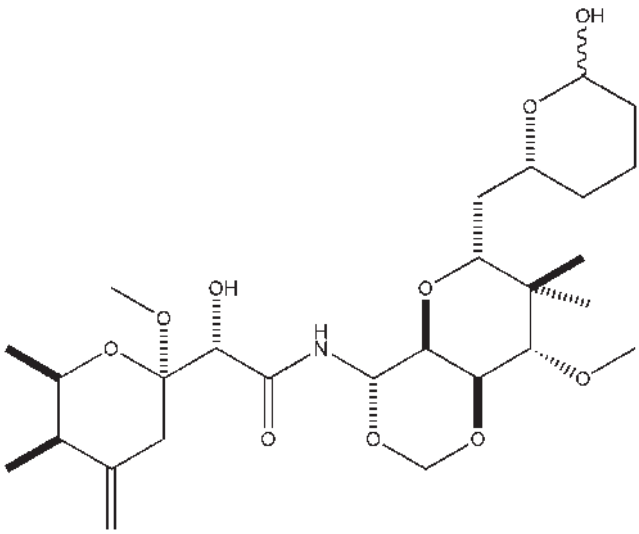
clinical studies as a potential new anticancer drug (Proksch *et al*., 2006). Kahalalides are also isolated from other *Elysia* spp. (e.g. Ashour *et al*., 2006) that feed on *Bryopsis* algae from which they sequester the kahalalides. Recently, Hill *et al*. (2005) described a kahalalide F producing *Vibrio* sp. as being associated with *Bryopsis* and the mollusc *E. rufescens*. It was hypothesized that the molluscs obtain *Vibrio* from the surface of the algae and maintain the bacteria as symbionts.

In spite of these encouraging results, it appears more likely at the moment that the quest for putative microbial producers of bioactive metabolites recovered from marine invertebrates will be successful using recombinant technology (as already discussed above for sponges of the genus *Dysidea* and their cyanobacterial symbionts) rather than attempting to isolate and cultivate respective microorganisms. Identification of biosynthetic gene clusters encoding for bioactive marine natural products of the polyketide or peptide type followed by detailed analysis of the gene architecture and sometimes even identification of the cellular localization of these genes by fluorescence in situ hybridization analysis (e.g. as described by Flatt *et al*., 2005) can be expected to shed light on the involvement of symbiotic microorganisms for natural product accumulation of their invertebrate hosts. This has been proven recently during investigations on bacterial biosynthetic gene clusters from *Paederus* beetles and from the marine sponge *Theonella swinhoei* (Piel, 2002; Piel *et al*., 2004). The two animals accumulate structurally very similar natural products which include the polyketide amide pederin (**67**) that is part of the defensive secretion of *Paederus* beetles (Kellner and Dettner, 1995) and the structurally related compounds theopederin A (**68**) and onnamide A (**69**) that are detected in *T. swinhoei* (Burres and Clement, 1989; Lee *at al*., 2005). 16 S rRNA analysis of *Paederus* beetles indicated the presence of a predominating symbiotic

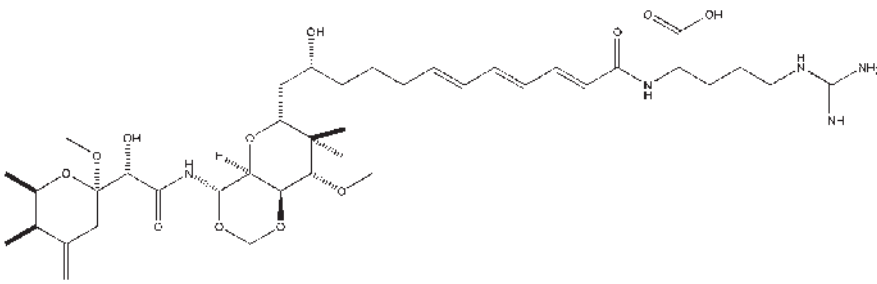
67 Pederin



68 Theopederin A



69 Onnamide



bacterium which shows similarity to *Pseudomonas aeruginosa* (Kellner, 2002). The putative biogenetic gene cluster encoding for pederin was cloned from the total DNA of the beetles. Through sequence analysis of the gene cluster that includes a mixed modular polyketide synthase/non-ribosomal peptide synthase and tailoring enzymes, the gene architecture was obtained that proved to be typical for bacteria and thereby proved bacterial origin of pederin (Piel, 2002). Using a similar strategy, closely related genes were identified in *T. swinhoei* (Piel *et al*., 2004). Analysis of their gene structure indicated that they are likewise prokaryotic and belong to so far uncultured sponge symbionts.

3.8 Conclusions and outlook

Marine chemical ecology has witnessed considerable growth and development since the first version of this chapter appeared in 1998 (Proksch, 1998). Whereas in earlier years most studies devoted to this field were still rather

descriptive, there is now a tendency to go beyond the mere observation of effects as highlighted by investigations on the mechanism of action by which furanones from the red alga *D. pulchra* interfere with bacterial quorum sensing and thus inhibit the formation of bacterial biofilms (Manefield *et al*., 2002; Steinberg and De Nys, 2002; Steinberg *et al*., 2002). A further major breakthrough came with the advent of molecular methods that finally allowed a vigorous experimental investigation of long-stated hypotheses on the contribution of microorganisms to natural product accumulation in sponges, bryozoans and other marine invertebrates as highlighted by recent studies on the origin of natural products in the sponge *T. swinhoei* (Piel, 2002; Piel *et al*., 2004). Again, with the help of molecular biology, it is now possible to unravel not only the source but furthermore also the biosynthesis of complex marine natural products that originate from polyketide synthases or non-ribosomal peptide synthases (e.g. Flatt *et al*., 2005). Some weaknesses, however, still remain. For example, very little is known about the physiological effects of deterrent natural products on potential consumers. How are these compounds perceived, how do they effect metabolic 'fitness' of consumers and how are they metabolized or excreted? A further aspect of marine chemical ecology that is in need of more detailed studies relates to the induction of defence in algae and invertebrates. As discussed earlier in this chapter, defence mechanisms of marine organisms are not necessarily static but can be highly dynamic as seen for Verongid sponges (Thoms *et al*., 2006a; Thoms and Schupp, 2008) and green algae (Paul and Van Alstyne, 1992; Jung *et al*., 2002). More research needs to be devoted into the induction of chemical defence as this phenomenon appears to be more widespread than initially thought.

Marine chemical ecology will continue to be a multidisciplinary effort. Whereas in the past marine ecologists have successfully collaborated with natural products chemists in order to structurally identify key metabolites that are responsible for certain biological phenomena, the future will witness an even wider incorporation of other disciplines such as biochemistry and molecular biology which will help the field to further move on from a descriptive level towards a more causative approach in elucidating the ecological roles, origin and modes of action of marine natural products.

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Chapter 4

PLANT–MICROBE INTERACTIONS AND SECONDARY METABOLITES WITH ANTIBACTERIAL, ANTIFUNGAL AND ANTIVIRAL PROPERTIES

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Abstract: Plants have developed effective defence strategies to protect themselves from phytopathogenic microbes and herbivores in their environment. Disease resistance in plants depends on the activation of coordinated, multicomponent defence mechanisms. One mechanism for disease resistance in plants is their ability to accumulate low-molecular-weight compounds (secondary metabolites) with high antimicrobial activities, such as alkaloids, coumarins, isoflavonoids, polyacetylenes, quinones, tannins and terpenes.

Based on this knowledge, there is every reason to believe that the plant kingdom is an important source of new antimicrobial agents with special biological targets. Thus, in the last two decades, hundreds of different new secondary metabolites were screened for their potential antibacterial, antifungal and antiviral activities. For instance, several secondary metabolites with antiviral properties have exhibited competitive in vitro and in vivo activities with those found for synthetic antiviral drugs. It has been shown that phyto-antiviral agents interfere with many viral targets, ranging from adsorption of the virus to the host cell via the inhibition of virus-specific enzymes (e.g. reverse transcriptase, protease) to release virus from the cells.

It is generally accepted that bioactive plant-derived secondary metabolites are useful leads to synthesize new and more active antimicrobial agents as well as substances with new pharmacological effects by repeated structural modification.

It is expected that structurally modified natural products will exhibit increased potency, selectivity, duration of action, bioavailability and reduced toxicity.

Keywords: plant defence system; secondary metabolites; antibacterial; antifungal; antiviral; antimicrobial agents; mode of antimicrobial action.

4.1 Introduction

Depending on the estimate, some 2×10^6 to 2×10^7 different interaction with species of bacteria, fungi and animals live on the earth. These require organic carbon from about 3×10^5 species of green, carbon dioxide-assimilating plants in order to live. As a consequence, green plants are subject to constant parasites, symbiotes, phytopathogenic microbes and herbivores.

If plants are attacked by bacteria or obligatory biotrophic fungi, various reactions may occur, depending on the nature of the organisms which are encountering each other. In a *compatible plant–pathogen interaction*, the attacking microbe is not rejected and subsequently host plant will be successfully infected by the pathogen. In an *incompatible plant–pathogen interaction*, the attacking microbe and the plant cannot coexist; the microbe is rejected by different plant defence responses. In principle, there are two types of incompatible plant–pathogen interaction named *specific plant disease resistance* and *non-specific plant disease resistance*.

4.1.1 Specific plant disease resistance (host resistance)

Specific resistance depends on the presence of a particular pathogen race (race-specific resistance), a particular host plant cultivar (cultivar-specific resistance), or both (race–cultivar-specific resistance). The race–cultivar-specific resistance results from the interaction of a particular pathogen race with a particular cultivar of the host plant. This incompatible plant–pathogen interaction is triggered by a so-called gene-for-gene resistance mechanism. It involves an interaction between a specific plant resistance gene product and a specific pathogen-derived avirulence gene product (race-specific elicitor) leading to the expression of plant defence genes. This finding means that the active host resistance is triggered by recognition of a pathogen race-specific elicitor by the plant cultivar (De Bruxelles and Roberts, 2001). A major feature of the activation of host resistance is the accumulation of several pathogen-related (PR) proteins (e.g. 1,3-β-glucanase, chitinase, chitin-binding protein). PR proteins, which are accumulate in the apoplast, are of low molecular weight, reveal highly resistant to proteolytic cleavage and exhibit extreme isoelectric points. For instance, 1,3-β-glucanase, chitinase and chitin-binding proteins were reported to inhibit the growth of various fungi, the PR protein osmotin inhibited the growth of *Phytophthora infestans* and defensin-like proteins from radish displayed antifungal effects (e.g. against *Alternaria*

longipes) when expressed in tobacco plants. In addition, there are mounting evidences that plant defence proteins can obviously act synergistically to inhibit pathogen growth (Glazebrook *et al.*, 1996; Hammerschmidt, 1999).

4.1.2 Non-specific plant disease resistance (non-host resistance)

Non-host resistance means that an entire plant species displays resistance to a specific pathogen. It is the most common one in the plant kingdom. The multicomponent complex of mechanisms for *non-specific plant disease resistance* functions by means of different defence strategies, which can be classified as *constitutive* and *infection-induced defence mechanisms*. The *constitutive defence mechanism* against bacteria, fungi and viruses includes the presence of a variety of preformed antimicrobial agents (e.g. more or less toxic low-molecular-weight secondary metabolites, such as cyanogenic glycosides, mustard oil glycosides, alkaloids, phenols, essential oils and tannins) and physical barriers (e.g. hairs, spikes, thorns, bark and bud scales), lignification, suberization and the formation of callose, agglutinins and enzyme inhibitors (e.g. extracellular microbial hydrolases). Of course, the maintenance of such a system of defence requires the investment of a considerable amount of energy, which is not then available for other plant functions (Nahrstedt, 1979).

In addition, plants have also developed an *infection-induced defence mechanism* that seems to be more economically than the constitutive one. Inducible defence responses associated with pathogen invasion are characterized by both a rapid and a delayed plant defence response (De Bruxelles and Roberts, 2001).

4.1.3 Rapid plant defence response

1. Changes in plasma membrane ion flux (e.g. Ca^{2+}, K^{+}, H^{+}).
2. Generation of active oxygen species (oxidative burst).
3. Protein phosphorylation cascades.
4. Cell wall crosslinking, lignification and production of hydroxyproline-rich glycoproteins to strengthen the cell wall barrier to pathogens.
5. Initiation of phytoalexin synthesis.
6. NO (nitric oxide) accumulation. NO has a key role in plant pathogen response.
7. Hypersensitive response. Hypersensitive cell death is a mechanism widely used by hosts to prevent the spread of pathogens, and in some cases, killing them.

4.1.4 Delayed plant defence response

In delayed plant defence response, inducible defence mechanisms offer the whole plant protection by physical responses, production of PR proteins and by systemic acquired resistance. Physical response is restricted to the site of

infection and the immediate vicinity, for example, through wound healing reactions; sealing of the cell walls with callose and lignin deposits. The ability to repair wounds can help protect the host from further infection by other, opportunistic pathogens (Kauss, 1987). PR proteins peak around 7–10 days after initial infection. Most of them reveal β-glucanase, chitinase or lysozyme activity. It is also known that chitinase and glucanase accumulate in the cell vacuols. After breakdown of the vacuol caused by the invading pathogen, different hydrolytic enzymes will be depleted with antibacterial, antifungal and antivirus activity. Systemic acquired resistance (SAR) is characterized by the increased resistance of a host to a wide range of pathogen following infection by one pathogen. SAR is a phenomenon wherein infection of a plant with a necrotizing pathogen leads to accumulation of PR proteins in the uninfected leaves far from the site of infection causing resistance to a variety of virulent pathogens. Salicylic acid has been reported to play an important role in signalling SAR (Glazebrook *et al.*, 1996).

The topic of future research will be the mechanisms by which plants activate different defence pathways in response to multiple and divergent attackers and the potential for crosstalk between the different defence pathways. In the present chapter, the phenomenon of induced local defence by means of phytoalexins is examined, together with the antimicrobial effects of preformed secondary metabolites.

4.2 Phytoalexins

In incompatible reactions, the attacking pathogen and the plant cannot coexist. The pathogen is rejected following the development of a hypersensitive reaction. In a hypersensitive reaction, affected cells or tissues are sacrificed by the plant in order to prevent the spread of the pathogen from one spot to the entire plant. After penetration of an incompatible pathogen (e.g. a fungus), the directly neighbouring cells turn brown and die off, often within a few hours. Within the resulting area of necrosis, which measures one or only a few millimetres in diameter, the pathogen cannot find any nutrition and dies (Schloesser, 1983). In such an area of necrosis, substances showing antibiotic effects can be found; namely, the phytoalexins. These compounds are synthesized by the adjacent living cells and subsequently released to the necrotic tissue.

4.2.1 Chemical structures and distribution of phytoalexins in the plant kingdom

Phytoalexins (Greek: phyton, meaning plant; alexis, meaning defence) are defined as low-molecular-weight and antibiotically effective substances of plant secondary metabolism, the synthesis and accumulation of which is induced by pathogens or herbivores (Müller and Borger, 1940). The phytoalexin

defence mechanism is not highly specific with regard to its induction, the products produced and the specificity of the products to inhibit the development of pathogens. Therefore, phytoalexin accumulation may be a primitive plant response to metabolic insult, stress or antimicrobial infection, which has persisted and remained effective as part of a multicomponent disease resistance mechanism (for comprehensive review, see Cline and Albersheim, 1981; Ebel *et al.*, 1985; Kombrink and Hahlbrock, 1985; Kuc and Rush, 1985; Ebel, 1986; Mayer, 1989; Lamb *et al.*, 1992; Niemann, 1993; Grayer and Harborne, 1994; Hammerschmidt, 1999; Grayer and Kokubun, 2001; Treutter, 2005).

The induction of phytoalexin synthesis in plant tissue has been studied mainly in pathogenic fungi; however, studies of attacks by viruses, bacteria, nematodes, arachnida and insects have also been conducted. Correspondingly, antibacterial, fungistatic and nematostatic phytoalexins have been discovered, as have those which deter insects from feeding. These substances usually demonstrate a biostatic or biocidal effect at relatively low concentrations (10^{-4} to 10^{-5} M/L). At present, we are aware of over 350 different phytoalexins in more than 100 plant species. Their molecular structures reflect the variation in secondary plant metabolic pathways, since phytoalexins can be found among the alkaloids, coumarins, dihydrophenanthrenes, flavonoids, isoflavonoids, phenols, polyacetylenes, steroids, stilbenes and terpenes (for selected phytoalexins, see Figs 4.1 and 4.2).

Phytoalexin production has been reported in Dicotyledones, rarely in Monocotyledones and Gymnosperms, and not at all in non-vascular plants (see Table 4.1). It seems that similarities are evident between phytoalexins from plants within a family. Accumulation of phytoalexins has been studied most carefully in the families of Fabaceae and Solanaceae, where the phytoalexins have chemosystematic properties. For Fabaceae, more than 100 phytoalexins have been reported belonging to isoflavonoids, furanoacetylenes, stilbenes, benzofurans, chromones and flavanones. Plants in the Fabaceae have not been reported to produce sesquiterpenoid phytoalexins. On the other hand, sesquiterpenes are typical phytoalexins for plants in the Solanaceae and Malvaceae (Sprecher and Urbasch, 1983; Wolters and Eilert, 1983). In addition, Orchidaceae synthesize dihydrophenanthrenes, Brassicaceae indole alkaloids and Poaceae stilbenes, deoxyanthocyanidins, avenanthramides and diterpenes as phytoalexins (Hammerschmidt, 1999). Furthermore, exact analysis has shown that an individual plant can often synthesize more than one and varying phytoalexins. In some cases, their chemical structures are very similar (e.g. several glyceollins synthesized by soya bean), but in others very different (e.g. rishitin and chlorogenic acid synthesized by the potato).

4.2.2 Elicitors

In order to trigger the genes necessary for phytoalexin synthesis and synthesis of the corresponding enzymes, the plant must receive information about

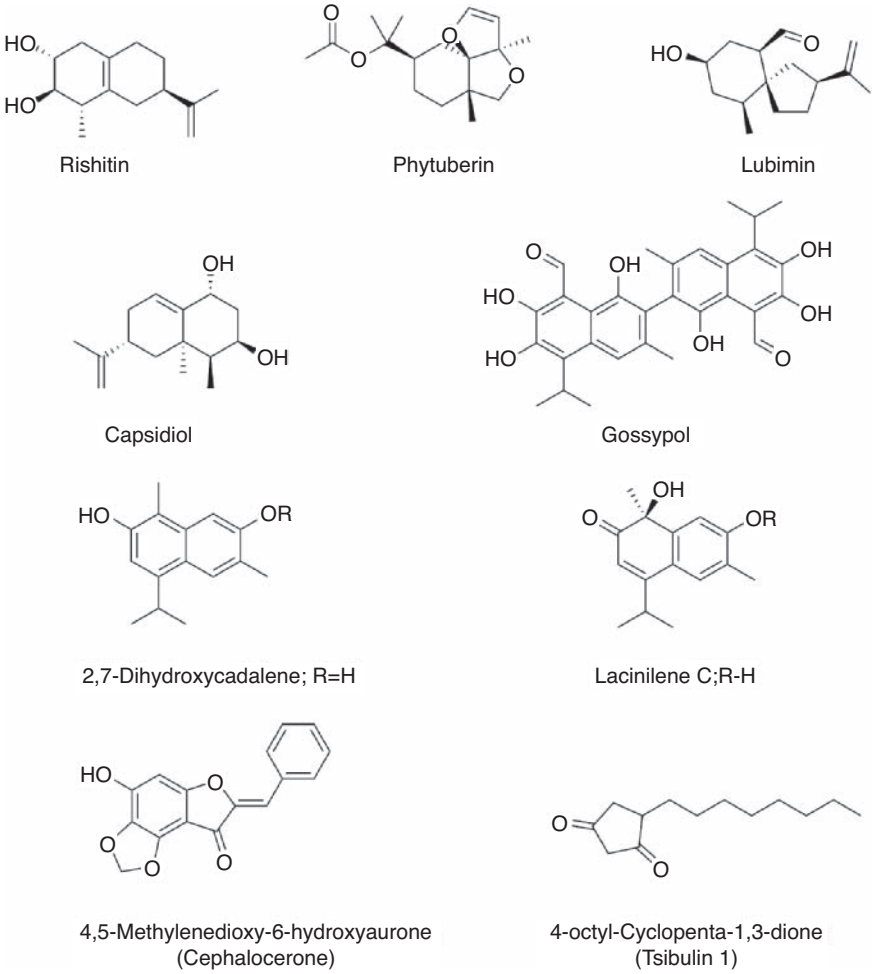


Figure 4.1 Chemical structures of selected phytoalexins.

the attack. The corresponding signals are called 'elicitors'. These are distinguished as biotic and abiotic elicitors.

4.2.2.1 Biotic elicitors

Biotic elicitors are organic substances, usually containing carbohydrates, which develop their signal effect at very low concentrations (about 10^{-9} M/L). They may originate from the attacking microbe (e.g. fungus) or the attacked plant. Such elicitors were first isolated from microbes in the cell walls of spores and hyphae of pathogenic and non-pathogenic fungi. There were, for example, oligosaccharides composed of the same saccharide units, such as β-1,3-glucan (Kombrink and Hahlbrock, 1985), oligosaccharides

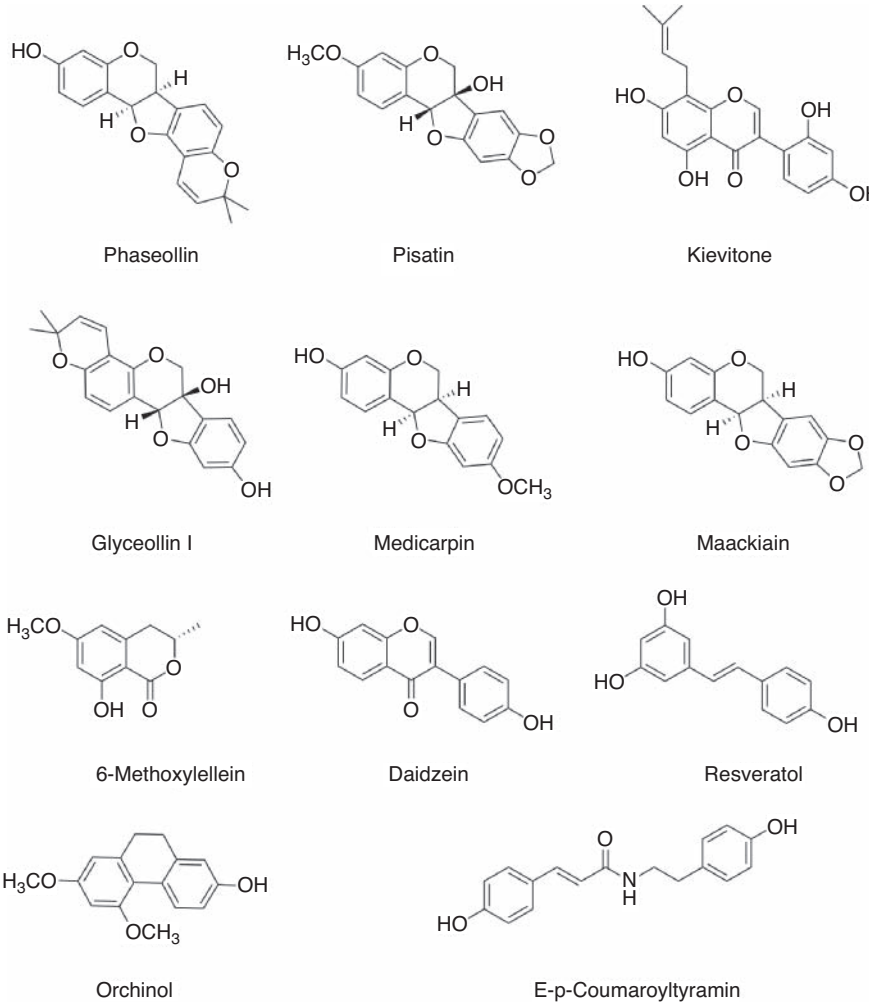


Figure 4.2 Chemical structures of selected phytoalexins.

composed of various saccharide units (Albersheim and Darvill, 1985; Davis *et al.*, 1986; Xavier *et al.*, 2004); glycoproteins (West, 1981) and fatty acids, such as eicosapentenoic acid and arachidonic acid (Bostock *et al.*, 1981; Preisig and Kuc, 1985).

At the site of infection, these elicitors are released either spontaneously or under the effect of plant cell wall enzymes from the wall of the fungus. In the interaction between soya bean and the fungus, *Phytophthora megasperma* f. sp. *glycinea*, for example, an endoglucanase and a glucosilase from the soya bean plant (Keen and Yoshikawa, 1983) induce the release of elicitors, which signal phytoalexin synthesis and a number of other defence reactions, such

Table 4.1 Phytoalexin accumulation in plants

Plant source	Phytoalexins	Fungi, bacteria and abiotic elicitors	References
Actinidiaceae			
Actinidia deliciosa	Triterpenes: actinidic acid	Fungus: *Colletotrichum musae*	Lahlou *et al.* (2001)
Apiaceae			
Apium graveolens	Furanocoumarins: angelicin; bergapten; columbianetin; isopimpinellin; psoralen; 4,5′,8-trimethylpsoralen	Fungus: *Fusarium oxysporum* f. sp. *apii*	Heath-Pagliuso *et al.* (1992)
Daucus carota	Isocoumarins: 6-methoxymellein	Fungus: *Chaetonium globosum*	Amin *et al.* (1988)
Glehnia littoralis	Furanocoumarins: bergapten; demethylsuberosin; psoralen; xanthotoxin	Bacterium: *Pseudomonas cichorii*	Masuda *et al.* (1998)
Asteraceae			
Carthamus tinctorius	Polyacetylenes: safinol; dehydrosafinol; Coumarins: ayapin	Fungi: *Phytophthora* sp.; *Alternaria carthami*	Nahrstedt (1979), Sprecher and Urbasch (1983), Wolters and Eilert (1983)
Cichorium intybus	Sesquiterpenes: cichoralexin	Bacterium: *Pseudomonas cichorii*	Monde *et al.* (1990b)
Lactuca sativa	Sesquiterpenes: lettucenin A; costunolid	Fungi: *Botrytis cinerea*; *Bremia lactucae*	Sprecher and Urbasch (1983), Wolters and Eilert (1983), Bestwick *et al.* (1995)
Helianthus annuus	Coumarins: ayapin; scopoletin	Abiotic elicitor: $CuCl_2$	Sprecher and Urbasch (1983), Wolters and Eilert (1983), Gutierrez *et al.* (1995)
Hypochoeris radicata	Sesquiterpenes: isohypoglabric acid methyl ester; hypochoeroside K Alkenals: 6-hydroxy-hexadienal; mucondi aldehyde	Abiotic elicitor: $CuCl_2$	Maruta *et al.* (1995)

(*Continued*)

Table 4.1 (*Continued*)

Plant source	Phytoalexins	Fungi, bacteria and abiotic elicitors	References
Polymnia sonchifolia	Acetophenones: 4′-hydroxy-3′-(3-methylbutanoyl)-acetophenone; 4′-hydroxy-3′-(3-methyl-2-butenyl)aceto-phenone; Benzofurans: 5-acetyl-2-(1-hydroxy-1-methylethyl)-benzofuran	Bacterium: *Pseudomonas cichorii*	Takasugi and Masuda (1996)
Brassicaceae			
Arabidopsis thaliana	Indole alkaloids: camalexin	Fungus: *Botrytis cinerea*	Kishimoto *et al.* (2006)
Brassica oleracea	Indole alkaloids: brassinin, *cyclo*-brassinin, methoxybrassinin A, methoxybrassinin B, 1-methoxybrassitin, spiroprassinin; isalexin, brassicanal C, caulilexins A–C	Bacterium: *Pseudomonas cichorii*; abiotic elicitor: UV light	Monde *et al.* (1990a, 1991), Pedras *et al.* (2006b)
Brassica napus L. ssp. *rapifera*	Indole alkaloids: brassinin, 1-methoxy-brassinin A, spirobrassinin, brassicanal C, isalexin, brassilexin, brassicanate, rutalexin	Fungus: *Albugo candida*; abiotic elicetor: UV light	Pedras *et al.* (2004), (2006a)
Cajanus cajan	Isoflavones: cajanin; genistein; 2′-hydroxy-genistein; formononetin. Isoflavanone: cajanol. Stilbenes: stilbene-2-carboxylic acid. Chalcon: pinostrobin chalcone	Fungi: *Helminthosporium carbonum*, *Botrytis cinerea*, *Fusarium udum*	Cooksey *et al.* (1982), Marley and Hillocks (2002)
Erucastrum gallicum	Indole alkaloids: indole-3-acetonitrile; arvelexin; 1-methoxy-spirobrassinin; erucalexin	Fungus: *Sclerotinia sclerotiorum*	Pedras and Ahiahonu (2004), Okinya and Pedras (2006), Pedras *et al.* (2006c)

Table 4.1 (*Continued*)

Plant source	Phytoalexins	Fungi, bacteria and abiotic elicitors	References
Thlapsi arvense	Indole alkaloids: wasalexin A, arvelexin	Fungus: *Leptosphaeria maculans*; abiotic elicitor: $CuCl_2$	Pedras *et al.* (2003)
Caryophyllaceae			
Dianthus caryophyllus	Dianthalexins, dianthramides, dianthramines, dianthanilides: e.g. hydroxydianthalexin B; hydroxydianthramide S methylester; methoxydianthramide S	Fungus: *Fusarium oxysporum* f. sp. *dianthi*	Niemann *et al.* (1991), Niemann (1993)
Convolvulaceae			
Ipomoea batatas	Furanoterpenoids: ipomoeamaron	Fungus: *Ceratocystis* sp.	Nahrstedt (1979), Sprecher and Urbasch (1983), Wolters and Eilert (1983)
Cucurbitaceae			
Cucumis sativus	C-glycosyl flavonoids	Fungus: *Podosphaera xanthii*	McNally *et al.* (2003)
Cupressaceae			
Cupressus sempervirens	Sesquiterpenes: 6-isopropyltropolone-β-glu-coside; 5-(3-hydroxy-3-methyltrans-1-butenyl)-6-isopropyltropolone-β-glucoside	Fungus: *Diplodia pinea* f. sp. *cupressi*	Madar *et al.* (1995)
Dioscoreaceae			
Dioscorea bulbifera	Dihydrostilbenes: demethylbatasin IV; dihydroresveratrol	Fungus: *Botryodiplodia theobromae*	Adesanya *et al.* (1989)
Dioscorea dumentorium	Dihydrostilbenes: demethylbatasin IV; dihydroresveratrol	Fungus: *Botryodiplodia theobromae*	Adesanya *et al.* (1989)
Euphorbiaceae			
Ricinus communis	Diterpenes: casbene	Fungi: *Rhizopus stolonifer*; *Aspergillus* sp.	Nahrstedt (1979), Lee and West (1981), Hill *et al.* (1996)

(*Continued*)

Table 4.1 (*Continued*)

Plant source	Phytoalexins	Fungi, bacteria and abiotic elicitors	References
Fabaceae			
Arachis hypogaea	Isoflavonoids: medicarpin, formononetin	Fungus: *Bradyrhizobium* sp.	Azpilicueta *et al.* (2004)
Cicer arietinum	Isoflavonoids: maackiain; medicarpin; (−)-vestitone	Fungi: *Ascochyta rabiei*; *Colletotrichum gloeosporioides*	Kessmann *et al.* (1988), Soby *et al.* (1997)
Glycine max	Isoflavonoids: glyceollin; glycinol; hydroxy-phaseollin; phaseollin; genistin; daidzin; genistein; daizein	Fungus: *Phytophthora megasperma* f. sp. g*lycinea*; nematode: *Maloidogyne incognita*	Hahn *et al.* (1985), Parniske *et al.* (1991), Carpentieri-Pipolo *et al.* (2005)
Medicago sativa	Isoflavonoids: daizein; maackiain; medicar-pin; trifolirhizin; (−)-vestitone; sativan	Fungi: *Verticillium alboatrium*; *Colletotrichum trifolii*; abiotic elicitor: $HgCl_2$	Gustine and Moyer (1982), Walton *et al.* (1993), Saunders and O'Neill (2004)
Phaseolus vulgaris	Isoflavonoids: phaseollin; phaseollidin; kievitone; wighteone; daidzein; genistein; 2′-hydroxygenistein; genistin; phaseoluteone; phaseollinisoflavan; dalbergioidin; coumestrol	Fungi: *Fusarium solani* f. sp. *phaseoli*; *Monilinia fructicola*; abiotic elicitor: $CuCl_2$	Kuc and Rush (1985), Turbek *et al.* (1992), Li *et al.* (1995), Durango *et al.* (2002)
Pisum sativum	Isoflavonoids: pisatin; (+)-2-hydroxypisatin	Abiotic elicitor: $CuCl_2$	Matthews and van Etten (1983), Miao *et al.* (1991), Kobayashi *et al.* (1993)
Trifolium pratense	Isoflavonoids: daidzein, medicarpin	Abiotic elicitor: $HgCl_2$	Gustine and Moyer (1982)
Vigna angularis	Isoflavonoids: daidzein; isoflavone; ligballinol	Abiotic elicitor: Actinomycin D	Kobayashi and Otha (1983)

Table 4.1 (*Continued*)

Plant source	Phytoalexins	Fungi, bacteria and abiotic elicitors	References
Vicia faba	Polyacetylenes: wyerone; wyerone acid; wyerone epoxide	Fungus: *Botrytis cinerea*	Nahrstedt (1979), Sprecher and Urbasch (1983), Wolters and Eilert (1983), Soylu *et al.* (2002), Kuti and Nawar (2003)
Iridaceae			
Iris pseudacorus	Isoflavones: ayamenin A; ayamenin B; ayamenin C; ayamenin D; biochanin A; irilin A; irilin B; irilin C; iristectorigenin A; iristectorigenin B; genistein, lupinalbin A; pratensein; tectorigenin	Abiotic elicitor: $CuCl_2$	Hanawa *et al.* (1991)
Juncaceae			
Juncus roemerianus	Dihydrophenanthrenes: juncusol		Boger *et al.* (1985)
Cactaceae			
Cephalocereus senilis	Aurones: 4,5-methylenedioxy-6-hydroxy-aurone	Abiotic elicitor: Chitin	Pare *et al.* (1991)
Alliaceae			
Allium cepa	5-octyl-cyclopenta-1,3-dione; 5-hexyl-cyclopenta-1,3-dione; alexin	Fungus: *Botrytis cinerea*; antibiotic elicitor: UV light	Tverskoy *et al.* (1991), Kodera *et al.* (2001)
Melanthiaceae			
Veratrum grandiflorum	Stilbenoids: resveratrol; oxyresveratrol; piceid; oxyresveratrol-3-*O*-glucoside	Antibiotic elicitor: $CuCl_2$	Hanawa *et al.* (1992)
Malvaceae			
Malva sylvestris	Naphthoquinone: malvone A	Fungus: *Verticillium dahliae*	Veshkurova *et al.* (2006)

(*Continued*)

Table 4.1 (*Continued*)

Plant source	Phytoalexins	Fungi, bacteria and abiotic elicitors	References
Gossypium hirsutum	Sesquiterpenes: gossypol; hemigossypol; deoxyhemigossypol; 2,7-dihydroxy-cadalene; 2-hydroxy-7-methoxycadalene; lacinilene C; lacinilene C 7-methyl ether; 7-hydroxycalamenene; 7-hydroxycalamenen-2-one	Bacterium: *Xanthomonas campestris pv malvacearum*; Fungi: *Aspergillus flavus*, *Verticillium dahliae*	Sprecher and Urbasch (1983), Wolters and Eilert (1983), Essenberg *et al.* (1990), Zeringue (1990), Davila-Huerta *et al.* (1995), Abraham *et al.* (1999), Bianchini *et al.* (1999)
Moraceae			
Morus alba	Phenylbenzofuran: moracin A	Fungus: *F. solani* f. sp. *mori*	Nahrstedt (1979)
Musaceae			
Musa acuminata	Phenalenones: methyl-2-benzimidazole carbamate; 2-(4′-hydroxyphenyl)-naphthalic anhydride; 2-hydroxy-9-(p-hydroxyphenyl)-phenalen-1-one	Fungi: *Colletotrichum musae*, *Fusarium oxysporum* f. sp. *Cubense*	Hirai *et al.* (1994), Borges *et al.* (2003)
Orchidaceae			
Orchis militaris	Dihydrophenanthrenes: orchinol; hircinol; loroglossol	Fungus: *Rhizoctonia* spp.	Nahrstedt (1979)
Papaveraceae			
Papaver somniferum	Alkaloids: sanguinarine	Fungus: *Sclerotina sclerotiorum*	Eilert *et al.* (1985)
Platanaceae			
Platanus acerifolia	Coumarins: scopoletin; umbelliferone	Fungus: *Ceratocystis fimbriata* f. sp. *platani*	Modafar *et al.* (1993)
Poaceae			
Avena sativa	*N*-cinnamoylanthranilic acids: avenanthramide G; avenanthramide B, bisavenanthramide B	Fungus: *Helminthosporium victoriae*; abiotic elicitor: hydrogen peroxide	Miyagawa *et al.* (1996), Okazaki *et al.* (2004)

Table 4.1 (*Continued*)

Plant source	Phytoalexins	Fungi, bacteria and abiotic elicitors	References
Oryza sativa	Diterpenes: oryzalexin A–F; phytocassanes A–E; Flavanones: sakuranetin	Fungus: *Pyricularia oryzae*; abiotic elicitor: UV light	Kato *et al.* (1993, 1994), Umemura *et al.* (2003)
Saccharum sp.	Stilbenes: piceatannol	Fungus: *Colletotrichum falcatum*	Brinker and Seigler (1991)
Zea mays	Several caffeoyl esters	Fungi: *Colletotrichum graminicola*; *Helminthosporium maydis*	Lyons *et al.* (1990)
Ranunculacea			
Thalictrum rugosum	Alkaloids: berberine	Fungus: *Saccharomyces cerevisiae*	Funk *et al.* (1987)
Rosaceae			
Mespilus germanica	Dibenzofurans: α-cotonefuran; 6-hydroxy-, 6-methoxy- and 7-hydroxy-6-methoxy-α-pyrufurans	Fungus: *Nectria cinnabarina*	Kokubun *et al.* (1995c)
Photinia davidiana	Dibenzofurans: eriobofuran; 9-hydroxyeriobofuran; 7-methoxyeriobofuran	Fungus: *Nectria cinnabarina*	Kokubun *et al.* (1995b)
Pyracantha coccinea	Dibenzofurans: eriobofuran; 9-hydroxyeriobofuran; 7-methoxyeriobofuran	Fungus: *Nectria cinnabarina*	Kokubun *et al.* (1995b)
Pyrus pyrifolia	Phenols: 3,5-di-*O*-caffeoylquinic acid	Fungus: *Alternaria alternata*	Kodoma *et al.* (1998)
Sanguisorba minor	2′,6′-Dihydroxy-4′-methoxyacetophenone	Fungus: *Botrytis cinerea*	Kokubun *et al.* (1994)
Sorbus aucuparia	Biphenyls: aucuparin, 2′-methoxyaucuparin; 4′-methoxyaucuparin; 2′-hydroxyaucuparin; isoaucuparin	Fungus: *Nectria cinnabarina*	Kokubun *et al.* (1995a)

(*Continued*)

Table 4.1 (*Continued*)

Plant source	Phytoalexins	Fungi, bacteria and abiotic elicitors	References
Rutaceae			
Citrus limon, *C. reticulata*, *C. tamurana*.	Coumarins: 6,7-dimethoxycoumarin (scoparone)	Fungus: *Botrytis cinerea*; abiotic elicitor: UV light	Kuniga *et al.* (2005), Kuniga and Matsumoto (2006)
Solanaceae			
Capsicum frutescens	Sesquiterpenes: capsidiol	Fungi: *Botrytis cinerea*; *Fusarium* sp.	Sprecher and Urbasch (1983), Wolters and Eilert (1983)
Datura stramonium	Sesquiterpenes: lubimin; hydroxylubimin	Fungi: *Monilinia fructicola*; *Phytophthora* sp.	Sprecher and Urbasch (1983), Wolters and Eilert (1983), Kuc and Rush (1985)
Lycopersicon esculentum	Sesquiterpenes: rishitin; Phenols: *p*-coumaroyltyramine, E-feruloyltyramine; Polyacetylenes: falcarinol; falcarindiol	Fungi: *P. megasperma*, *Phytium oligandrum*; abiotic elicitors: chitosan; mechanical wounding	Sprecher and Urbasch (1983), Gross (1987), Pearce *et al.* (1998), Le Floch *et al.* (2005)
Nicotiana spp.	Sesquiterpenes: capsidiol; glutinosone; oxyglutinosone; 5-epi-aristolochene; phytuberol; phytuberin	Bacteria: *Pseudomonas solanacereum*; *P. syringae*	Tanaka and Fujimori (1985)
Solanum tuberosum	Sesquiterpenes: C-1′ epimers of (2*R*,5*S*,10*R*)-2-(1′,2′-dihydroxy-1′-methylethyl)-6,10-dimethyl-spiro dec-6-en-8-one and their 2′-*O*-β-D-glucopyranosides	Fungi: *Phoma foveata*, *Fusarium* spp.	Engström (1998)
Solanum tuberosum	Sesquiterpenes: anhydro-β-rotunol; hydroxylubimin; lubimin; phytuberin; phytuberol; rishitin; rishitinol; solavetivone	Fungus: *Phytophthora infestans*	Bostock *et al.* (1981), Kuc and Rush (1985)
Vitaceae			
Vitis vinifera	Stilbenes: α-viniferin, ε-viniferin; resveratrol; piceatannol; ochratoxin A	Fungi: *Botrytis cinerea*, *Aspergillus japonicus*, *A. ochraceus*, *A. carbonarius*	Nahrstedt (1979), Bavaresco *et al.* (2003)

as local accumulation of chitinase (Roby *et al.*, 1986), local accumulation of lignin (Dean and Kuc, 1987) and systemic production of proteinase inhibitors (Walker-Simmons *et al.*, 1984).

Elicitors can also be derived from the cell wall of the infected plant. There they are broken down under the effect of enzymes of varying origin ('endogenous elicitors'). The activating enzymes may originate from the microbe; for example, an endopolygalacturonic acid lyase from the bacterium, *Erwinia carotovora* (Davis *et al.*, 1986), and an endo-1,4-polygalacturonase from the fungus, *Rhizopus stolonifer* (Lee and West, 1981) release cell wall fragments, which serve as elicitors for phytoalexin synthesis in the infected plant. In addition, the plant tissue itself may deliver the enzymes, which then release the elicitors from the plant cell wall (Albersheim and Darvill, 1985).

4.2.2.2 Abiotic elicitors

Chemical or physical factors that put a plant under stress and then also trigger the production of phytoalexins are called abiotic elicitors. These are heavy metal salts, such as $CuCl_2$ or $HgCl_2$ (Adesanya *et al.*, 1984), agents which interact with DNA, such as actinomycin D or ultraviolet rays (Hardwiger and Schwochau, 1971a, 1971b), and metabolic inhibitors, such as trichloroacetic acid, monoiodoacetate or 2,4-dinitrophenol (Cruickshank, 1966).

Although these elicitors have nothing to do with the defence of a primary attack by a foreign organism, they still provoke suitable defence reactions and protect the plant that has been weakened or injured by abiotic stress from subsequent parasitic attack, which is deterred directly by the high concentrations of phytoalexins. The observation that abiotic stimuli induce the synthesis and accumulation of phytoalexins in plant tissues can easily be explained through the stress-determined release of endogenous, constitutive elicitors from the cell wall (Albersheim and Darvill, 1985; Smith and Banks, 1986).

4.2.3 Specificity of phytoalexin accumulation

The simple definition of phytoalexins given by Müller and Borger (1940) leaves open the question whether the accumulation of phytoalexins is defensive or only a response to pathogen attack. If phytoalexins play actually a role in plant defence, there must be some measurable effects on the growth, survival and reproduction of the plant that can be attributed to the phytoalexins. So, Hammerschmidt and others (1999) formulated some additional claims to establish a role for phytoalexins in plant disease resistance:

1. 'Localization and timing of phytoalexin accumulation in infected tissue in relation to pathogen development.'
2. 'Phytoalexins must accumulate to antimicrobial levels at the infection site in resistance plants in sufficient concentrations to inhibit the pathogen at the time pathogen development is stopped.'

3. 'Strong positive correlation of rapid phytoalexin production with incompatible interactions in gene-for-gene plant pathogen systems.'
4. 'Association of rapid phytoalexin accumulation with resistance genes that condition restriction of pathogen development.'
5. 'Use of metabolic inhibitors that enhance susceptibility and block phytoalexin production.'
6. 'A positive relationship between pathogen virulence and tolerance of phytoalexins.'
7. 'An increase of plant tissue resistance by stimulation of phytoalexin production prior to inoculation.'
8. 'There must be evidence that the phytoalexins are directly involved in defence, and that this defence role has a measurable benefit for the plant.'

The majority of phytoalexins are found in or immediately adjacent to the browned, necrotic, infected tissues at concentrations that are inhibitory to the development of fungi and bacteria (Hahn *et al.*, 1981, 1985; Keen, 1986; Smith and Banks, 1986; Hammerschmidt, 1999; McNally *et al.*, 2003). In addition, at the site of the hypersensitive reaction, there is decreased phytoalexin degradation. At the same time, new synthesis is transiently greatly increased (Keen, 1986; Hammerschmidt, 1999; McNally *et al.*, 2003).

In general, the levels of phytoalexin in the plant tissue are regulated by new synthesis and degradation of secondary metabolites. Phytoalexins are synthesized relative quickly after contact with the attacking pathogen. After a lag phase, at a minimum of 2 h, the bioactive substance can be measured and the amounts increase during the following hours and days for up to about 96 h, sometimes longer, until maximum accumulation has been achieved. Subsequently, the levels of phytoalexin decrease to those which existed before the attack. This means that high levels of phytoalexin accumulation do not persist in plants once a pathogen or stress has been contained and plant metabolism has returned to normal.

The phytoalexin synthesis is set into motion by chemical signals of the attacking pathogen. In resistant plants, the chemical signal causes a rapid increase in the synthesis of key enzymes of the plant secondary metabolism. It has been shown that, prior to enzyme synthesis, the corresponding messenger ribonucleic acid (mRNA) has developed (Chappell and Hahlbrock, 1984; Cramer *et al.*, 1985; Grab *et al.*, 1985). For example, before the phytoalexins, glyceollin I–III, accumulate in the soya bean plant, the activity of a number of metabolic isoflavonoid enzymes greatly increases, namely that of phenyalanine ammonia lyase (PAL), *p*-coumaric acid- coenzyme A (CoA)-ligase, chalcone synthase and chalcone isomerase. Simultaneously, an increase in PAL, *p*-coumaric acid-CoA-ligase and chalcone synthase mRNA is seen (Ebel *et al.*, 1985; Kombrink and Hahlbrock, 1985; Smith and Banks, 1986). In seedlings of *Medicaco sativa* that has been challenged with the fungal pathogen *Colletotrichum trifolii*, the activity and transcript level of three enzymes involved

in isoflavonoid biosynthesis (PAL, cinnamic acid 4-hydroxylase, isoflavone reductase) were raised significantly. These enhanced enzyme activities were accompanied by the synthesis of the two phytoalexins medicarpin and sativa; both antimicrobial agents are biosynthetic end products of the induced enzyme cascade. So, the idea is obvious that the accumulation of medicarpin and sativa is attributable to increased expression of genes of flavonoid biosynthesis (Saunder and O'Neill, 2004). While enzymes of secondary metabolism are being newly synthesized in response to the pathogen's signal, the activities of many other enzymes of primary metabolism remain unchanged.

The synthesis and accumulation of a certain phytoalexins are induced by very different species of microbes. Cruickshank and Perrin (1961) name, for example, 19 species of fungi that induce the development of pisatin on the pods of peas. Furthermore, different races of the same fungus that are compatible with the host plant induce phytoalexin development, just like those races that are incompatible with the host and cause a hypersensitive reaction. Therefore, the phytoalexins are not a specific means of defence directed towards a certain microbe (as, for example, antibodies are); their effect is non-specific and directed against numerous microbes.

On the other hand, the difference between compatible and incompatible reaction is of interest. Here, it is seen that various races of a fungus can affect the extent and kinetics of phytoalexin accumulation to completely different degrees. Incompatible races usually cause phytoalexin synthesis to begin very quickly and the amount of phytoalexin accumulated is relatively high. It takes longer for phytoalexin synthesis to commence after an attack by a compatible race and the final levels are considerably lower. As an example, both compatible and incompatible races of *P. megasperma* f. sp. *glycinea* induce the accumulation of glyceollines in soya bean. Twelve hours after infection, the same levels of glyceollines are reached. After this time, the levels of glyceollines continue to increase greatly in the incompatible race, whereas they are likely to remain constant in the compatible race (Hahn *et al.*, 1985).

An exact analysis shows that incompatible and compatible microbes alike deliver elicitor molecules. The varying levels of phytoalexin accumulation that have been observed might be due to the following factors: (1) various species and races of microbes send differing, specific elicitor signals; (2) phytoalexins are degraded by enzymes of penetrating microbes (Maloney and van Etten, 1994; Li *et al.*, 1995). For example, among the antimicrobial phytoalexins produced by *Phaseolus vulgaris* is the prenylated isoflavonoid, kievitone. The bean pathogen, *Fusarium solani* f. sp. *phaseoli*, secretes the glycoenzyme, kievitone hydrolase, which catalyzes conversion of kievitone to a less toxic metabolite (Li *et al.*, 1995); (3) compatible species or races of microbes synthesize suppressor molecules, which annul or reduce the effect of the elicitors of different origin. Such suppressor molecules have been found in strains of the pea pathogens, *Mycosphaerella pinodes* and *Erysiphe pisi* (Schloesser, 1983).

4.2.4 Transgenic plants

Transgenic plants cloned by genes specific for phytoalexin biosynthesis may be useful tools to get more insight on the role of phytoalexins in plant defence (Hammerschmidt, 1999; He and Dixon, 2000; Essenberg, 2001; Szankowski *et al.*, 2003; Liu *et al.*, 2006; Morelli *et al.*, 2006). In recent years, several enzymes that are thought to be phytoalexin pathway-specific have been identified, and the corresponding phytoalexin-specific genes have been cloned (e.g. sesquiterpene cyclase from Solanaceae: converting farnesyl diphosphate into cyclic hydrocarbone; 5-epi-aristolochene synthase from tobacco and green pepper plants: converting farnesyl diphosphate into aristolochene).

Reveratrol (trans-3,5,4′-trihydroxystilbene) is a typical phytoalexin that evokes resistance to fungal diseases in several plant species. It is synthesized by the enzyme stilbene synthase, which appears to be deficient or lacking in susceptible plants (Jeandet *et al.*, 2002). Zhu *et al.* (2004) isolated from grapevine (*Vitis vinifera*) the stilbene gene Vst1 and introduced the construct pVst1 into papaya (*Carica papaya*) which is originally susceptible against root, stem and fruit rot caused by the fungus *Phytophthora palmivora*. The construct pVst1 contains the Vst1 gene and its pathogen-inducible promoter. In some transformed plant lines, RNA transcripts of stilbene synthase and resveratrol glycoside were induced shortly after pathogen inoculation. The authors reported that the positive transformed papaya lines revealed increased resistance to *P. palmivora*.

Ruhmann *et al.* (2006) transferred a stilbene synthase gene via *Agrobacterium tumefaciens* into apple plant (*Malus domestica*). It was shown on a molecular base that the stilbene synthase was expressed in some transgenic plant lines as well as in the skin and flesh of transgenic apple fruit. In addition, resveratrol glucoside, named piceid, was synthesized.

Plants of tomato and rice that were transformed with a grapevine stilbene synthase were more resistant to *P. infestans* and *Magnaporthe grisea* than non-transformed plants (Hammerschmidt, 1999). Similar experiments with papaya revealed high resistance against the fungal pathogen *P. palmivora* in comparison to non-transformed controls (Zhu *et al.*, 2005).

Another approach to study the relative contribution of phytoalexins in plant defence would be to produce plants that are specifically blocked in phytoalexin synthesis. For instance, Glazebrook and co-workers (1996) demonstrated for *Arabidopsis* mutants a decreased plant resistance to the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* by genetic blockage of particular defence response (e.g. camalexin synthesis).

4.3 Antibacterial and antifungal agents of higher plants

The indiscriminate use of antibiotics has resulted in the emergence of a number of resistant bacteria and bacterial strains. To overcome the increasing resistance of nosocomial bacteria, more effective antimicrobial agents with

novel modes of action must be developed. Medicinal plants used in traditional medicines to treat infectious diseases seem to be an abundant source of new bioactive secondary metabolites (see Table 4.2). Therefore, in the last few years, a variety of medicinal plants and plant extracts have been screened for their antimicrobial activity (Rios *et al.*, 1987; Greger *et al.*, 1993; Nick *et al.*, 1995; Salie *et al.*, 1996; Cantrell *et al.*, 1998; Cowan, 1999). In addition, using the bioassay-guided fractionation of bioactive plant extracts, different secondary metabolites exhibiting antimicrobial activities have been isolated. The results obtained by this method have clearly revealed that the antimicrobial activity is mainly due to alkaloids, flavonoids, phenolic compounds, terpenoids and tannins. Furthermore, essential oils have also been reported to be active against gram-positive and gram-negative bacteria, as well as against yeasts and fungi. Bioactive secondary metabolites and essential oils are considered to be part of the preformed defence system of higher plants. It is assumed that all families of higher plants possess more or less bioactive secondary metabolites involved in the comprehensive plant defence system. For the distribution of antimicrobial secondary metabolites in the plant kingdom, see Grayer and Harborne (1994).

Whilst it is beyond the scope of the present chapter to review this expanding scientific field extensively, its progress will be documented by the most important results published in the last two decades. The essential oils studied and secondary metabolites isolated were preferably tested in vitro against some of the following microorganisms: (1) gram-positive bacteria, e.g. *Bacillus cereus*, *Bacillus subtilis*, *Mycobacterium intracellulare*, *Sarcinia flava*, *Sarcinia lutea*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus faecalis*, *Streptococcus hemolyticus* and *Streptococcus pneumoniae*; (2) gram-negative bacteria, e.g. *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus morgani*, *Proteus rettgeri*, *Pseudomonas aeruginosa*. *Salmonella enteritidis*, *Salmonella typhosa*, *Salmonella typhimurium*, *Shigella flexneri* and *Shigella sonnei*; (3) yeasts, e.g. *Candida albicans*, *Candida kruzei*, *Candida tropicalis*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Torula glabrata*, *Torulopsis utilis*, *Torulopsis glabrata* and *Trichosporon capitatum*; and (4) fungi, e.g. *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Epidermophyton flocosum*, *Fusarium sporotrichoides*, *Fusarium tricintum*, *Microsporum canis*, *Penicillium rubrum*, *Penicillium spinulosum*, *Trichophyton rubrum* and *Trichophyton mentagrophytes*.

4.3.1 Essential oils with antimicrobial activity

Essential oils are widely distributed in certain plant families, e.g. Alliaceae, Apiaceae, Asteraceae, Brassicaceae, Lamiaceae, Myrtaceae and Rutaceae. They are mixtures of different lipophilic and volatile substances, such as monoterpenes, sesquiterpenes and/or phenylpropanoids, and have a pleasant odour. Therefore, testing and evaluation of antimicrobial activity is difficult because of their volatility and insolubility in water. The assay technique,

Table 4.2 Selected essential oils with antimicrobial activity

Origin of essential oil	Bacteria gram (+)	Bacteria gram (y)	Yeasts (y)	Fungi (+)	MIC (μg/mL)	References
Abies alba	+					Schales *et al.* (1993)
Achillea fragrantissima	+	y	y		850–1900	Barel *et al.* (1991)
Aegle marmelos	+	y	y	+		Pattnaik *et al.* (1996)
Ageratum conyzoids	+	y				Pattnaik *et al.* (1996)
Allium sativum			y	+	*64.0*	Aboul Ela *et al.* (1996), Pyun and Shin (2006)
Artemisia douglasiana	+	y	y		*156–625*	Setzer *et al.* (2004)
Artemisia parviflora	+	y	y			Mehrotra *et al.* (1993)
Artemisia rhoxburghiana	+	y	y			Mehrotra *et al.* (1993)
Bystropogon pulmosus	+	y	y	+		Economou and Nahrstedt (1991)
Calamintha nepeta	+	y	y		*5.0–20.0*	Panizzi *et al.* (1993)
Cinnamomum camphora	+	y	y			Hili *et al.* (1997)
Cinnamomum zeylanicum	+	y	y			Hili *et al.* (1997)
Citrus aurantium	+	y	y	+		Pattnaik *et al.* (1996)
Cochleospermum regium	+				*1500–5000*	Brum *et al.* (1997)
Commiphora mukul	+	y			*0.31–5%* of oil	Saeed and Sabir (2004)
Coriandrum sativum	+	y	y			Hili *et al.* (1997)
Cryptomeria japonica			y	+	EC_{50}: *39–110*	Cheng *et al.* (2005)
Cymbopogon citratus	+	y	y			Hili *et al.* (1997), El-Kamali *et al.* (1998)
Cymbopogonflexuosus	+	y	y	+	*0.16–11.6*	Pattnaik *et al.* (1995a,b, 1996)
Cymbopogon martini	+	y	y	+	*0.5–8.3*	Pattnaik *et al.* (1995a,b, 1996)
Cymbopogon winterianus	+	y	y	+		Pattnaik *et al.* (1995a,b, 1996)
Daucus carota	+	y			120–18500	Kilibarda *et al.* (1996)
Ellaieria cardamomum	+	y	y			Hili *et al.* (1997)
Eucalyptus globulus	+	y	y		1000–5000	Hajji and Fkih-Tetouani (1993), Chand *et al.* (1994)
Eucalyptus citriodora	+	y	y	+	*0.16–10.0*	Pattnaik *et al.* (1996)
Foeniculum vulgare	+	y			*0.25–2.0%* of oil	Hammer *et al.* (1999)

Helichrysum amorginum	+	y			750–1250	Chinou *et al.* (1996)
Helichrysum italicum	+	y			3250–3750	Chinou *et al.* (1996)
Jasona candicans	+	y				Hammerschmidt *et al.* (1993)
Jasona montana	+	y				Hammerschmidt *et al.* (1993)
Juniperus communis	+	y			*1.0–2.0%* of oil	Hammer *et al.* (1999)
Lavandula angustifolia			y		*0.69–1.8%* of oil	D'Auria *et al.* (2005)
Leptospermum scoparium	+	y			*0.12–2.0%* of oil	Reichling *et al.* (2002)
Melaleuca alternifolia	+	y	y	+	500–6000	Chand *et al.* (1994), Nenoff *et al.* (1996), Hili *et al.* (1997)
Melaleuca alternifolia	+	y			*0.12–2.0%* of oil	Carson and Riley (1994, 1995), Carson *et al.* (1995)
Melaleuca alternifolia			y		*0.03–0.125*	Oliva *et al.* (2003)
Mentha arvensis	+	y		+	*400–800*	Singh *et al.* (1992), Imai *et al.* (2001)
Mentha piperita	+	y	y	+	*0.27–10.0*	Pattnaik *et al.* (1996)
Mentha spicata	+	y			*400–800*	Imai *et al.* (2001)
Micromeria thymifolia	+	y	y	+	*0.1–60.0%* of oil	Kalodera *et al.* (1994)
Nigella sativa	+		y	+	2500	Aboul Ela *et al.* (1996), El-Kamali *et al.* (1998)
Ocimum gratissimum	+	y	y	+	312.5–625	Ndounga and Ouamba (1997)
Ocimum basilicum	+	y	y	+	1250–5000	Ndounga and Ouamba (1997)
Pelargonium graveolens	+	y	y	+		Pattnaik *et al.* (1996)
Peumus boldus	+	y	y		*0.9–58.0*	Vila *et al.* (1999)
Picea abies	+		y		1.6–100	Kartnig *et al.* (1991), Schales *et al.* (1993)
Pimpinella anisum			y	+	*0.78–1.56%* of oil	Kosalec *et al.* (2005)
Pinus sylvestris	+	y				Schales *et al.* (1993)
Paper angustifolia	+	y	y	+	10–100	Tirillini *et al.* (1996)
Pogostemon patchouli	+	y	y	+		Pattnaik *et al.* (1996)
Pulicaria undulata	+	y				El-Kamali *et al.* (1998)
Rosmarinus officinalis	+	y	y		*5.0–40.0*	Panizzi *et al.* (1993), Hili *et al.* (1997)

(*Continued*)

Table 4.2 (*Continued*)

Origin of essential oil	Bacteria gram (+)	Bacteria gram (y)	Yeasts (y)	Fungi (+)	MIC (μg/mL)	References
Salvia officinalis	+	y	y	+		Hili *et al.* (1997)
Salvia sclarea				+	*EC_{50}: 493–584 μL/L*	Pitarokili *et al.* (2002)
Satureja montana	+	y	y		*5.0–40.0*	Panizzi *et al.* (1993)
Sideritis cladestina	+	y	y			Gergis *et al.* (1990)
Sideritis sipylea	+	y	y			Gergis *et al.* (1990)
Syzygium aromatikum	+	y	y			Hili *et al.* (1997)
Tagetes patula			y	+	*1.25–10.0 μL/mL*	Romagnoli *et al.* (2005)
Thymbra capitata			y	+	0.08–0.32 μL/mL	Salgueiro *et al.* (2004)
Thymus vulgaris	+	y	y		*1.0–5.0*	Panizzi *et al.* (1993)
Thymus vulgaris	+	y	y		125–8000	Janssen *et al.* (1988)
Thymus masticina	+	y	y			Hili *et al.* (1997)
Thymus pulegioides			y	+	0.16–0.64 μL/mL	Pinto *et al.* (2006)
Ziziphora clinopodioides	+	y			*3750*	Sonboli *et al.* (2006)

Note: Values in italics indicate agar/broth dilution method.
MIC, minimum inhibitory concentration; gram(+), gram-positive; gram(−), gram-negative. EC_{50}, effective concentration of the test compound which inhibit the growth of fungi by 50%.

the growth medium and the microorganisms used are especially important when testing essential oils. Depending on its insolubility in water, an emulsifying agent, e.g. Tween 80, dimethyl sulphoxide (DMSO), has to be used to disperse the essential oil in broth liquid or agar medium. All these facts should be taken into account when estimating the antimicrobial activity of essential oils examined by different researchers.

4.3.1.1 Medicinal plants with antimicrobial active essential oils

Essential oils derived from different plant species have been used for a variety of purposes ranging from the use of rosewood in perfumery, to flavouring drinks with fennel or juniper berry oil, and the application of lemongrass oil for the preservation of stored food crops. During the last two decades, a large number of studies have been performed to assess the antimicrobial activity of different essential oils (Jalsenjak *et al.*, 1987; Gergis *et al.*, 1990; Barel *et al.*, 1991; Economou and Nahrstedt, 1991; Kartnig *et al.*, 1991; Hammerschmidt *et al.*, 1993; Panizzi *et al.*, 1993; Schales *et al.*, 1993; Carson and Riley, 1994, 1995; Kalodera *et al.*, 1994; Carson *et al.*, 1995; Pattnaik *et al.*, 1995a, b, 1996, 1997; Kilibarda *et al.*, 1996; Nenoff *et al.*, 1996; Tirillini *et al.*, 1996; Hili *et al.*, 1997; Hammer *et al.*, 1999; Dorman and Deans, 2000; Horne *et al.*, 2001; Hammer *et al.*, 2004; Setzer *et al.*, 2004; Carson *et al.*, 2006; Reichling *et al.*, 2009). All essential oils investigated worldwide exhibited antimicrobial activity against at least one of the microorganisms tested (see also Table 4.2). The antimicrobial activity of plant-derived essential oils formed the basis of many applications, especially in food preservation, aromatherapy and in complementary medicine.

4.3.1.2 Essential oils with anti-Helicobacter activity

Helicobacter pylori is a gram-negative bacterium that colonizes the epithelial surface of gastric mucosa. Nowadays, there is no doubt that *H. pylori* is a major etiological agent of acute and chronic gastritis. The role of the bacterium in the pathogenesis of peptic ulcer as well as in the development of adenocarcinoma of the distal stomach has been well established.

To cure a *H. pylori* infection, a combined treatment of proton pump inhibitor with two antibiotics has shown to be successful. Since recent reports on antibiotic resistance, it is also necessary to find new agents against this type of bacterium as alternatives to existent antibiotics or as adjuvant agents in combination with established and still effective antibiotics.

Recently, isolated plant substances (e.g. alkaloids, flavonoids, polysaccharides) as well as plant extracts have been shown to be effective against *H. pylori*. In the last decade, several research groups have investigated essential oils from different plant origin for their anti-*Helicobacter* activity using a broth microdilution/macrodilution method (Bergonzelli *et al.*, 2003; Ohno *et al.*, 2003; Weseler *et al.*, 2005). All essential oils tested exhibited a high anti-*Helicobacter* activity in vitro with MIC/MBC values of 20.0–589.4 μg/mL. Of all essential oils tested, carrot seed oil was the most active one with an MBC

Table 4.3 Essential oils with antibacterial activity against *Helicobacter pylori*

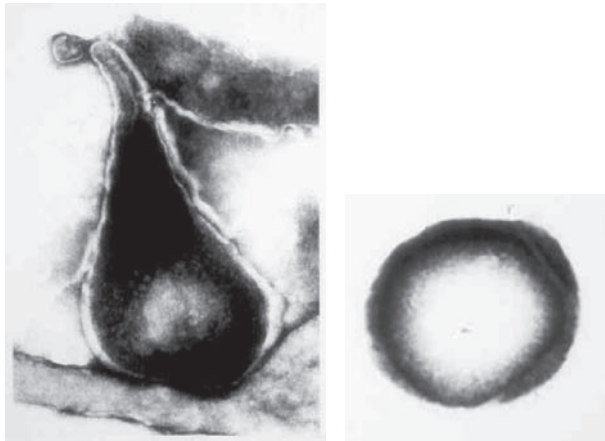
Origin of essential oil	MIC/MBC (μg/mL)	References
Daucus carota	*20.0*	Bergonzelli *et al*. (2003)
Cinnamomum zeylanicum	*40.0*	Bergonzelli *et al*. (2003)
Satureja montana	*40.0*	Bergonzelli *et al*. (2003)
Matricaria chamomilla	35.7–70.4	Weseler *et al*. (2005)
Nepeta argolica	64.0	Kalpoutzaki *et al*. (2001)
Citrus aurantium	65.1	Weseler *et al*. (2005)
Mentha spicata	50.0–100.0	Imai *et al*. (2001)
Zingiber officinalis	65.4–130.9	Weseler *et al*. (2005)
Eugenia caryophyllus	*100.0*	Bergonzelli *et al*. (2003)
Mentha arvensis	*100.0*	Imai *et al*. (2001)
Nepeta camphorata	*128.0*	Kalpoutzaki *et al*. (2001)
Melissa officinalis	*135.7*	Weseler *et al*. (2005)
Menta piperita	*135.7*	Weseler *et al*. (2005)
Salvia officinalis	*137.6*	Weseler *et al*. (2005)
Rosmarinus officinalis	*137.0*	Weseler *et al*. (2005)
Leptospermum scoparium	*140.0*	Weseler *et al*. (2005)
Elettaria cardamomum	*130.0–278.0*	Weseler *et al*. (2005)
Thymus vulgaris	*275.2*	Weseler *et al*. (2005)
Coriandrum sativum	*259.3*	Weseler *et al*. (2005)
Foeniculum vulgare	*288.3*	Weseler *et al*. (2005)
Carum carvi	*273.1*	Weseler *et al*. (2005)
Ocimum basilicum	*286.7–573.4*	Weseler *et al*. (2005)
Illicium verum	*294.7–589.4*	Weseler *et al*. (2005)
Melaleuca alternifolia	*539.0*	Weseler *et al*. (2005)

Note: Values in italics indicate MBC values.
MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

value of 20.0 μg/mL (see Table 4.3). Moreover, recent studies reported the in vivo (e.g. mice and rats) efficiency of different essential oils against antibiotic-susceptible and -resistant *H. pylori* strains. It was also of interest that the bactericidal activities of the essential oils tested were enhanced at acidic pH values (Bergonzelli *et al.*, 2003; Ohno *et al.*, 2003; Tzakou and Skaltsa, 2003). So, some scientists speculate that the anti-*Helicobacter* activities of several essential oils are not irrelevant if one intends to use them as food supplement to complement standard therapy (Bergonzelli *et al.*, 2003).

4.3.1.3 Tea tree (*Melaleuca alternifolia*) oil with anti-*Mycoplasma pneumoniae* activity

Mycoplasmas are bacteria without a rigid cell wall. Their physiological habitats are plants and animals but in various circumstances they may become pathogen for human too. *M. pneumoniae* is spread all over the world. It frequently causes atypical courses of pneumonia, particularly in children between 5 and 15 years and adults between 30 and 35 years. As a result of lung inflammations, myocarditis, arthritis, polyneuritis and other chronical diseases may appear. Tetracyclines and macrolides are the preferred antibiotics



(a) (b)

Figure 4.3 Cell shape of *Mycoplama pneumoniae* before treatment (a) and after treatment (b) with 0.25% TTO.

in the treatment of mycoplasmal infections. However, in recent years, bacterial strains emerged with a resistance to macrolide antibiotics.

The most common morphological shape of *M. pneumoniae* is the typical 'pear shape' with a tip structure at one end of the cell (see Fig. 4.3a). There are specific protein filaments inside the tip structure which are taken to be a cytoskeleton (Harkenthal *et al.*, 2000).

If cells of *M. pneumoniae* were treated with 0.25% tea tree oil (TTO) in ethanol (1%) for 12 h, the cells lost their typical 'pear-shaped' appearance and became rounded (see Fig. 4.3b). The rounded shape resembles mutants which have lost their virulence as a result of this morphological change and the loss of its attachment site. TTO seems to affect the intracellular cytoskeletal structure in a way that *M. pneumoniae* cells become rounded and lose their virulence. On the other hand, the integrity of cell membrane was not impaired by TTO (Harkenthal *et al.*, 2000).

In a recent in vitro experiment, Furneri *et al.* (2006) exposed 25 clinically isolated strains and 1 reference strain of *Mycoplasma hominis* (from vagina, urethra, cervix), 1 clinically isolated strain and 1 reference strain of *M. pneumoniae* and 4 clinically isolated strains (from vagina) and 2 reference strains of *Mycoplasma fermentans* to TTO. The MIC values were determined by a broth microdilution assay (see Table 4.4).

4.3.1.4 Mode of antimicrobial action

While essential oils were extensively tested against a broad spectrum of bacteria, yeasts and fungi, the interaction between essential oils and microbes which ultimately induce the antimicrobial activity is not well understood. Essential oils are complex mixtures of different compounds having diverse chemical structures and low molecular weights. Against this background, it

Table 4.4 Susceptibility of different *Mycoplasma* species against tea tree oil (TTO)

Bacteria	MIC (% v/v)
Mycoplasma hominis (26 isolates)	0.06–0.12
Mycoplasma fermentans (6 isolates)	0.01–0.06
Mycoplasma pneumoniae (2 isolates)	0.01

Note: All *Mycoplama* species tested revealed, independently of the origin, a high susceptibility against TTO in vitro.

seems unlikely that there should be only one mechanism of action or that only one compound should be responsible for the antimicrobial activity of essential oils. Therefore, different target sites and modes of action are discussed (see Table 4.5). Recently, Takaisi-Kikuni and co-workers (1996) studied the effect of various amounts of the essential oil of *Cymbopogon densiorus* (lemongrass oil) on the metabolic activity, growth and morphology of *S. aureus*. Relatively high concentrations of the oil impaired staphylococcal growth in a bacteriostatic manner (chloramphenicol-type), and in low doses metabolism became ineffective due to energy losses in the form of heat. Ultrastructural data revealed morphological changes characteristic of the induction of bacteriolysis by bactericidal antibiotics (penicillin-type). Hammer and co-workers (2004) investigated the antifungal effects of tea tree (*M. alternifolia*) oil and several of its components on *C. albicans*, *C. glabrata* and *Saccharomyces cerevisea*. TTO and components were reported to alter both permeability and membrane fluidity of the yeasts tested. Based on these results, it was assumed that the essential oils may have antimicrobial activity by influencing bacterial and fungal targets involved in cytoplasmatic and cell wall metabolism. It is stated by several researchers that especially monoterpenes will increase cytoplasmic membrane fluidity and permeability, disturb the order of membrane embedded proteins, inhibit cell respiration and alter ion transport processes (Sikkema *et al.*, 1994; Reichling *et al.*, 2006). In addition, Pattnaik and co-workers (1995a, b, 1996) showed that palmarosa oil and peppermint oil induced the formation of an elongated, filamentous form of *E. coli* cells at concentrations of 1.66 μg/mL.

4.3.2 Isolated secondary plant metabolites with antimicrobial properties

4.3.2.1 Alkaloids

The antimicrobial activity of alkaloids has been extensively reviewed (Clark and Hufford, 1992; Wink, 1993). Recently, Verpoorte (1998) published another comprehensive review on this subject. The review considered about 300 alkaloids with antimicrobial activity and reported that bioactive alkaloids could be found within acridone-, aporphine-, benzophenanthridine-, bisbenzylisoquinoline-, indole-, isoquinoline-, piperidine-, protoberberine-,

Table 4.5 Cell targets and physiological effects of selected essential oils and individual oil components

Targets	Bacteria/Fungi	Substances	References
Cell morphology			
Forming elongated filamentous forms after treatment with essential oil; normal cells: 3–5 μm in length; elongated cells: 10–25 μm in length	*Escherichia coli*	Palmrose oil; peppermint oil	Pattnaik *et al.* (1995a)
Alteration of cell shape: cells of wild type exhibit a flask-shaped morphology, whereas TTO treated strains form ovoid or round cells.	*Mycoplasma pneumoniae*	Tea tree oil (TTO)	Harkenthal *et al.* (2000)
Cytoplasmic membrane (alteration of integrity and permeability)			
Inhibition of cell respiration	*Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*	TTO	Cox *et al.* (1998), Cox *et al.* (2000), Carson *et al.* (2006)
Inhibition of oxygen uptake, respiratory electron flow and oxidative phosphorylation	*Rhodopseudomonas sphaeroides*	Thymol, carvacrol and other monoterpene alcohols	Knobloch *et al.* (1986)
K^+ leakage	*Escherichia coli*, *Staphylococcus aureus*	TTO; TTO, farnesol, nerolidol	Inoue *et al.* (2004), Cox *et al.* (1998), Cox *et al.* (2000)
Depletion of intracellular ATP concentration	*Escherichia coli*, *Listeria monocytogenes*	Oregano oil, cinnamon oil, savory oil; carvacrol, thymol	Helander *et al.* (1998), Oussalah *et al.* (2006)
Formation of multilamellar, mesosome-like structures	*Staphylococcus aureus*	TTO; terpinen-4-ol	Carson *et al.* (2002), Reichling *et al.* (2002)
Changes in membrane permeability	*Candida albicans*, *Candida glabrata*, *Saccharomyces cerevisiae*	TTO; terpinen-4-ol; α-terpineol; 1,8-cineol; γ-terpinene; α-terpinene	Hammer *et al.* (2004)

(*Continued*)

Table 4.5 (*Continued*)

Targets	Bacteria/Fungi	Substances	References
Changes in membrane fluidity	*Candida albicans*, *Candida glabrata*, *Saccharomyces cerevisiae*	TTO; 1,8-cineol; terpinen-4-ol; α-terpinene	Hammer *et al.* (2004)
Lesion of cytoplasmic membrane; reduction of ergosterol content in the cell membrane	*Candida albicans*, *Aspergillus fumigatus*	*Thymus pulegioides* oil	Pinto et al. (2006)
Cell wall			
Formation of extra cellular blebs	*Escherichia coli*	TTO; lemongrass oil	Ogunlana *et al.* (1987); Gustafson *et al.* (1998)
Disintegration of outer membrane (OM) and OM-associated LPS release	*Escherichia coli*	Thymol, carvacrol	Helander *et al.* (1998)
Cell lysis	*Streptococcus pneumoniae*, *Escherichia coli*, *Bacillus subtilis*	Oregano oil, thyme oil; oregano oil, glove oil	Horne *et al.* (2001), Rhayour *et al.* (2003)
Cell division			
Total inhibition of cell division	*Staphylococcus aureus*	TTO	*Reichling et al.* (2002)
Anti-R-plasmid activity			
Elimination of R-plasmids	*Escherichia coli*	Peppermint oil, rose mary oil, eucalyptus oil; menthol	Schelz *et al.* (2006)
Cell cytoplasm/cytosol			
Formation of condensed, filamentous, electron dense material in the cytoplasm/cytosol	*Staphylococcus aureus*	TTO	*Reichling et al.* (2002)

Note: Bacteria: *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Streptococcus pneumoniae, Rhodopseudomonas sphaeroides, Listeria monocytogenes, Mycoplasma pneumoniae.* Fungi: Candida albicans.

quinoline-, terpenoid- and steroid-type alkaloids. The studies on the antimicrobial activity of alkaloids published in the last decade are summarized in Table 4.6 (for more detail, see Verpoorte, 1998).

Dictamnine, a furoquinoline alkaloid, isolated from the root bark of *Dictamnus dasycarpus* (a traditional Chinese medicine), exhibited strong antifungal activity against the pathogenic fungus, *Cladosporium cucumerium* (minimal concentration required to cause 50% inhibition [MIC_{50} 25.0 μg/mL]). It is an interesting fact that Grayer and Harborne (1994) have previously suggested

Table 4.6 Alkaloids with antimicrobial activity

Alkaloids	Bacteria gram (+)	Bacteria gram (y)	Yeasts (y)	Fungi (+)	MIC (μg/mL)	References
Diterpenoid alkaloids						
8-Acetylheterophyllisine				+	100–250	Rahman *et al.* (1997)
Panicutin				+	75–200	Rahman *et al.* (1997)
Vilmorrianone				+	100–225	Rahman *et al.* (1997)
Terpenoid indole alkaloids						
Yohimbine-heteroyohimbine-type						
Usambarensine and derivatives						Caron *et al.* (1988)
Dihydro-(3α)	+				64.0	Caron *et al.* (1988)
Tetrahydro-(3α,17α)	+					Caron *et al.* (1988)
Tetrahydro-(3α,17β)	+				32.0	Caron *et al.* (1988)
Tetrahydrousambarensine derivatives						
10′-OH-(3α,17α)	+					Caron *et al.* (1988)
10′-OH-(3α,17β)	+					Caron *et al.* (1988)
10,10′-diOMe-(3α,17α)	+					Caron *et al.* (1988)
10,10′-diOMe,NMe-(3α,17α)	+					Caron *et al.* (1988)
10′-OH,10-OMe,NMe-(3α,17α)	+					Caron *et al.* (1988)
10,10′-diOH,NME-(3α,17α)	+					Caron *et al.* (1988)
10-OH,10′-OMe,NMe-(3α,17α)	+					Caron *et al.* (1988)
Cinchophylline (3α,17α)	+					Caron *et al.* (1988)
Cinchophylline (3α,17β)	+					Caron *et al.* (1988)
Cinchophylline (3β,17α)	+				16.0	Caron *et al.* (1988)
Cinchophylline (3β,17β)	+				32.0	Caron *et al.* (1988)

(*Continued*)

Table 4.6 (*Continued*)

Alkaloids	Bacteria gram (+)	Bacteria gram (y)	Yeasts (y)	Fungi (+)	MIC (μg/mL)	References
Other derivatives						
17,4′-Didehydro (3α)	+					Caron *et al.* (1988)
18,19-Dihydro (3α,17α)	+					Caron *et al.* (1988)
18,19-Dihydro (3α,17β)	+					Caron *et al.* (1988)
18,19-Dihydro (3β,17α)	+					Caron *et al.* (1988)
18,19-Dihydro (3β,17β)	+					Caron *et al.* (1988)
19-OH,18,19-Dihydro (3α,17α)	+					Caron *et al.* (1988)
19-OH,18,19-Dihydro (3α,17β)	+				32.0	Caron *et al.* (1988)
19-OH,18,19-Dihydro (3β,17α)	+					Caron *et al.* (1988)
19-OH,18,19-Dihydro (3β,17β)	+					Caron *et al.* (1988)
Ochrolifuanine E	+					Caron *et al.* (1988)
Ochrolifuanine F	+	y			32.0	Caron *et al.* (1988)
18,19-Dehydroo-chrolifuanine F	+	y				Caron *et al.* (1988)
Iboga-type						
Conoduramine	+	y			15–400	Munoz *et al.* (1994)
Conodurine	+	y			4–400	Munoz *et al.* (1994)
Miscellaneous terpenoid indols						
Stemmadenine	+	y	y		*1.2–37.5*	Mariee *et al.* (1988)
Various indols						
Clausenal	+	y	y	+	*3.0–25.0*	Chakraborty *et al.* (1995)
Cryptoheptine	+	y			*6.2–100*	Paulo *et al.* (1994, 1997)

Cryptolepine	+	y	y		*1.5–500*	Paulo *et al.* (1994, 1997); Sawer *et al.* (1995)
Cryptolepine	+	y	y		*6.3–500*	Cimanga *et al.* (1996)
Cryptoquindoline	+	y			*100*	Paulo *et al.* (1994, 1997)
Harman				+	*25–100*	Quetin-Leclercq *et al.* (1995)
Harmaline	+	y	y	+		Ahmad *et al.* (1992)
Harmalol	+	y		+		Ahmad *et al.* (1992)
Harmine	+	y	y	+		Ahmad *et al.* (1992)
Harmine			y	+	*12.5–100*	Quetin-Leclercq *et al.* (1995)
Harmol	+	y	y	+		Ahmad *et al.* (1992)
Harmol				+	100	Ahmad *et al.* (1992)
Norharman			y	+	*12.5–100*	Quetin-Leclercq *et al.* (1995)
N_b-Methylharmalan			y		*50.0*	Quetin-Leclercq *et al.* (1995)
Melinonine F			y		*12.5–100*	Quetin-Leclercq *et al.* (1995)
Hydroxycryptoleptine	+	y			*100*	Paulo *et al.* (1994)
Quindoline	+	y			*100*	Paulo *et al.* (1994)
Quindoline	+	y	y		*8–500*	Cimanga *et al.* (1996)
Quadrigemine B	+	y			*125*	Mahmud *et al.* (1993)
Yuehchukene	+				20.0–25.0	Waterman (1990)
Pyrrolidinoindoline-type						
Isopsychotridine E	+		y	+	5.0–100	Saad *et al.* (1995)
Hodgkinsine A	+		y	+	5.0–100	Saad *et al.* (1995)
Quadrigemine C	+		y	+	5.0–50	Saad *et al.* (1995)
Quadrigemine H	+		y	+	10.0–75	Saad *et al.* (1995)
Psychotridine E	+		y	+	25.0–100	Saad *et al.* (1995)
Vatine	+		y	+	10.0–100	Saad *et al.* (1995)
Vatine A	+		y	+	25.0–100	Saad *et al.* (1995)
Vatamine	+		y	+	10.0–100	Saad *et al.* (1995)

(*Continued*)

Table 4.6 (*Continued*)

Alkaloids	Bacteria gram (+)	Bacteria gram (y)	Yeasts (y)	Fungi (+)	MIC (μg/mL)	References
Vatamidine	+		y	+	25.0–100	Saad *et al.* (1995)
Isoquinoline alkaloids						
Bisbenzylisoquinoline-type						
Dehatrine	+			+	*300–1000*	Tsai *et al.* (1989)
Aporphine-type						
Actinodaphnine	+	y	y	+	*50–1000*	Tsai *et al.* (1989)
Anhydroushinsunine			y	+	*125–1000*	Tsai *et al.* (1989)
Anhydroushinsunine methoiodide			y	+	*62.5–1000*	Tsai *et al.* (1989)
Anonaine	+	y	y	+	*3.0–125*	Tsai *et al.* (1989); Simeon *et al.* (1990), Paulo *et al.* (1992)
Asimilobine	+		y		*12.0–100*	Simeon *et al.* (1990)
Vbulbocapnine	+	y	y		*1000*	Abbasoglu *et al.* (1991)
O-Methylbulbocapnine			y		*500–1000*	Tsai *et al.* (1989)
O-Methylbulbocapnine methoiodide	+				*300*	Tsai *et al.* (1989)
Dicentrine methoiodide	+				*50.0*	Tsai *et al.* (1989)
Glaucine methoiodide	+				*4300*	Tsai *et al.* (1989)
Glaziovine	+				*12.0–50.0*	Simeon *et al.* (1990)
Isoboldine	+	y		+	*500*	Abbasoglu *et al.* (1991); Paulo *et al.* (1992)
Lanuginosine	+			+	*25.0–100*	Simeon *et al.* (1990), Ferdous *et al.* (1992)
Laurelliptine				+	*500*	Paulo *et al.* (1992)
Laurotetanine	+	y	y		*100–1000*	Tsai *et al.* (1989)
Liriodenine	+	y	y	+	*0.4–100*	Simeon *et al.* (1990), Pabuccuoglu *et al.* (1991)

Liriodendronine			y		*25.0*	Pabuccuoglu *et al.* (1991)
Liriodenine methoiodide	+		y		*0.4–6.0*	Pabuccuoglu *et al.* (1991)
Lysicamine	+		y	+	*12.0–26.0*	Simeon *et al.* (1990), Pabuccuoglu *et al.* (1991)
Lysicamine methoiodide			y		*0.8–6.2*	Pabuccuoglu *et al.* (1991)
Magnoflorine			y		*250*	Tsai *et al.* (1989)
N-Methylxylopine methoiodide	+	y			*50–300*	Tsai *et al.* (1989)
Norushinsunine	+		y		*6.0–100*	Simeon *et al.* (1990)
N-Methylasimilobine	+				*25.0*	Simeon *et al.* (1990)
Nuciferine	+				*50–1000*	Simeon *et al.* (1990)
N-Methylactinodaphnine	+	y	y	+	*50–1000*	Tsai *et al.* (1989)
N-Methyllaurotetanine	+	y	y		*100–1000*	Tsai *et al.* (1989)
Roemerine methoiodide	+				*50–100*	Tsai *et al.* (1989)
2-*O*-Methylliriodendronine			y		*50.0*	Pabuccuoglu *et al.* (1991)
Oxostephanine	+	y			*50–150*	Ferdous *et al.* (1992)
Xylopine	+		y		*25.0–100*	Tsai *et al.* (1989), Simeon *et al.* (1990)
Benzophenanthridine-type						
Chelerythrine	+	y	y	+	*6.3–100*	Abbasoglu *et al.* (1991)
Sanguinarine	+	y	y	+	*0.5–100*	Abbasoglu *et al.* (1991)
Protoberberine-type						
β-Allocryptopine	+	y				Abbasoglu *et al.* (1991)
Berberine	+	y	y	+	*0.31–100*	Okunade *et al.* (1994)
Canadine	+	y				Abbasoglu *et al.* (1991)
Corydaline	+	y				Abbasoglu *et al.* (1991)
Cryptopine	+	y				Abbasoglu *et al.* (1991)
Ophiocarpine	+	y				Abbasoglu *et al.* (1991)
Palmatine	+	y	y		*1000*	Abbasoglu *et al.* (1991)

(*Continued*)

Table 4.6 (*Continued*)

Alkaloids	Bacteria gram (+)	Bacteria gram (y)	Yeasts (y)	Fungi (+)	MIC (μg/mL)	References
Protopine	+	y			*100*	Abbasoglu *et al.* (1991)
Scoulerine	+	y				Abbasoglu *et al.* (1991)
Stylopine	+	y				Abbasoglu *et al.* (1991)
Tetrahydropalmatine	+				*50.0*	Simeon *et al.* (1990)
Miscellaneous isoquinoline alkaloids						
Adlumidine	+	y				Abbasoglu *et al.* (1991)
Bicuculline	+	y				Abbasoglu *et al.* (1991)
Corydaldine	+	y				Abbasoglu *et al.* (1991)
Crinamine	+				10.0	Adesanya *et al.* (1992)
Diacetylcrinamine	+				10.0	Adesanya *et al.* (1992)
Diacetalhamayne	+				10.0	Adesanya *et al.* (1992)
Eupolauridine			y		*1.56*	Liu *et al.* (1990)
Hydrastinine	+	y				Abbasoglu *et al.* (1991)
3-Methoxysampangine			y	+	*0.2–3.1*	Liu *et al.* (1990)
Steroidal alkaloids						
α-Chaconine				+	*60–100* μM	Fewell and Roddick (1993)
α-Solanine				+	*80–100* μM	Fewell and Roddick (1993)
Miscellaneous types of alkaloids						
Furoquinoline-type						
Dictamnine				+	25.0	Zhao *et al.* (1998)
Piperidine-type						
Julifloricine	+	y	y		0.5–100	Aqeel *et al.* (1989), Tawara *et al.* (1993)
Euphococcinine		y			*1000*	Aqeel *et al.* (1989), Tawara *et al.* (1993)

Pyrrolizidine-type					
9-Angeloylretronecine		y	y	+	Marquina *et al.* (1989)
Heliotrine			y		Marquina *et al.* (1989)
Lasiocarpine	+		y	+	Marquina *et al.* (1989)
Supinine			y		Marquina *et al.* (1989)
Miscellaneous-types					
Antofine	+			+	Baumgartner *et al.* (1990)
Ficuseptine	+			+	Baumgartner *et al.* (1990)
Illukumbin B				+	Greger *et al.* (1992, 1993)
Methylillukumbin B				+	Greger *et al.* (1992, 1993)
Methylillukumbin A				+	Greger *et al.* (1992, 1993)
N-Methylsinharine				+	Greger *et al.* (1992, 1993)
Sinharine				+	Greger *et al.* (1992, 1993)

Note: Values in italics indicate agar/broth dilution method.
MIC, minimum inhibitory concentration; gram (+), gram-positive bacteria; gram(−), gram-negative bacteria.

that furoquinoline alkaloids may play an important role in the defence of plants against potentially pathogenic fungi.

Recently, Colombo and Bosisio (1996) reported on the pharmacological activity of *Chelidonium majus* (Papaveraceae). The plant has a long history of use in the treatment of several diseases in European countries. It contains various isoquinoline alkaloids with protopine, protoberberine and benzophenanthridine structures, e.g. sanguinarine, chelidonine, chelerythrine, berberine and coptisine. *C. majus* extracts and their purified compounds exhibited interesting antiviral, antitumoral and antimicrobial properties both in vitro and in vivo. Sanguinarine and chelerythrine have been reported to display antibacterial activity, with an MIC value of 6.25 μg/mL. Sanguinarine is used in oral health products, such as mouthwashes and toothpastes. In vitro studies have indicated that the antiplaque action of sanguinarine is due to its ability to inhibit the adherence of bacteria to newly formed pellicle. The MIC values of the compound ranged 1–32 μg/mL for most species of plaque bacteria (Godowski, 1989; Grenby, 1995).

Polymorphonuclear neutrophils (PMNs) represent an important defence mechanism against bacterial infection. Superoxide is one of the most important factors released by PMNs following a variety of stimulations, including that of bacteria. It was found that 25–200 μg/mL of ofloxacin (1-[5-isoquinolinesulfonyl]-2-methylpiperazine) augmented superoxide production of PMNs. This augmentation was assumed to be due to the enhancement of leukocyte protein kinase C (Nagafuji *et al.*, 1993) (see Fig. 4.4).

4.3.2.2 Aliphatic aldehydes

Olive oil derived from *Olea europaea* (Oleaceae) has been used worldwide in traditional medicine to treat skin diseases. Recently, olive oil mixed with honey and beeswax was reported to be effective in an open pilot study against skin fungal infection (e.g. *C. albicans*, *Pityriasis versicolor*, *Tinea cruris*, *Tinea corpis*) after topical application (Al-Waili, 2004). Oleuropein and hydroxytyrosol, two secoiridoids contained in olive oil, are known for their antibacterial activities. Noteworthy is also the activity of saturated and unsaturated aldehydes (e.g. hexanal, E-2-heptanal, nonanal, E-2-octanal) from olive fruit and oil against different fungi. In addition, unsaturated aldehydes revealed a broad spectrum of antimicrobial effects against gram-positive and gram-negative bacteria. It was hypothesized that these phytoagents act not only on the plasmatic membrane but also on intracellular targets. Finally, α,β-unsaturated aldehydes are considered to be involved in the resistance of olive plants to microbe and insect attack (Battinelli *et al.*, 2006).

4.3.2.3 Anthraquinones

Anthraquinonic compounds, traditionally used as laxatives, possess many other pharmacological properties, including microbiological action (see Table 4.7). Of several pure anthraquinones tested, aloe-emodin, emodin and rhein

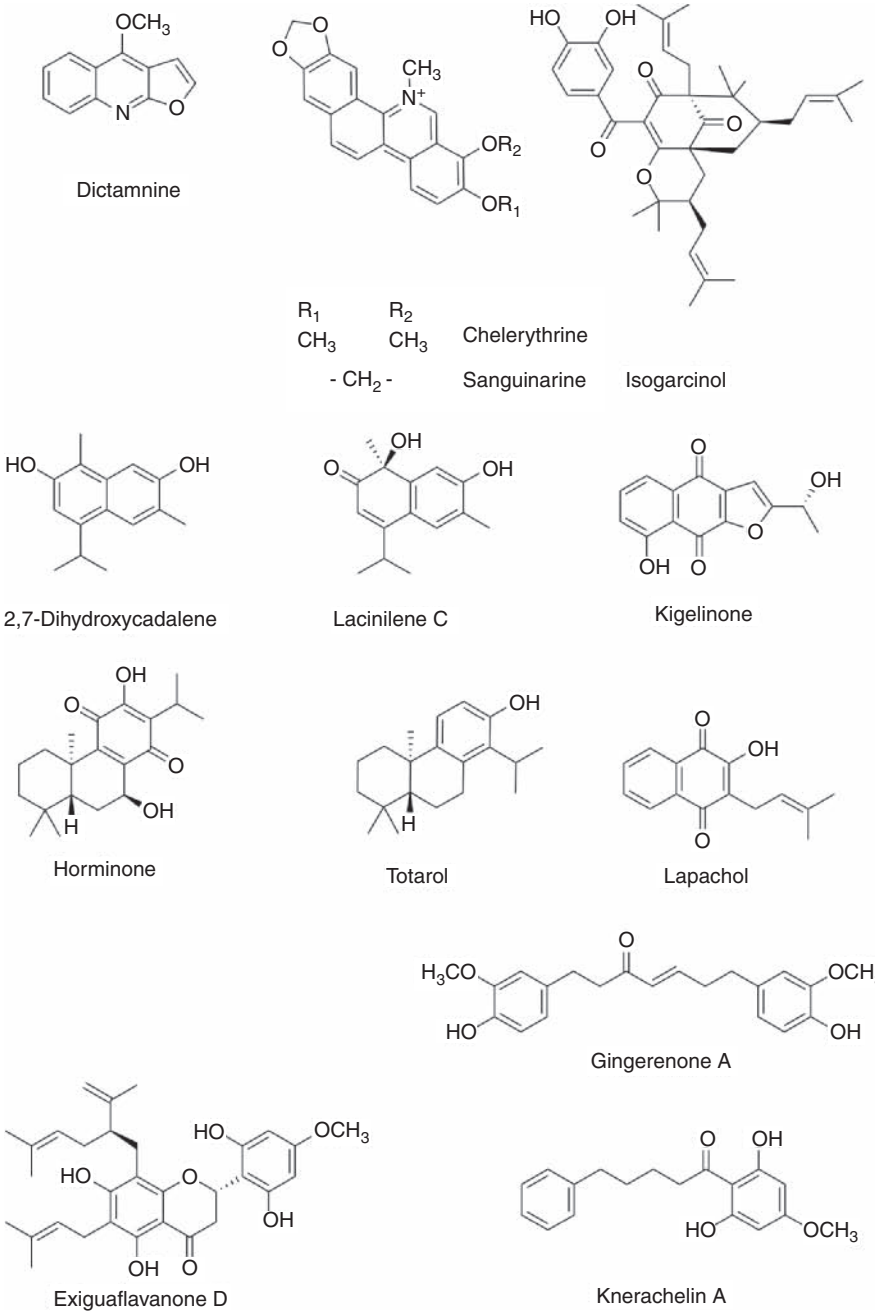


Figure 4.4 Chemical structures of several plant-derived compounds with antimicrobial properties.

Table 4.7 Secondary metabolites with antimicrobial activity

Compounds	Bacteria gram (+)	Bacteria gram (y)	Yeasts (y)	Fungi (+)	MIC (μg/mL)	References
Acetophenones						
Xanthoxyline	+	y				De Godoy *et al.* (1991)
Aliphatic aldehydes						
Hexanal			y	+	3.9–125	Battinelli *et al.* (2006)
E-2-Hexanal			y	+	15.6–62.5	Battinelli *et al.* (2006)
E-2-Heptanal			y	+	1.9–125	Battinelli *et al.* (2006)
Nonanal			y	+	1.9–15.6	Battinelli *et al.* (2006)
E-2-Octenal			y	+	1.9–250	Battinelli *et al.* (2006)
Anthraquinones						
Aloe-emodin	+				*2–64*	Hatano *et al.* (1999)
Emodin	+					Hatano *et al.* (1999)
Rhein	+				*2–125*	Didry *et al.* (1994), Hatano *et al.* (1999)
Apocarotenoids						
Cochloxanthin		y	y	+	*500* (total inhibition)	Diallo *et al.* (1991)
Dihydrocochloxanthin		y	y	+	*500* (total inhibition)	Diallo *et al.* (1991)
Benzophenones						
Garcinol	+				*6.3–25.0*	Iinuma *et al.* (1996)
Isogarcinol	+				*12.5–25.0*	Iinuma *et al.* (1996)
Xanthochymol	+				*3.1–12.5*	Iinuma *et al.* (1996)
Benzoquinones						
2-Hydroxy-5-methoxy-3-(8′*Z*,11,14′-pentadecatrienyl) 1,4-benzoquinone				+	6.0	Suzuki *et al.* (1998)

Biflavonoids						
Calodenin B	+				*8.0–64.0*	Tang *et al.* (2003)
Dihydrocalodenin B	+				*8.0*	Tang *et al.* (2003)
Chromenes						
Methylripariochromene A				+		Bandara *et al.* (1992)
Coumarins						
Angelicin				+	4160–6030	Afek *et al.* (1995)
Bergapten				+	4090–5950	Afek *et al.* (1995)
Columbianetin				+	25.0–48.0	Afek *et al.* (1995)
Herniarin	+	y	y	+		Ceska *et al.* (1992)
8-Methoxyporalen	+	y	y	+		Ceska *et al.* (1992)
Psoralen				+	3850–5160	Afek *et al.* (1995)
Umbelliferone	+	y	y	+		Ceska *et al.* (1992)
Xanthotoxin				+	3920–4510	Afek *et al.* (1995)
Furanocoumarin-type						
Oxypeucedanin				+	1 μg (minimum quantity for inhibition)	Marston *et al.* (1995)
Oxypeucedanin hydrate				+	10 μg (minimum quantity for inhibition)	Marston *et al.* (1995)
Dihydrochalcones						
Asebogenin	+	y			0.1–5.0 μg/TLC	Orjala *et al.* (1994)
2′,6′-Dihydroxy-4′-methoxy-dihydrochalcone	+	y			0.3–5.0 μg/TLC	Orjala *et al.* (1994)
3′-Formyl-2′,4′,6′-trihydroxy-dihydrochalcone				+		Miles *et al.* (1991)
Piperaduncin A	+	y			1.6–5.0 μg/TLC	Orjala *et al.* (1994)
Piperaduncin B	+	y			0.2–5.0 μg/TLC	Orjala *et al.* (1994)
Piperaduncin C	+	y			1.5–5.0 μg/TLC	Orjala *et al.* (1994)

(*Continued*)

Table 4.7 (*Continued*)

Compounds	Bacteria gram (+)	Bacteria gram (y)	Yeasts (y)	Fungi (+)	MIC (μg/mL)	References
Diterpenes						
Abietane-type						
2,3-Dehydrosalvipisone	+				10.5	Ulubelen *et al.* (1994)
7α,12-Dihydroxy-17(15→16)-abeo-abieta-8,12,16-trien-11,14-dione	+	y	y		*15.6–31.2*	Batista *et al.* (1994)
7,11-Dihydroxy-12-methoxy-abietatriene	+				*10.0–60.0*	Dellar *et al.* (1996)
Forskalinone	+				*168–670*	Ulubelen *et al.* (1996)
Horminone	+	y	y		*7.8–250*	Batista *et al.* (1994)
11-Hydroxy-12-oxo-abietatriene	+				*10.0–40.0*	Dellar *et al.* (1996), Batista *et al.* (1994)
Hypargenin A	+				15.6	Ulubelen *et al.* (1988)
Hypargenin B	+				125.0	Ulubelen *et al.* (1988)
Hypargenin C	+				15.6–125	Ulubelen *et al.* (1988)
Hypargenin D	+				62.5	Ulubelen *et al.* (1988)
Hypargenin F	+	y			62.5–125	Ulubelen *et al.* (1988)
Lanigerol	+				1000	El-Lakany *et al.* (1995)
Manool	+				13.8	Ulubelen *et al.* (1994)
Sclareol	+				48.3	Ulubelen *et al.* (1994)
Totarol	+				*0.39–1.56*	Kubo *et al.* (1992)
Totaradiol	+				*25–200*	Kubo *et al.* (1992)
Labdane-type						
Labdane-14-ene-8,13-diol	+	y	y			Chinou *et al.* (1994)
Labdane-13(*E*)-ene-8α,15-diol	+	y	y			Chinou *et al.* (1994)
Labdane-13(*E*)-ene-8α-ol-15-yl acetate	+	y	y			Chinou *et al.* (1994)

Labdane-7,13(*E*)-dien-15-ol	+	y	y			Chinou *et al.* (1994)
13-Episclareol	+	y	y			Chinou *et al.* (1994)
8,13-Epoxylabdane-14-en	+	y	y			Chinou *et al.* (1994)
8,13-Epoxy-13-epi-labdane-14-en	+	y	y			Chinou *et al.* (1994)
Beyerenane-type						
1-Acetyljativatriol	+				25.0–50.0	Diaz *et al.* (1988)
ent-Beyer-15-en-19-ol	+	y	y		*3.1–104.0*	Drewes *et al.* (2006)
ent-Beyer15-en-18-ol	+	y	y		*3.6–250*	Drewes *et al.* (2006)
15β,16β-Epoxide-ent-beyeran-19-ol	+	y	y		*19.5–625*	Drewes *et al.* (2006)
Clerodane-type						
Clerodin				+		Cole *et al.* (1991)
Hardwickic acid	+		y	+		McChesney and Clark (1991)
Jodrellin A				+		Cole *et al.* (1991)
Jodrellin B				+		Cole *et al.* (1991)
16α-Hydroxy-cleroda-3,13(14)Z-diene-15,16-olide	+	y	y		*0.78–12.5*	Murthy *et al.* (2005)
16-oxo-Cleroda-3,13(14)-*E*-diene-15-oic acid	+	y	y		*3.12–25.0*	Murthy *et al.* (2005)
12-oxo-ent-3,13(16)-Cleroden-15-oic acid	+				6.3–25.0	Habtemariam *et al.* (1990)
3,4-Secotrachylobanoic acid	+		y	+		McChesney and Clark (1991)
Seco-kaurane-type						
Trichorabdal A	+				*12.5–100*	Osawa *et al.* (1994)

(*Continued*)

Table 4.7 (*Continued*)

Compounds	Bacteria gram (+)	Bacteria gram (y)	Yeasts (y)	Fungi (+)	MIC (μg/mL)	References
Trichorabdal B	+				*12.5–100*	Osawa *et al.* (1994)
Trichorabdal C	+				*100–>200*	Osawa *et al.* (1994)
Trichorabdal H	+				*50–200*	Osawa *et al.* (1994)
Miscellaneous-type						
Pseudolaric acid B			y		*0.78–12.5*	Li *et al.* (1995)
Flavonoids						
Catechin-type						
(−)-Epigallocatechin	+	y			*50–100*	Mori *et al.* (1987)
Flavonol-type						
5,7-Dihydroxy-3,8-dimethoxyflavone	+	y			20–50	Tomas-Lorente *et al.* (1989, 1991)
3,5-Dimethoxy-6,7-methylene-dioxyflavone	+				20.0	Pomilio *et al.* (1992)
3,6-Dimethoxy-5,7-dihydroxyflavone	+				15.0	Pomilio *et al.* (1992)
3′-7-Di-*O*-methylquercetin				+		Miles *et al.* (1993)
5-Hydoxy-3-methoxy-6,7-methylenedioxyflavone	+				75.0	Pomilio *et al.* (1992)
3,5,6,7,8-Pentamethoxyflavone				+	5.0 μg per TLC	Tomas-Barberan *et al.* (1988)
3,5,6,7-Tetramethoxyflavone	+			+	20.0	Tomas-Barberan *et al.* (1988); Pomilio *et al.* (1992)
Datisetin	+	y			*100*	Mori *et al.* (1987)
Galangin	+				*50.0*	Pomilio *et al.* (1992)
Isorhamnetin-3-*O*-robinobioside	+				*50.0*	Pomilio *et al.* (1992)

Kaempferol	+	y			*25.0–50.0*	Pomilio *et al.* (1992), Martini *et al.* (2004)
3-*O*-Methylquercetin	+	y	y	+	*6.3–100*	Van Puyvelde *et al.* (1989)
Morin	+	y			*100*	Mori *et al.* (1987)
Myricetin	+	y			*50–100*	Mori *et al.* (1987)
Papyriflavonol A	+	y	y		*5.0–30.0*	Sohn *et al.* (2004)
Platanoside	+	y				Mitrokotsa *et al.* (1993)
Quercetagetin	+	y			*100*	Mori *et al.* (1987)
Quercetagetin-7-arabinosyl-galactoside	+	y			38–130	Tereschuk *et al.* (1997)
Quercetin 3-*O*-rhamnoside	+	y				Hasan and Ahmad (1996)
Quercetin 3-*O*-glucosyl-(1→4)-galactoside	+	y				Hasan and Ahmad (1996)
Quercetin-5,3′-dimethylether	+	y			*25.0–50.0 μg/mL*	Martini *et al.* (2004)
Robinetin	+	y			*100*	Mori *et al.* (1987)
Rutin		y			32.0	Bernard *et al.* (1997)
Tiliroside	+	y				Mitrokotsa *et al.* (1993)
Flavone-type						
5-Hydroxy-7,4′-dimethoxyflavone	+	y			*25.0–50.0 μg/mL*	Martini *et al.* (2004)
7,8-Dihydroxyflavone	+	y			*100*	Mori *et al.* (1987)
5,7-Dihydroxy-6-methoxyflavone	+				15.0	Pomilio *et al.* (1992)
5,6-Dimethoxy-7-hydroxyflavone	+				75.0	Pomilio *et al.* (1992)
5,6,7,8-Tetramethoxyflavone				+	2.0 μg per TLC plate	Tomas-Barberan *et al.* (1988)
Dimethylchrysin				+	1.0 μg per TLC plate	Tomas-Barberan *et al.* (1988)

(*Continued*)

Table 4.7 (*Continued*)

Compounds	Bacteria gram (+)	Bacteria gram (y)	Yeasts (y)	Fungi (+)	MIC (μg/mL)	References
Flavone				+		Weidenbörner *et al.* (1990a)
Kuwanon C	+	y	y		*5.0–30.0*	Sohn *et al.* (2004)
Trimethylgalangin				+	1.0 μg per TLC plate	Tomas-Barberan *et al.* (1988)
Flavanone-type						
Abyssinone-V	+	y			2.9–26.4	Ratsimamanga-Urverg *et al.* (1994)
7,4′-Dihydroxyflavan				+		Achenbach *et al.* (1988)
Euchrestaflavanone A	+	y			5.58–23.53	Ratsimamanga-Urverg *et al.* (1994)
Exiguaflavanone B	+				50.0	Iinuma *et al.* (1994)
Exiguaflavanone D	+				1.56–6.25	Iinuma *et al.* (1994)
Flavanone				+		Weidenbörner *et al.* (1990a)
Naringenin	+					Osawa *et al.* (1992)
Sophoraflavanone D	+	y	y		*5.0–30.0*	Sohn *et al.* (2004)
Flavanonol-type						
(+)-Dihydrorobinetin	+	y			*200*	Mori *et al.* (1987)
3′,4′-Dihydroxy-7-methoxyflavan				+		Achenbach *et al.* (1988)
7,4′-Dihydroxy-3′-methoxyflavan				+		Achenbach *et al.* (1988)
7,3′-Dimethoxy-4′-hydroxyflavan				+		Achenbach *et al.* (1988)
Obtustyrene				+		Achenbach *et al.* (1988)
Isoflavan-type						

5,7-Dihydroxy-4′-hydroxyisoflavan				+		Weidenbörner *et al.* (1990a,b)
6,7-Dihydroxy-4′-methoxyisoflavan				+		Weidenbörner *et al.* (1990a,b)
5,7-Dihydroxy-4′-methoxyisoflavan				+		Weidenbörner *et al.* (1990a,b)
Isoflavanone-type						
Biochanin A				+		Weidenbörner *et al.* (1990a,b)
Darbergioidin	+					Osawa *et al.* (1992)
Desmodianone A	+		y		*1.0–100*	Delle Monache *et al.* (1996)
Desmodianone B	+	y	y		*1.0–100*	Delle Monache *et al.* (1996)
Dihydrobiochanin A	+					Osawa *et al.* (1992)
Dihydrogenistein	+					Osawa *et al.* (1992)
Dihydrocajanin	+					Osawa *et al.* (1992)
2,7-Dihydroxy-3(3′-methoxy-4′-hydroxy)-5-methoxy-isoflavone				+		Miles *et al.* (1993)
Ferreirin	+					Osawa *et al.* (1992)
Sophoraisoflavanone	+	y	y		*5.0–30.0*	Sohn *et al.* (2004)

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Table 4.7 (*Continued*)

Compounds	Bacteria gram (+)	Bacteria gram (y)	Yeasts (y)	Fungi (+)	MIC (μg/mL)	References
Pterocarpene-type						
Erycristagallin	+				*3.13–6.25*	Tanaka *et al.* (2002)
Glycyrrhizol A	+				*1*	He *et al.* (2006)
Glycyrrhizol B	+				*32.0*	He *et al.* (2006)
5-*O*-Methylglycyrol	+				*500.0*	He *et al.* (2006)
Isoglycyrol	+				*500.0*	He *et al.* (2006)
Isolupalbigenin	+				*1.56–3.13*	Sato *et al.* (2006)
Gancaonin G	+				*125.0*	He *et al.* (2006)
Orientanol	+				*3.13–6.25*	Tanaka *et al.* (2002)
Lignans						
Aryltetralin-type						
4′-*O*-Demethyldehydropodophyllotoxin				+		Rahman *et al.* (1995)
Picropodophyllone				+		Rahman *et al.* (1995)
Cyclolignan-type						
Galbulin				+		Sartorelli *et al.* (1998)
Oleiferin-B				+		Sartorelli *et al.* (1998)
Oleiferin-G				+		Sartorelli *et al.* (1998)
Oleiferin-F				+		Sartorelli *et al.* (1998)
Oleiferin-H				+		Sartorelli *et al.* (1998)
Verrucosin				+		Sartorelli *et al.* (1998)
Limonoids						
Limonoid glycoside	+	y				Srivastava (1986)
Mahmoodin	+	y				Siddiqui *et al.* (1992)
Naheedin	+	y				Siddiqui *et al.* (1992)

Monoterpenes						
Borneol	+	y			*0.03–0.5%* of oil	Reichling *et al.* (2006)
Camphene	+		y	+		Tirillini *et al.* (1996)
Carvacrol	+	y	y	+	*1.0–5.0 mM*; *0.016–0.13*% of oil; 0.04–0.16 μL/mL	Lucchini *et al.* (1990), Didry *et al.* (1993), Reichling *et al.* (2006), Pinto *et al.* (2006)
Carveol	+	y			*0.03–0.25%* of oil	Reichling *et al.* (2006)
Carvone	+	y	y	+	*1.56–100* μL/mL	Hinou *et al.* (1989), Naigre *et al.* (1996)
Carvone	+	y	y	+	2500–12500	Aboul Ela *et al.* (1996)
1,8-Cineole	+	y	y	+	*0.4–6.6*	Pattnaik *et al.* (1997)
1,8-Cineole	+	y	y		6254–25000	Aboul Ela *et al.* (1996)
Citral	+	y	y	+	*0.1–6.6*	Pattnaik *et al.* (1997), Reichling *et al.* (2006)
Citral	+	y			*0.016–>1.0%* of oil	Hinou *et al.* (1989), Reichling *et al.* (2006)
β-Citronellol	+	y			*0.016–1.0%* of oil	Reichling *et al.* (2006)
Cuminal	+	y			*0.03–1.0%* of oil	Reichling *et al.* (2006)
p-Cymene			y	+	*1.25–20.0 μL/mL*	Pinto *et al.* (2006)
Geraniol	+	y			*125–375*	Hinou *et al.* (1989), Chinou *et al.* (1996)
Geraniol	+	y	y	+	*0.3–5.0*	Pattnaik *et al.* (1997)
Geranyl acetate	+				*250–750*	Hinou *et al.* (1989), Chinou *et al.* (1996)

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Table 4.7 (*Continued*)

Compounds	Bacteria gram (+)	Bacteria gram (y)	Yeasts (y)	Fungi (+)	MIC (μg/mL)	References
Isobornyl acetate	+					Hinou *et al.* (1989)
Isolimonene	+	y	y	+	*0.78–100 μL/mL*	Naigre *et al.* (1996)
Isomenthone	+	y				Economou and Nahrstedt (1991)
Isopulegol	+	y	y	+	*0.78–12.5 μL/mL*	Naigre *et al.* (1996)
Limonene			y		*100 μL/mL*	Naigre *et al.* (1996)
Linalool	+	y			*0.06–0.5%* of oil	Hinou *et al.* (1989), Reichling *et al.* (2006)
Linalool	+	y	y	+	2500–6250	Aboul Ela *et al.* (1996)
Linalool	+	y	y	+	*0.2–6.6*	Pattnaik *et al.* (1997)
Linalyl acetate	+	y				Hinou *et al.* (1989)
Menthene	+	y				Hinou *et al.* (1989)
Menthenefuran	+					Hinou *et al.* (1989)
Menthone	+	y			*0.25–1.0%* of oil	Hinou *et al.* (1989), Economou and Nahrstedt (1991), Reichling *et al.* (2006)
Menthol	+	y			*0.06–0.25%* of oil	Reichling *et al.* (2006)
Menthyl acetate	+					Hinou *et al.* (1989)
Myrtenol	+	y			*0.008–0.5%* of oil	Reichling *et al.* (2006)
Neryl acetate	+	y			*250–750*	Hinou *et al.* (1989), Chinou *et al.* (1996)
α-Pinene	+	y			*0.03–1.0%* of oil	Reichling *et al.* (2006)
Piperitone	+					Hinou *et al.* (1989)
Pulegone	+	y	y	+	*0.4–85.0%* of oil	Hinou *et al.* (1989), Economou and Nahrstedt (1991), Kalodera *et al.* (1994)

Sabinene	+					Hinou *et al.* (1989)
γ-Terpinene			y	+	1.25–20.0 μL/mL	Pinto *et al.* (2006)
Terpinolene	+	y				Hinou *et al.* (1989)
Terpineol	+	y				Hinou *et al.* (1989)
Terpinyl acetate	+	y				Hinou *et al.* (1989)
Tetrahydrogeraniol	+					Hinou *et al.* (1989)
Thymol	+	y	y	+	*1.0–4.0 mM*; 0.08–0.32 μL/mL	Lucchini *et al.* (1990), Didry *et al.* (1993), Pinto *et al.* (2006)
Thymol	+				*50–100*	Osawa *et al.* (1994)
Verbenol	+	y			*0.016–0.5%* of oil	Reichling *et al.* (2006)
Naphthoquinones						
Cassiaside B	+				10.0	Messana *et al.* (1991)
Dehydro-α-lapachone	+				100–200	Binutu *et al.* (1996)
Dehydro-α-lapachone			y	+	200–400	Binutu *et al.* (1996)
Isopinnatal	+				100–200	Binutu *et al.* (1996)
Isopinnatal			y	+	200–400	Binutu *et al.* (1996)
Juglone	+				*125–500*	Didry *et al.* (1994)
Kigelinone	+				100	Binutu *et al.* (1996)
Kigelinone			y	+	100–200	Binutu *et al.* (1996)
Lapachol	+				100–200	Binutu *et al.* (1996)
			y	+	200–400	Binutu *et al.* (1996)
Lawsone	+				*125–500*	Didry *et al.* (1994)
Plumbagin	+				*1.0–250*	Didry *et al.* (1994)
Quinquangulin-6-*O*-apiofuranosyl-(1→6)-*O*-glucopyranoside	+		y		100	Messana *et al.* (1991)

(*Continued*)

Table 4.7 (*Continued*)

Compounds	Bacteria gram (+)	Bacteria gram (y)	Yeasts (y)	Fungi (+)	MIC (μg/mL)	References
Rubrofusarin-6-*O*-glucopyranoside	+				10.0	Messana *et al.* (1991)
Phenanthrenes						
9,10-Dihydrophenanthrene type						
Desvinyljuncusol	+		y		*6.25–50.0*	Boger *et al.* (1985)
2-Hydroxy-3-methyl-9,10-dihydrophenanthrene	+		y		*3.12–25.0*	Boger *et al.* (1985)
Juncusol	+		y		*12.5–25.0*	Boger *et al.* (1985)
Phenolic compounds						
Caffeic acid	+		y	+	100–200	Binutu *et al.* (1996)
Ferulic acid	+		y	+	100–200	Binutu *et al.* (1996)
Gingerenone A				+	10.0 ppm	Endo *et al.* (1990)
4-Hydroxystyrene	+	y		+	32–500	Kobayashi *et al.* (1996)
Knerachelin A	+				*8.0–32.0*	Zahir *et al.* (1993)
Knerachelin B	+				*4.0–16.0*	Zahir *et al.* (1993)
3-Methoxy-4-acetoxystyrene	+	y		+	32–500	Kobayashi *et al.* (1996)
	+	y		+	65–1000	Kobayashi *et al.* (1996)
p-Coumaric acid	+		y	+	200–400	Binutu *et al.* (1996)
Phenylpropanoids						
1-Allyl-2,6-dimethoxy-3,4-methylenedioxybenzene	+	y			100 ppm	Masuda *et al.* (1991)
Allylanisole	+	y			*0.06–1.0%* of oil	Reichling *et al.* (2006)
Anisaldehyde	+	y			*0.06–0.25%* of oil	Reichling *et al.* (2006)
Cinnamaldehyde	+	y			*0.016%* of oil	Reichling *et al.* (2006)
Cinnamic acid	+	y			*0.03–0.13%* of oil	Reichling *et al.* (2006)
Cinnamic alcohol	+	y			*0.03–0.25%* of oil	Reichling *et al.* (2006)
Colenemal	+					Brader *et al.* (1997)
Elemicin				+	20 μg (minimum quantity for inhibition)	Marston *et al.* (1995)

Eugenol	+	y			*0.03–0.13%* of oil	Reichling *et al.* (2006)
Honokiol	+	y			*20.0–80.0*	Chang *et al.* (1998)
trans-Isoelemicin				+	8.0 μg (minimum quantity for inhibition)	Marston *et al.* (1995)
Isoeugenol	+	y			*0.03–0.13%* of oil	Reichling *et al.* (2006)
Magnolol	+	y			*20–160*	Chang *et al.* (1998)
Methyleugenol	+	y			*0.06–1.0%* of oil	Reichling *et al.* (2006)
Myristicin				+	8.0 μg (minimum quantity for inhibition)	Marston *et al.* (1995)
Plicatin B	+		y		12.5–50.0	Schmitt *et al.* (1991)
Precolpuchol	+			+		Brader *et al.* (1997)
Phloroglucinols						
Hyperbrasilol A	+					Rocha *et al.* (1995)
Isouliginosin B	+					Rocha *et al.* (1995)
Japonicine A	+					Rocha *et al.* (1995)
Uliginosin A	+					Rocha *et al.* (1995)
Resorcinol						
Malabaricone B	+		y		1.0–16.0	Orabi *et al.* (1991)
Malabaricone C	+		y		2.0–32.0	Orabi *et al.* (1991)
Secoiridoids						
Hydroxytyrosol	+	y			*31.25–250*	Battinelli *et al.* (2006)
Oleuropein	+	y			*0.24–31.25*	Battinelli *et al.* (2006)
Sesquiterpenoids						
alloAromadendrane-10β,14-diol	+	y				De Siqueira *et al.* (1997)

(*Continued*)

Table 4.7 (*Continued*)

Compounds	Bacteria gram (+)	Bacteria gram (y)	Yeasts (y)	Fungi (+)	MIC (μg/mL)	References
Argentone				+		Maatooq *et al.* (1996)
Bisabolol	+	y			*0.016–1.0%* of oil	Reichling *et al.* (2006)
Carisone				+		Maatooq *et al.* (1996)
β-Caryophyllene	+	y			*0.016–1.0%* of oil	Reichling *et al.* (2006)
Caryophyllene oxide	+				13.8	Ulubelen *et al.* (1994)
Cedrene	+	y			*0.016–1.0%* of oil	Reichling *et al.* (2006)
Cernuol	+	y	y	+	*5.0–200*	Smirnov *et al.* (1998)
Dentatin A	+	y				Gören *et al.* (1990)
Farnesol	+	y			*0.008–1.0%* of oil	Reichling *et al.* (2006)
Glaucolide A	+					Montanaro *et al.* (1996)
8α-Hydroxyanhydroverlotorin	+	y				Gören *et al.* (1990)
15-Hydroxyargentone				+		Maatooq *et al.* (1996)
Hymenin	+	y				Montanaro *et al.* (1996)
6-*O*-Isobutyroylplenolin	+				*38–300*	Taylor and Towers (1998)
Isospeciformin	+	y				Gören *et al.* (1990)
6-*O*-Methylacrylylplenolin	+				*75–300*	Taylor and Towers (1998)
Nerolidol	+	y			*0.008–1.0%* of oil	Reichling *et al.* (2006)
15-nor-Argentone				+		Maatooq *et al.* (1996)
8-oxo-Argentone				+		Maatooq *et al.* (1996)
8-oxo-15-nor-Argentone				+		Maatooq *et al.* (1996)
6-*O*-Angeloylplenolin	+				*75–300*	Taylor and Towers (1998)
Polygodial			y	+	*0.78–100.0*	Lee *et al.* (1999)
Spathulenol	+				136.0	Ulubelen *et al.* (1994)
Tabulin	+	y				Gören *et al.* (1990)
Tanachin	+	y				Gören *et al.* (1990)
Vernodalin	+	y	y	+		Al Magboul *et al.* (1997)
Vernoleptin	+	y	y	+		Al Magboul *et al.* (1997)

Steroids						
Cryptanoside A	+					Vasanth *et al.* (1997)
Cryptanoside C	+	y				Vasanth *et al.* (1997)
20-Hydroxyecdysone	+	y		+		Ahmad *et al.* (1996)
Stilbenes						
(*E*)-3-Chloro-4-stilbenol				+	40–43 ppm	Schultz *et al.* (1992)
3,5-Dihydroxy-trans-stilbene	+	y	y		*62.5–250*	Lee *et al.* (2005)
(*E*)-3,5-Dimethoxy-4-stilbenol				+	49–60 ppm	Schultz *et al.* (1992)
(*E*)-3,5-Dimethoxystilbene				+	90 ppm	Schultz *et al.* (1992)
(*E*)-3-Methoxy-4-stilbenol				+	40 ppm	Schultz *et al.* (1992)
(*Z*)-4-Methoxy-3-stilbenol				+	8–64 ppm	Schultz *et al.* (1992)
(*E*)-5-Methoxy-3-stilbenol				+	42–163 ppm	Schultz *et al.* (1992)
(*E*)-4-Stilbenol				+	12–31 ppm	Schultz *et al.* (1992)
(*E*)-3-Stilbenol				+	35–54 ppm	Schultz *et al.* (1992)
(*Z*)-3-Stilbenol				+	25–75 ppm	Schultz *et al.* (1992)
(*E*)-3,4-Stilbenediol				+	33–34 ppm	Schultz *et al.* (1992)
(*E*)-3,5-Stilbenediol				+	29–140 ppm	Schultz *et al.* (1992)
3,5,4′-Trihydroxy-trans-stilbene	+	y	y		*250*	Lee *et al.* (2005)
Tannins						
1,2,3,4,6-Penta-galloylglucose	+	y			*256–1024*	Burapadaja and Bunchoo (1995)
1,2,3,4,6-Penta-galloylglucose			y		*512*	Burapadaja and Bunchoo (1995)
1,3,6-Tri-galloylglucose	+	y			*460–1024*	Burapadaja and Bunchoo (1995)

(*Continued*)

Table 4.7 (*Continued*)

Compounds	Bacteria gram (+)	Bacteria gram (y)	Yeasts (y)	Fungi (+)	MIC (μg/mL)	References
Chebulagic acid	+	y			*256–1024*	Burapadaja and Bunchoo (1995)
Corilagin	+	y			*128–1024*	Burapadaja and Bunchoo (1995)
Punicalagin	+	y			*256–1024*	Burapadaja and Bunchoo (1995)
Thiophenes						
5′-Methyl-5-[4-(3-methyl-1-oxobutoxy)-1-butynyl]-2,2′-bithiophene				+	*200*	Ahmad *et al.* (1995)
5′-Hydroxymethyl-5-[butyl-3-en-1-yn]-2,2′-bithiophene isovaleroxy ester				+	*200*	Ahmad *et al.* (1995)
Triterpenes						
Cucurbitane-type						
Mormodicine I				+		Chandravadana *et al.* (1997)
Mormodicine II				+		Chandravadana *et al.* (1997)
Cycloartane-type						
Astrasieversianin II	+	y			2.0–10.0	Calis *et al.* (1997a, b)
Astragaloside I	+	y			20–30	Calis *et al.* (1997a, b)
Astragaloside II	+	y			20.0	Calis *et al.* (1997a, b)
Astrasieversianin X	+	y			20–50	Calis *et al.* (199a,b7)
Astragaloside VI		y			50.0	Calis *et al.* (1997a,b)
Cyclocanthoside G		y			10.0	Calis *et al.* (1997a)
(24*R*)-24,25-Epoxycycloartan-3-one	+				*8.0*	Cantrell *et al.* (1996)
(3β,24*R*)-24,25-Epoxycycloartan-3-ol	+				*8.0*	Cantrell *et al.* (1996)

Lupane-type						
Ceanothic acid	+	y			*42–250*	Li *et al.* (1997)
27-Hydroxy ceanothic acid	+	y			*562–875*	Li *et al.* (1997)
Ceanothetric acid	+	y			*406–525*	Li *et al.* (1997)
Miscellaneous-tritepenes						
Argentatine A	+	y	y			Martinez-Vazquez *et al.* (1994)
Oleanane-type						
Aegicerin	+				*1.6–3.1*	Rojas *et al.* (2006)
Arjungenin	+	y			*17.5*	Nandy *et al.* (1997)
Arjungenin methylester	+	y	y		*3.0–6.4*	Nandy *et al.* (1997)
Arjunglucoside	+	y			*38.0*	Nandy *et al.* (1997)
Belleric acid	+	y			*3.0–10.0*	Nandy *et al.* (1997)
Belleric acid methylester	+	y	y		*6.4–33.3*	Nandy *et al.* (1997)
Bellericagenin A	+	y	y		*6.0–40.0*	Nandy *et al.* (1997)
Bellericagenin B	+	y	y	+	*6.2–17.0*	Nandy *et al.* (1997)
Bellericaside A	+	y	y		*7.0–25.0*	Nandy et a1. (1997)
Bellericaside B	+	y	y	+	*1.5–10.0*	Nandy *et al.* (1997)
Bellericaside	+	y	y		*12.5–22.0*	Nandy *et al.* (1997)
Cyclamin			y		*80–160*	Calis *et al.* (1997b)
Cyclaminorin			y		*80–160*	Calis *et al.* (1997b)
Deglucocyclamin			y		*80–160*	Calis *et al.* (1997b)
Dillinic acid A	+	y				Nick *et al.* (1994)
Dillinic acid B	+	y				Nick *et al.* (1994)
Dillinic acid C		y				Nick *et al.* (1994)

(*Continued*)

Table 4.7 (*Continued*)

Compounds	Bacteria gram (+)	Bacteria gram (y)	Yeasts (y)	Fungi (+)	MIC (μg/mL)	References
3β-Hydroxyolean-12-en-28-oic acid				+	500	Verma *et al.* (1998)
3β-Hydroxyolean-12-en-28-oic acid methyl ester				+	600	Verma *et al.* (1998)
3β-24-Dihydroxyolean-12-en-28-oic acid				+	500–600	Verma *et al.* (1998)
3β-24-Dihydroxyolean-12-en-28-oic acid methyl ester				+	600–1000	Verma *et al.* (1998)
Pulsatilla saponin D			y	+	*8.0–250*	Ekabo and Farnsworth (1996)
Salzmannianoside A			y	+	*8.0–125*	Ekabo and Farnsworth (1996)
Salzmannianoside B			y	+	*8.0–250*	Ekabo and Farnsworth (1996)
Tomentosic acid methylester	+	y			*8.3–36.5*	Nandy *et al.* (1997)
Phenol nortriterpene-type						
3-*O*-Methyl-6-oxo-tingenol	+				*35–39*	Gonzalez *et al.* (1996)
6-oxo-lguesterol	+				*25.0*	Gonzalez *et al.* (1996)
6-oxo-Tingenol	+				*12.0–14.0*	Gonzalez *et al.* (1996)

Ursane-type						
22β-Acetoxylantic acid	+	y	y	+		Barre *et al.* (1997)
Rubrinol	+	y			*130–200*	Akhtar *et al.* (1994)
Xanthones						
BR-xanthone A				+		Gopalakrishnan *et al.* (1997)
Formoxanthone A	+	y			18.7–37.5	Bonsri *et al.* (2006)
Formoxanthone C	+	y			*2.3–18.7*	Bonsri *et al.* (2006)
Garcinone D				+		Gopalakrishnan *et al.* (1997)
Gartanin				+		Gopalakrishnan *et al.* (1997)
Laurentixanthone A	+	y	y		*1.2–78.12*	Nguemeving *et al.* (2006)
Laurentixanthone B	+	y	y		*2.44–78.12*	Nguemeving *et al.* (2006)
Mangostin				+		Gopalakrishnan *et al.* (1997)
γ-Mangostin				+		Gopalakrishnan *et al.* (1997)

Note: Values in italics indicate agar/broth dilution method; the others using disc/agar diffusion method.
MIC, minimum inhibitory concentration; gram (+), gram-positive bacteria; gram (−), gram-negative bacteria; TLC, thin layer chromatographic plate.

showed significant antibacterial activity. Chrysophanic acid, physcion, aloin, sennoside A and sennoside B were found to be inactive (Didry *et al.*, 1994; Hatano *et al.*, 1999).

4.3.2.4 Diterpenoids

Many diterpenoids exhibit antimicrobial activity (see Table 4.7). Some of these compounds may be involved in the resistance of higher plants (e.g. conifers).

Abietane-type: lanigerol and forskalinone, isolated from the roots of *Salvia lanigera* and *Salvia forskahlei*, respectively, demonstrated moderate antibacterial activity against gram-positive bacteria (El-Lakany *et al.*, 1995; Ulubulen *et al.*, 1996). Additional antibacterial diterpenoids were isolated from *Salvia hypargeia* and *Salvia sclarea*. Hypargenins A, B, C, D and F, as well as 2,3-dehydrosalvipisone, sclareol, manool and 7-oxoroyleanone, were active against *S. aureus*, while hypargenin F was also active against *Mycobacterium tuberculosis* (Ulubelen *et al.*, 1988, 1994). Horminone, 11-hydroxy-12-oxo-abietatriene and 7α,11-dihydroxy-12-methoxy-abietatriene, isolated from the roots and aerial parts of *Plectranthus hereroensis* (Lamiaceae), were active against several gram-positive and gram-negative bacteria (Batista *et al.*, 1994; Dellar *et al.*, 1996). Totarol, isolated from the bark of *Podocarpus nagi* (Podocarpaceae), exhibited potent bactericidal activity against gram-positive bacteria, among which *Propionibacterium acnes* was the most sensitive. Totarol also showed strong activity both against penicillin-resistant and penicillin-susceptible strains of *S. aureus* (Kubo *et al.*, 1992). Later, Haragüchi and co-workers (1996) studied the biological mechanism of totarol in *P. aeruginosa*. It was shown that the compound inhibited oxygen consumption and respiratory-driven proton translocation in whole cells and oxidation of nicotinamide adenine dinucleotide (reduced form) (NADH) in membrane preparations. NADH-cytochrome c reductase, NADH-2,6-dichlorophenol indophenol (DCIP) reductase and NADH-coenzyme Q (CoQ) reductase were also inhibited.

Clerodane-type: from the leaves of *Premna schimperi* (Verbenaceae), 12-oxo-ent-3,13-clerodien-15-oic acid and, from the roots of *Croton sonderianus* (Euphorbiaceae), hardwickic acid and 3,4-*seco*-trachylobanoic acid were obtained. The diterpenes were active against *S. aureus*, and the two latter compounds were also active against *B. subtilis* (McChesney and Clark, 1991). From a hexane extract of seeds of *Polyalthia longifolia* (Annonaceae), 16α-hydroxy-cleroda-3,13(14)-Z-diene-15,16-olide and 16-oxo-cleroda-3,13(14)-E-diene-15-oic acid were isolated. Both diterpenoids demonstrated significant activity against gram-positive and gram-negative bacteria as well as against yeasts (Murthy *et al.*, 2005)

Ent-beyerenane-type: nine of the natural diterpenes, isolated from *Sideritis pusilla*, and five semisynthetic compounds were tested for antimicrobial activity. The natural compound, 1-acetyljativatriol, as well as the semisynthetic substances showed antimicrobial activity against gram-positive bacteria. All compounds were inactive against gram-negative bacteria. Studies on the

structure–activity relationship revealed that the 12,17-dihydroxy group is responsible for the antimicrobial activity of these compounds. In contrast, if a hydroxy group was present at C-1, no antimicrobial activity was observed (Diaz *et al.*, 1988).

Labdane-type: some labdane-type diterpenes, isolated from *Aframomum aulacocarpos* and *Cistus incanus*, demonstrated only weak activity against gram-positive and gram-negative bacteria (Chinou *et al.*, 1994).

Seco-kaurane-type: trichorabdal A and B, isolated from the leaves of *Rabdosia trichocarpa*, have antimicrobial effects against gram-positive and gram-negative periodontopathic bacteria. Both compounds completely inhibited the growth of the gram-negative bacterium, *Porphyromonas gingivalis*, at 12.5 μg/mL (Osawa *et al.*, 1994).

4.3.2.5 Monoterpenoids

A selection of structurally diverse monoterpenoids were tested for their antimicrobial activity against two gram-positive bacteria (*S. aureus*; *Streptococcus pneumoniae*) and two gram-negative bacteria (*E. coli*, *Haemophilus influenzae*) in order to investigate the antibacterial activity as well as a potential structure–activity relationship (Reichling *et al.*, 2006). The acyclic and monocyclic hydrocarbons myrcene and limonene and the aromatic hydrocarbon p-cymen revealed the weakest antibacterial activity against *S. aureus*, *S. pneumoniae* and *E. coli* (MIC values: 0.5 to >1%). In contrast, the bicyclic monoterpene hydrocarbon α-pinene was active against *S. aureus*, *S. pneumoniae* and *H. influenzae* (MIC values: 0.03–0.25%). The relative good antibacterial activity of α-pinene against three out of four bacteria species tested may be based on an interaction with the cytoplasmic membrane. The introduction of either a hydroxyl or carbonyl function into the monoterpene C-skeleton led clearly to an increase in the antibacterial activity in the following ascending order: ketones < aldehydes < alcohols < phenols. Among the monoterpenes tested, the phenols thymol and carvacrol exhibited the highest antibacterial activity (MIC values: 0.008–0.13%). The monoterpene phenols as well as the monoterpene alcohols displayed bactericidal rather than bacteriostatic activity. In addition, phenols are known for their membrane-disturbing activities, as well as cell lysis. Monoterpene alcohols are thought to act either as protein denaturing agents or as dehydrating substances. The relatively good antibacterial effects of monoterpene aldehydes may be retraced to the electronegative feature of the aldehyde group. It is assumed that aldehydes may interfere with biological processes in bacteria cells such as electron transfer or binding to molecules like proteins and nucleic acids (see also Table 4.5).

4.3.2.6 Flavonoids

Flavonoids are known to demonstrate a variety of biological activities, including antithrombic effects, anti-inflammatory and antispasmodic actions, antiviral, antifungal, antibacterial and antitumoral activities and diuretic properties. They also play an important role in normal plant growth and

development and defence against infection and injury (Cody *et al.*, 1986, 1988). Whereas the antibacterial and antifungal activities of the flavonoids have been reported repeatedly, little is known about the mode of action of the bioactive flavonoids. Mori and co-workers (1987) reported antibacterial activity, a structure–activity relationship and the effects of several flavonoids (e.g. flavones, flavonols, flavanones, flavanonols and catechins) on DNA and RNA synthesis in *Proteus vulgaris* (gram-negative) and *S. aureus* (gram-positive). Certain flavonols (e.g. myricetin, robinetin) were the most effective antibacterial agents. Flavanones and flavanonols were not at all effective. A free 3′,4′,5′-trihydroxy B-ring and a free 3-OH group are necessary for antibacterial activity (Table 4.7).

The antimicrobial activities of extracts and constituents of *Gomphrena martiona* and *Gomphrena boliviana* (Amaranthaceae) were determined, in order to identify the compounds responsible for the traditional medicinal use of these plants. A bioassay-guided fractionation of a petroleum ether extract yielded five 5,6,7-trisubstituted flavones showing high activity against *Mycobacterium phlei*, MIC_{50} 15.0–75.0 μg/mL, which approaches that of the commercial antibiotic streptomycin sulphate (MIC_{50} 10.0 μg/mL). Other natural and synthetic flavonoids with diverse structures were tested to define structure–activity relationships. For example, 5,6,7-trisubstituted flavones exhibited minor MIC_{50} values as compared to the 5,7-disubstituted flavones. The occurrence of the rare 5,6,7-trisubstituted flavones in both *Gomphrena* species may explain the medicinal use of these plants against bacterial diseases. Furthermore, the lipophilic flavonoids, located in the epidermis and/or cuticula of the leaves, may be responsible for the resistance of these species to plant diseases (Pomilio *et al.*, 1992).

Recently, Bernard *et al.* (1997) described, for the first time, a DNA topoisomerase inhibitor specific for topoisomerase IV. Three flavonoids were isolated from cottonseed flour, which promoted *E. coli* topoisomerase IV-dependent DNA cleavage. Rutin, the most active, inhibited topoisomerase IV-dependent decatenation activity (concentration required to produce half maximum inhibition IC_{50} 64 μg/mL). None of the flavonoids isolated had any stimulatory activity on *E. coli* DNA gyrase-dependent or calf thymus topoisomerase II-dependent DNA cleavage.

The isoflavanones, dihydrobiochanin A, ferreirin, darbergioidin and dihydrocajanin, isolated from *Swartzia polyphylla* (Leguminosae), revealed potent antibacterial activity against cariogenic bacteria (e.g. *Streptococcus cricetus*, *Streptococcus rattus*, *Streptococcus mutans*) (Osawa *et al.*, 1992).

Multidrug-resistant efflux pumps (MDRs) are widely spread among the gram-positive and gram-negative bacteria as well as among yeasts. MDRs are responsible for the increasing number of multidrug-resistant bacteria worldwide. The problematic group of bacteria include methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE), β-lactamase-producing enteric bacteria (*E. coli*, *Salmonella*, *Klebsiella*, *Shigella* spp.) as well as *Pseudomonas* spp., *Campylobacter* spp. and *M. tuberculosis* (Aqil *et al.*, 2006). Using a

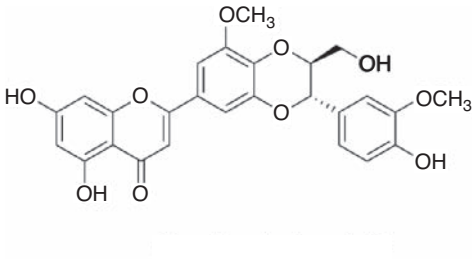

Figure 4.5 5′-methoxyhydnocarpin.

bioassay-guided purification, Stermitz *et al.* (2000) isolated from a chloroform extract of *Berberis fremontii*, a potent MDR inhibitor. Its chemical structure was elucidated as 5′-methoxyhydnocarpin (5′-MHC), a so-called flavonolignane derivative. 5′-MHC (see Fig. 4.5) was also found in *Berberis repens* and *Berberis aquifolia*. The compound potentiated the antibacterial activity of berberine, a cationic alkaloid which is synthesized in a variety of plant species, especially in the family Berberidaceae. In vitro berberine displays a relatively weak antibiotic activity against *S. aureus* because of its efflux by MDRs. 5′-MHC inhibited the efflux of berberine from the pathogenic bacteria *S. aureus* (wild type) expressing the NorA MDR pump. The MIC value of berberine dropped dramatically from 256 μg/mL (MIC value without 5′-MHC) to 16 μg/mL (MIC value with 5′-MHC).

4.3.2.7 Miscellaneous phenolic compounds

Plants release a variety of organic compounds into the environment by leaf leachates and root exudates and through decomposition of litter. The growth of microorganisms in the rhizosphere may be profoundly controlled by these compounds. Certain classes of these compounds, predominantly phenols, phenolic acids and aromatic alcohols, have been reported in the literature to exert an antibacterial effect (see also Table 4.7). Ferulic, isovanillic, *p*-hydroxycinnamic, *p*-hydroxybenzoic, syringic, caffeic, gentisic, protocatechuic, *p*-coumaric, vanillic and *p*-hydroxybenzoic acid, isolated from different plant sources, have exhibited potent antibacterial activities. The mechanism of action of phenolic compounds is described as being non-specific and resulting in alterations of the cytoplasmic membrane (Lucchini *et al.*, 1990; Binutu *et al.*, 1996; Fernandez *et al.*, 1996). Caffeic acid inhibited aflatoxin production of *Aspergillus flavus* without inhibiting the fungal growth. The compound also showed bactericidal activity towards *P. aeruginosa* and *S. aureus* (Paster *et al.*, 1988).

In a further study, Kobayashi *et al.* (1996) identified 4-hydroxystyrene, 3-methoxy-4-hydroxystyrene and 3-methoxy-4-acetoxystyrene as exudate components from wheat roots in sterile hydroponic culture. This indicates that

these antimicrobial components may play a significant role in the defence system as allelochemicals for the rhizosphere.

The benzophenone derivatives, garcinol and isogarcinol, both isolated from the pericarps of *Garcinia purpurea* (Guttiferae), as well as xanthochymol, isolated from the pericarp of *Garcinia subelliptica*, exhibited antibacterial properties against methicillin-resistant and methicillin-sensitive *S. aureus*. Xanthochymol inhibited the methicillin-resistant bacterium in the concentration range 3.1–12.5 μg/mL. This MIC_{50} value was almost equal to that of the antibiotic vancomycin (6.26 μg/mL), which is currently used to treat methicillin-resistant infections (Iinuma *et al.*, 1996).

The naphthoquinones, plumbagin, juglone, lawsone, kigelinone, isopinnatal, dehydro-α-lapachone and lapachol, displayed a wide spectrum of antimicrobial activity (Didry *et al.*, 1994; Binutu *et al.*, 1996).

The dimeric phenylpropanoids, magnolol and honokiol, both isolated from stem bark of *Magnolia obovata*, displayed strong antimicrobial activity against the periodontopathic microorganisms, *P. gingivalis*, *Prevotella gingivalis*, *Actinobacillus actinomycetemcomitans*, *Capnocytophaga gingivalis* and *Veillonella disper*. Their activities were similar to that of listerine (MIC: 10–80 μg/mL), a known oral antiseptic (Chang *et al.*, 1998).

4.3.2.8 Triterpenoids

During the search for antimicrobial compounds from higher plant sources, several bioactive triterpenes were isolated by bioassay-guided fractionation and purification of aqueous or alcohol plant extracts (see Table 4.7). A methanol extract of *Ceanothus americanus* demonstrated antimicrobial activity against selected oral pathogens. Ceanothic acid and ceanothetric acid isolated from the extract demonstrated growth inhibitory effects against *S. mutans*, *Actinomyces viscosus*, *P. gingivalis* and *Prevotella intermedia*, with MIC values ranging 42.0–625.0 μg/mL (Li *et al.*, 1997). In a further study, Perese and co-workers (1997) isolated acetyl aleuritolic acid from the aqueousethanolic extract of *Croton urucurana* (Euphorbiaceae), a triterpene exhibiting antibacterial activity both against *S. aureus* and *S. typhimurium*. Furthermore, 22β-acetoxylantic acid, a triterpene derived from *Lantana camara*, was active against *S. aureus* and *Salmonella typhyi* (Barre *et al.*, 1997).

4.3.3 Mode of antimicrobial action

The mode of antimicrobial action thought to be responsible for the toxicity of plant secondary metabolites (= phytoantimicrobial agents; see Table 4.8) includes inhibition of different microbial-based enzymes, MDR pumps, synthesis of microbial nucleic acids, direct binding to microbial cell wall, disruption of cell membrane and alteration of cell membrane integrity (Cowan, 1999).

Table 4.8 Compilation of important bacterial/fungal targets attacked by selected phyto-antimicrobial agents (see also Table 4.6)

Bacterial/fungal targets	Bacteria	Selected compounds	Selected plants	References
Enzymes				
DNA topoisomerase IV inhibitor	*Escherichia coli*	*Flavonoids*: rutin	*Gossypium arboreum*	Bernard *et al.* (1997)
β-Lactamase inhibitor	*Staphylococcus aureus*	*Tannins*: epigallocatechin gallate	*Camelia sinensis*	Zhao *et al.* (2001)
	E. coli; *Pseudomonas aeruginosa*, *Enterobacter cloacae*	Anacardic acid	*Spondias mombin*	Coates *et al.* (1994)
	S. aureus	*Isoflavones*: Lycoricidin	*Glycyrrhiza glabra*	Hatano *et al.* (2000)
	Bacillus cereus, *S. aureus*	*Anthraquinones*: aloe-emodin; emodin; rhein		Liang *et al.* (2003)
MDR pumps				
NorA inhibitor	*S. aureus*	*Flavonolignans*: 5′-methoxyhydnocarpin	*Berberis fremontii*, *B. repens*, *B. aquifolia*	Stermitz *et al.* (2000)
Nucleic acid				
Inhibition of DNA synthesis	*Proteus vulgaris*, *S. aureus*, *Bacillus subtilis*	*Flavonoids*: myricetin; robinetin. *Triterpenes*: β-glycyrrhitinic acid	*G. glabra*	Mori *et al.* (1987), Kim *et al.* (2004)
Inhibition of RNA synthesis	*P. vulgaris*, *S. aureus*, *B. subtilis*	*Flavonoids*: myricetin; robinetin; *Triterpenes*: β-glycyrrhitinic acid	*G. glabra*	Mori *et al.* (1987), Kim *et al.* (2004)
Cell membrane				
Change of membrane integrity	*B. cereus*, *B. subtilis*, *Clostridium sporogenes*	Capsaicin	*Capsicum annuum*, *Capsicum frutescens*	Cichewicz *et al.* (1996)
	Liposomes; artificial membranes	*Tannins*: epicatetechin; epigallocatechin gallate	*Camelia sinesis*	Ikigai *et al.* (1993)
	Candida albicans	*Triterpenes*: rotunic acid	*Ilex integra*	Haragüchi *et al.* (1999)
Cell wall				
Change of cell wall integrity; direct binding to peptidoglycan	*S. aureus*	*Tannins*: epigallocatechin gallate	*C. sinensis*	Zhao *et al.* (2001)

4.4 Secondary metabolites from higher plants with antiviral properties

The search for selective antiviral agents, focused mainly on anti-human immunodeficiency virus (HIV) agents, has been vigorous in recent years but progress in the development of useful new antivirals has been slow. Meanwhile, the frequency of viral resistance to the relatively few antiviral drugs currently used is increasing (Mohrig, 1996). Furthermore, the treatment of viral infections is often unsatisfactory and new viral pathogens are likely to be discovered. There is a need to find new substances with not only intracellular but also extracellular virucidal properties. Most of the known antiseptics and disinfectants fail to kill all pathogens in a given time at room temperature (Vlietinck and Vanden Berghe, 1991). The methods commonly used for the evaluation of in vitro antiviral activities of synthetic and natural substances are based mainly on the inhibition of cytopathic effects, the reduction or inhibition of plaque formation and reduction in the virus yield, but also on other viral functions in selected host cells cultures (Vlietinck and Vanden Berghe, 1991).

Facing novel antiviral agents, there have been numerous broad-based programmes initiated worldwide in the last two decades to evaluate the antiviral activity of several hundred medicinal plants for in vitro assays. Especially such countries known for its rich rainforests reached the centre of special scientific interest. For instance, different aqueous and alcoholic extracts of *Sanicula europaea*, *Nepeta tuberose*, *Sanguisorba minor* as well as other medicinal plants worldwide exhibited clear antiviral activity against DNA and RNA viruses such as herpes simplex virus type 1 and type 2, varicella-zoster virus, vesicular stomatitis virus, influenza A virus and poliovirus type 1. Furthermore, a leaf extract of *Azadirachta indica* was found to be active against a number of viruses such as smallpox virus, chicken pox virus, poliomyelitis virus and herpesvirus. An extract of the cactus plant *Opuntia streptacantha* inhibited intracellular DNA and RNA virus replication and inactivated extracellular virus such as herpes simplex virus, equine herpesvirus, pseudorabies virus and influenza virus (for comprehensive reviews, see Nawawi *et al.*, 1999; Hsiang *et al.*, 2001; Jassim and Naji, 2003; Lipipun *et al.*, 2003; Li *et al.*, 2004; Tolo *et al.*, 2006). These examples may show that there are innumerable potentially useful medicinal plants waiting to be evaluated and exploited for therapeutical applications against genetically and functionally diverse virus families such as *Retroviridae*, *Hepadnaviridae*, *Herpesviridae*, *Orthomyxoviridae* and *Picornaviridae*.

Using bioassay-guided fractionation of bioactive plant extracts, it was found that various proteins, glycoproteins, polysaccharides and secondary metabolites are the most active antiviral substances of plant extracts. Secondary metabolites with antiviral properties originate in a whole range of substance classes, such as alkaloids, lignans, phenols, phenolic glycosides, quinones, flavonoids, coumarins, tannins, sesquiterpenes and saponins (Van

Den Berghe *et al.*, 1978, 1986; Van Hoof *et al.*, 1989; De Rodriguez and Chulia, 1990; Bender *et al.*, 1992; Stevenson and Lenard, 1993; Amoros *et al.*, 1994; Vlietinck *et al.*, 1998). These compounds are able to inhibit different stages in the replication cycle of various viruses (see Tables 4.11–4.14 and Figs 4.6–4.9).

In the following review, the progress on this expanding scientific field is documented by the most important results published in the last decade. In the following sections, those substances whose mode of action is known are discussed in more detail.

4.4.1 Compilation of viruses and host cells mentioned in this review

Tables 4.9 and 4.10 comprise the abbreviations of the viruses and host cell systems reviewed.

4.4.2 Isolated secondary metabolites with anti-HIV properties

In the last 10 years, much effort has been made to find effective anti-HIV agents. The acquired immunodeficiency syndrome (AIDS) is a pandemic immunosuppressive disease, which results in life-threatening, opportunistic infections. HIV, a retrovirus, has been identified as the aetiological agent of AIDS. HIV infects human $CD4^+$ lymphocytes, monocytes and macrophages. Direct binding between the viral envelope gp120 glycoprotein and the $CD4^+$ receptor is necessary to initiate a productive infection. The HIV-1 infected cells express viral gp120 glycoproteins on their surface, which bind with the $CD4^+$ receptors of infected cells, leading to the formation of giant cell complexes (syncytia). Subsequently, the syncytia undergo cytolysis and cells die within a few days.

Any novel synthetic or plant-derived anti-HIV compound that selectively interferes with HIV-1 replication, virus adsorption, syncytium formation, inhibition of HIV-1 reverse transcriptase (HIV-1 RT) or viral protease should be considered relevant in the treatment of AIDS. In a review, Vlietinck and co-workers (1998) reported that many compounds of plant origin inhibit different stages in the replication cycle of HIV: virus adsorption, e.g. chromone alkaloids, isoquinoline alkaloids, polyphenolics, flavonoids, coumarins, phenols, tannins and triterpenes; virus-cell fusion, e.g. triterpenes; reverse transcription, e.g. benzophenanthridine alkaloids, protoberberine alkaloids, isoquinoline and quinoline alkaloids, coumarins, flavonoids, lactones, tannins, iridoids and triterpenes; proteolytic cleavage (protease inhibition), e.g. triterpenes, xanthones and coumarins; glycosylation, e.g. indolizidine alkaloids, piperidine alkaloids and pyrrolizidine alkaloids; integrase inhibition, e.g. flavonoids, tannins; and virus assembly and release, e.g. hypericin and pseudohypericin. The most important secondary metabolites with antiviral properties against HIV are summarized in Table 4.11 (see also Fig. 4.6).

Table 4.9 Abbreviations of viruses reviewed

Double-stranded deoxyribonucleic acid (dsDNA) viruses	
Adenoviridae	
ADV	adenovirus
ADV31	adenovirus type 31
Herpesviridae	
BHV 1	bovine herpes virus type 1
BMV	bovine mammalitis virus
CMV	cytomegalovirus
EHV-1	equine herpes virus type 1
HCMV	human cytomegalovirus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
MCMV	murine cytomegalovirus
PRV	pseudorabies virus
VZV	varicella-zoster virus
Poxviridae	
VV	vaccinia virus
Double-stranded ribonucleic acid (dsRNA) viruses	
Birnaviridae	
IPNV	infectious pancreatic necrosis virus
Reoviridae	
BRV	bovine rotavirus
(−)-Single-stranded DNA (ssDNA)/(+)-ssDNA	
Hepadnaviridae	
HBV	hepatitis B virus
(+)-Single-stranded ribonucleic acid (ssRNA) viruses	
Coronaviridae	
BCV	bovine coronavirus
Flaviviridae	
DV-4	dengue virus type 4
JEV	Japanese encephalitis virus
YFV	yellow fever virus
Picornaviridae	
COXB1	coxsackievirus B type 1
COXB2	coxsackievirus B type 2
EMCV	encephalomyocarditis virus
HRV-1B	human rhinovirus type 1B
HRV-2B	human rhinovirus type 2B
PCV	picornavirus
PMV	poliomyelitis virus
POLIO 1	poliovirus 1
RV-2	rhinovirus type 2
RV-1B	rhinovirus type 113
Retroviridae	
AMV	avian myeloblastosis virus
EAV	equine anaemia virus
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
MMLV	Moloney murine leukaemia virus
Mo-MuLV	Moloney murine leukaemia virus

(*Continued*)

Table 4.9 (*Continued*)

Togaviridae	
SINV	Sindbis virus
SFVL10	Semliki forest L10 virus
SV	Sindbis virus
VEEV	Venezuelan equine encephalomyelitis virus
(−)-Single-stranded ribonucleic acid (ssRNA) viruses	
Bunyaviridae	
PTV	Punta Toro virus
RVFV	Rift Valley fever virus
SFV	sandfly fever virus
Orthomyxoviridae	
FPVA	avian influenza (fowl plague) virus A
INFA	influenza A virus
INFB	influenza B virus
Paramyxoviridae	
BPIV 3	bovine parainfluenza virus type 3
BRSV	bovine respiratory syncytial virus
MEVA	measles Edmonston A virus
Para-3	parainfluenza virus type 3
RSV	respiratory syncytial virus
Rhabdoviridae	
IHNV	infectious haematopoietic necrosis virus
VSV	vesicular stomatitis virus
OMV	Onorhynchus masou virus

Table 4.10 Abbreviations of host cells reviewed

A-549	human adenocarcinoma A-549 cells
BHK-21	baby hamster kidney cells
BSC-1	monkey kidney epithelial cells
C-8166	human lymphoblastic cells
CEM-SS	human lymphoblastoid cells
CER	chicken embryo related cells
CV-1	African green monkey kidney cells
HEF	human embryonic fibroblast cells
HeLa	(Helen Lake) human epithelial cervical carcinoma cells
HEL	human embryonic lung cells
MA 104	monkey kidney cells
MDBK	Madin–Darby bovine kidney cells
MDCK	Madin–Darby canine kidney cells
MOLT-4	human leukaemic T cells
MRC-5	human embryo lung cells
MT-4	human T cell leukaemia virus-I (HTLV-I)-carrying cells
PLC/PRF/5	human hepatoma cells
PBM	primary human peripheral blood mononuclear cells
SC-1	mouse cells
3T3-L1	mouse cells
Vero	African green monkey kidney cells
VK	primary vervet monkey kidney cells

Table 4.11 Secondary metabolites with anti-human immunodeficiency virus (HIV) activity

Compounds	Viruses	Host cells/ biological targets	Evaluation of antiviral activity	EC_{50}/IC_{50} (μM)	References
Alkaloids					
Colchicine-type					
Colchicine	HIV-1	H9 lymphocytes	p24	0.01	Tatematsu *et al.* (1991)
Piperidone-type					
N-Methylschummannificine	HIV-1	C8166	gp120	5.0	Houghton *et al.* (1994)
Anhydroschummannificine	HIV-1	C8166	gp120	0.4	Houghton *et al.* (1994)
N-Methylanhydroschummannificine	HIV-1	C8166	gp120	20.0	Houghton *et al.* (1994)
Rohitukine	HIV-1	C8166	gp120	30.0	Houghton *et al.* (1994)
Schummanificine	HIV-1	C8166	gp120	1.6	Houghton *et al.* (1994)
Pyridino-type					
Schummanniophytine	HIV-1	C8166	gp120	8.0	Houghton *et al.* (1994)
Isoschummanniophytine	HIV-1	C8166	gp120	80.0	Houghton *et al.* (1994)
N-Methylschummanniophytine	HIV-1	C8166	gp120	80.0	Houghton *et al.* (1994)
Quinoline-type					
Buchapine	HIV-1	CEM-SS	XTT assay	0.94	McCormick *et al.* (1996)
Buchapine	HIV-1	RT		12.0	McCormick *et al.* (1996)

3-(3-Methyl-2-butenyl)-4-(3-methyl-2-butenyl)-oxyl-2-quinolinone	HIV-1	CEM-SS	XTT assay	1.64	McCormick *et al.* (1996)
	HIV-1	RT		8.0	McCormick *et al.* (1996)
Coumarins					
Calanolide A	HIV-1	CEM-SS	XTT assay	0.1	Currens *et al.* (1996)
	HIV-1	C8166	XTT assay	2.0	Currens *et al.* (1996)
Inophyllum B	HIV-1	RT		0.042	Taylor *et al.* (1994)
Diterpenes					
Abietane-type					
Carnosolic acid	HIV-1	Protease		0.08 μg/mL (IC_{90})	Paris *et al.* (1993)
Rosmanol	HIV-1	Protease		0.60 μg/mL (IC_{90})	Paris *et al.* (1993)
7-*O*-Methylrosmanol	HIV-1	Protease		1.5 μg/mL (IC_{90})	Paris *et al.* (1993)
7-*O*-Ethylrosmanol	HIV-1	Protease		1.7 μg/mL (IC_{90})	Paris *et al.* (1993)
Kauren-type					
Tripterifordin	HIV-1	H9 lymphocytes	p24	1.0	Chen *et al.* (1992a)
Flavonoids					
Flavonol-type					
Fisetin	HIV-1	H9 lymphocytes	p24	122	Hu *et al.* (1994)
Galangin	HIV-1	H9 lymphocytes	p24	28.0	Hu *et al.* (1994)
Hesperidin	HIV-1	H9 lymphocytes	p24	> 164	Hu *et al.* (1994)
Kaempferol	HIV-1	Protease		2.0 μg/mL	Mahmood *et al.* (1996)
	HIV-1	C8166	gp120	4.0 μg/mL	Mahmood *et al.* (1996)
Kaempferol-3-glucoside	HIV-1	C8166	gp120	8.0 μg/mL	Mahmood *et al.* (1996)
Kaempferol-3-(6 *p*-coumaroyl)-β-glucoside	HIV-1	C8166	gp120	8.0 μg/mL	Mahmood *et al.* (1996)

(*Continued*)

Table 4.11 (*Continued*)

Compounds	Viruses	Host cells/ biological targets	Evaluation of antiviral activity	EC_{50}/IC_{50} (μM)	References
Morin	HIV-1	H9 lymphocytes	p24	> 331	Hu *et al.* (1994)
Myricetin	HIV-1	H9 lymphocytes	p24	35.0	Hu *et al.* (1994)
Quercetin	HIV-1	Protease		20.0 μg/mL	Mahmood *et al.* (1996)
	HIV-1	H9 lymphocytes	p24	132	Hu *et al.* (1994)
	HIV-1	C8166	gp120	20.0 μg/mL	Mahmood *et al.* (1996)
Quercetin-3-*O*-β-galactopyranoside	HIV-1	Integrase		64.6 μg/mL	Kim *et al.* (1998)
Quercetin-3-*O*-α-rhamnopyranoside	HIV-1	Integrase		75.2 kg/mL	Kim *et al.* (1998)
Quercetin-3-*O*-(2″-galloyl)-α-arabinopyranoside	HIV-1	Integrase		18.1 μg/mL	Kim *et al.* (1998)
Quercetin-3-*O*-(2″-galloyl)-β-galactopyranoside	HIV-1	Integrase		27.9 kg/mL	Kim *et al.* (1998)
Quercetin-3-*O*-(2″,6″-digalloyl)-β-galactopyranoside	HIV-1	Integrase		24.2 lig/mL	Kim *et al.* (1998)
Flavone-type					
Acacetinrhamnoglucosid	HIV-1	H9 lymphocytes	p24	> 231	Hu *et al.* (1994)
Acacetingalactoside	HIV-1	H9 lymphocytes	p24	8.0	Hu *et al.* (1994)
Apigenin	HIV-1	H9 lymphocytes	p24	9.0	Hu *et al.* (1994)
	HIV-1	RT		443	Lin *et al.* (1997)
Apigeningalactosid	HIV-1	H9 lymphocytes	p24	61.0	Hu *et al.* (1994)
Baicalin	HIV-1	CEM-SS	focal syncytium formation	25.0	Li *et al.* (1993a)
	HIV-1	RT		0.015	Li *et al.* (1993a)
Chrysin	HIV-1	H9 lymphocytes	p24	5.0	Hu *et al.* (1994)
7,8-Dihydroxyflavone	HIV-1	H9 lymphocytes	p24	10.0	Hu *et al.* (1994)

3-Hydroxyflavone	HIV-1	H9 lymphocytes	p24	13.0	Hu *et al.* (1994)
4′,5,7-Trihydroxyflavone	HIV-1	H9 lymphocytes	p24	92.0	Hu *et al.* (1994)
Flavone	HIV-1	H9 lymphocytes	p24	50.0	Hu *et al.* (1994)
Luteolin	HIV-1	H9 lymphocytes	p24	10.0	Hu *et al.* (1994)
Flavanone-type					
(+)-Catechin	HIV-1	H9 lymphocytes	p24	> 345	Hu *et al.* (1994)
Flavanone	HIV-1	H9 lymphocytes	p24	45.0	Hu *et al.* (1994)
Biflavonoid-type					
Agathisflavone	HIV-1	RT		100	Lin *et al.* (1997)
Agathisflavone	HIV-1	PBM		33.6	Lin *et al.* (1997)
Amentoflavone	HIV-1	RT		119	Lin *et al.* (1997)
	HIV-1	PBM		94.0	Lin *et al.* (1997)
Hinokiflavone	HIV-1	RT		62.0	Lin *et al.* (1997)
	HIV-1	PBM		4.1	Lin *et al.* (1997)
Morelloflavone	HIV-1	RT		116.0	Lin *et al.* (1997)
	HIV-1	PBM		6.9	Lin *et al.* (1997)
Robustaflavone	HIV-1	RT		65.0	Lin *et al.* (1997)
	HIV-1	PBM		> 100	Lin *et al.* (1997)
Swertifrancheside	HIV-1	Nucleic acid polymerase		42.9	Pengsuparp *et al.* (1995)
	HIV-2	Nucleic acid polymerase		56.6	Pengsuparp *et al.* (1995)
Isoflavonoids					
Pterocarpan-type					
3-*O*-Methylcalopocarpin	HIV-1	CEM-SS	pra	0.2 μg/mL	McKee *et al.* (1997)
Sandwicensin	HIV-1	CEM-SS	pra	2.0 μg/mL	McKee *et al.* (1997)
Isoflavan-type					
5-Deoxyglyasperin F	HIV-1	NIC primary anti-HIV screen	XTT assay		McKee *et al.* (1997)
5-Hydroxyneobavaisoflavanone	HIV-1	NIC primary anti-HIV screen	XTT assay		McKee *et al.* (1997)

(*Continued*)

Table 4.11 (*Continued*)

Compounds	Viruses	Host cells/ biological targets	Evaluation of antiviral activity	EC_{50}/IC_{50} (μM)	References
Lignans					
Anolignan A	HIV-1	RT		60.4 μg/mL	Rimando *et al.* (1994)
Anolignan B	HIV-1	RT		1072.0 μg/mL	Rimando *et al.* (1994)
Interiotherin A	HIV-1	H9 lymphocytes	p24	3.1 μg/mL	Chen *et al.* (1996)
Mal.4	HIV-1	PBM	p24	1–10 μg/mL	Gnabre *et al.* (1995, 1996)
Phyllamycin B	HIV-1	RT		3.5	Chang *et al.* (1995)
	HIV-1	Human DNA polymerase-α		289	Chang *et al.* (1995)
Retrojusticin B	HIV-1	RT		5.49	Chang *et al.* (1995)
	HIV-1	Human DNA polymerase-α		989	Chang *et al.* (1995)
Schisantherin D	HIV-1	H9 lymphocytes	p24	0.5 μg/mL	Chen *et al.* (1996)
Phenolics					
Curcumin	HIV-1	Integrase		40.0	Mazumder *et al.* (1995)
Protolichesterinic acid	HIV-1	Nucleic acid polymerase		24.3	Pengsuparp *et al.* (1995)
	HIV-2	Nucleic acid polymerase		29.6	Pengsuparp *et al.* (1995)
Putranjivain	HIV-1	RT		3.9	El-Mekkawy *et al.* (1995)
Sesquiterpenes					
Peyssonol A	HIV-1	RT		38.7	Loya *et al.* (1995)
	HIV-2	RT		23.7	Loya *et al.* (1995)

Peyssonol B	HIV-1	RT	34.5	Loya *et al.* (1995)
	HIV-2	RT	28.0	Loya *et al.* (1995)
Tannins				
Gallic acid methyl ester	HIV-1	Integrase	35.8 μg/mL	Kim *et al.* (1998)
1.6-Digalloyl-glucose	HIV-1	RT	270	El-Mekkawy *et al.* (1995)
Digallic acid	HIV-1	RT	200	El-Mekkawy *et al.* (1995)
Punicalin	HIV-1	RT	8.0	Nonaka *et al.* (1990)
Punicacortein	HIV-1	RT	5.0	Nonaka *et al.* (1990)
Sanguin H-11	HIV-1	RT	20.0	Nonaka *et al.* (1990)
Caffeoylquinate-type				
3,4-Di-caffeoylquinic acid	HIV-1	RT	19.4	Chang *et al.* (1995)
	HIV-1	Human DNA polymerase-α	11.6	Chang *et al.* (1995)
3,5-Di-caffeoylquinic acid	HIV-1	RT	1.16	Chang *et al.* (1995)
	HIV-1	Human DNA polymerase-α	2.32	Chang *et al.* (1995)
Methyl 3,4-di-caffeoyl-quinic acid	HIV-1	RT	94.0	Chang *et al.* (1995)
	HIV-1	Human DNA polymerase-α	45.0	Chang *et al.* (1995)
Methyl 3,5-di-caffeoyl-quinic acid	HIV-1	RT	1.7	Chang *et al.* (1995)
Methyl 3,5-di-caffeoyl-quinic acid	HIV-1	Human DNA polymerase-α	3.77	Chang *et al.* (1995)
Galloylglucose-type				
1,2,6-Tri-*O*-galloyl-β-glucose	HIV-1	Integrase	28.3 μg/mL	Kim *et al.* (1998)
1,2,3,4,6-Penta-*O*-galloyl-β-glucose	HIV-1	Integrase	28.0 μg/mL	Kim *et al.* (1998)
Galloylquinate-type				
3-Galloylquinic acid	HIV-1	RT	72.6	Chang *et al.* (1995)

(*Continued*)

Table 4.11 (*Continued*)

Compounds	Viruses	Host cells/ biological targets	Evaluation of antiviral activity	EC_{50}/IC_{50} (μM)	References
	HIV-1	Human DNA polymerase-α		8.72	Chang *et al.* (1995)
3,4-Di-galloylquinic acid	HIV-1	RT		7.81	Chang *et al.* (1995)
	HIV-1	Human DNA polymerase-α		0.61	Chang *et al.* (1995)
3,5-Di-galloylquinic acid	HIV-1	RT		1.31	Chang *et al.* (1995)
	HIV-1	Human DNA polymerase-α		0.48	Chang *et al.* (1995)
3,5-Di-galloyl-4-di-galloylquinic acid	HIV-1	RT		10.0	Nishizawa *et al.* (1989)
	HIV-1	H9 lymphocytes	p24	6.25	Nishizawa *et al.* (1989)
3,4-Di-galloyl-5-di-galloylquinic acid	HIV-1	RT		10.0	Nishizawa *et al.* (1989)
	HIV-1	H9 lymphocytes	p24	6.25	Nishizawa *et al.* (1989)
3-Di-galloyl-4,5-di-galloylquinic acid	HIV-1	RT		10.0	Nishizawa *et al.* (1989)
	HIV-1	H9 lymphocytes	p24	6.25	Nishizawa *et al.* (1989)
1,3,4,5-Tetra-galloylquinic acid	HIV-1	RT		10.0	Nishizawa *et al.* (1989)
	HIV-1	H9 lymphocyte cells	p24	6.25	Nishizawa *et al.* (1989)
3,4,5-Tri-galloylquinic acid	HIV-1	H9 lymphocyte cells	p24	6.52	Nishizawa *et al.* (1989)

	HIV-1	RT		0.08	Chang *et al.* (1995)
	HIV-1	Human DNA polymerase-α		0.17	Chang *et al.* (1995)
Galloylshikimate-type					
3,4-Di-galloylshikimic acid	HIV-1	RT		1.77	Chang *et al.* (1995)
	HIV-1	Human DNA polymerase-α		1.05	Chang *et al.* (1995)
3,5-Di-galloylshikimic acid	HIV-1	RT		0.52	Chang *et al.* (1995)
	HIV-1	Human DNA polymerase-α		0.31	Chang *et al.* (1995)
3,4,5-Tri-galloylshikimic acid	HIV-1	RT		0.10	Chang *et al.* (1995)
	HIV-1	Human DNA polymerase-α		0.22	Chang *et al.* (1995)
2-Phenylethanol-(6-galloyl)-glucoside	HIV-1	C8166	gp120	40.0 μg/mL	Mahmood *et al.* (1996)
Triterpenes					
1β-Hydroxyaleuteriolic acid 3-*p*-hydroxybenzoate	HIV-1	RT		3.7	Pengsuparp *et al.* (1995)
	HIV-2	RT		59.0	Pengsuparp *et al.* (1995)
Betulinic acid	HIV-1	H9 lymphocytes	p24	1.4	Fujioka and Kashiwada (1994)
Celasdine-B	HIV-1	H9 lymphocytes	p24	0.8 μg/mL	Kuo and Kuo (1997)
Epipomolic acid	HIV-1	Protease		17.9 μg/mL (inhibition: 42%)	Xu *et al.* (1996)

(*Continued*)

Table 4.11 (*Continued*)

Compounds	Viruses	Host cells/ biological targets	Evaluation of antiviral activity	EC_{50}/IC_{50} (μM)	References
Gleditsia saponin C	HIV-1	H9 lymphocytes	p24	1.1	Konoshima *et al.* (1995)
Glycyrrhizin	HIV-1	MT-4	pra	150.0	Ito *et al.* (1987)
Gymnocladus saponin G	HIV-1	H9 lymphocytes	p24	2.7	Konoshima *et al.* (1995)
Maslinic acid	HIV-1	Protease		17.9 μg/mL (inhibition: 100%)	Xu *et al.* (1996)
Platanic acid	HIV-1	H9 lymphocytes	p24	6.5	Fujioka and Kashiwada (1994)
Salaspermic acid	HIV-1	H9 lymphocytes	p24	10.0	Chen *et al.* (1992b)
Salaspermic acid	HIV-1	RT		16.0 μg/mL	Chen *et al.* (1992b)
Soyasaponin II	HIV-1	MT-4	pra	112.0	Hayashi *et al.* (1997)
Suberosol	HIV-1	H9 lymphocytes	p24	3.0 μg/mL	Li *et al.* (1993b)
Tormetic acid	HIV-1	Protease		17.9 μg/mL (inhibition: 49%)	Xu *et al.* (1996)
Ursolic acid	HIV-1	Protease		17.9 μg/mL (inhibition: 85%)	Xu *et al.* (1996)

EC_{50}/IC_{50}, effective/inhibition concentration causing a 50% reduction in the viral cytopathic effect or in viral replication; p24, p24 antigen capture assay; gp120, reduction of gp120 production in cells; RT, reverse transcriptase; pra, plaque reduction assay; XTT, tetrazolium metabolic assay; NCI, National Cancer Institute. For abbreviations of the viruses and host cell systems reviewed, see Tables 4.9 and 4.10.

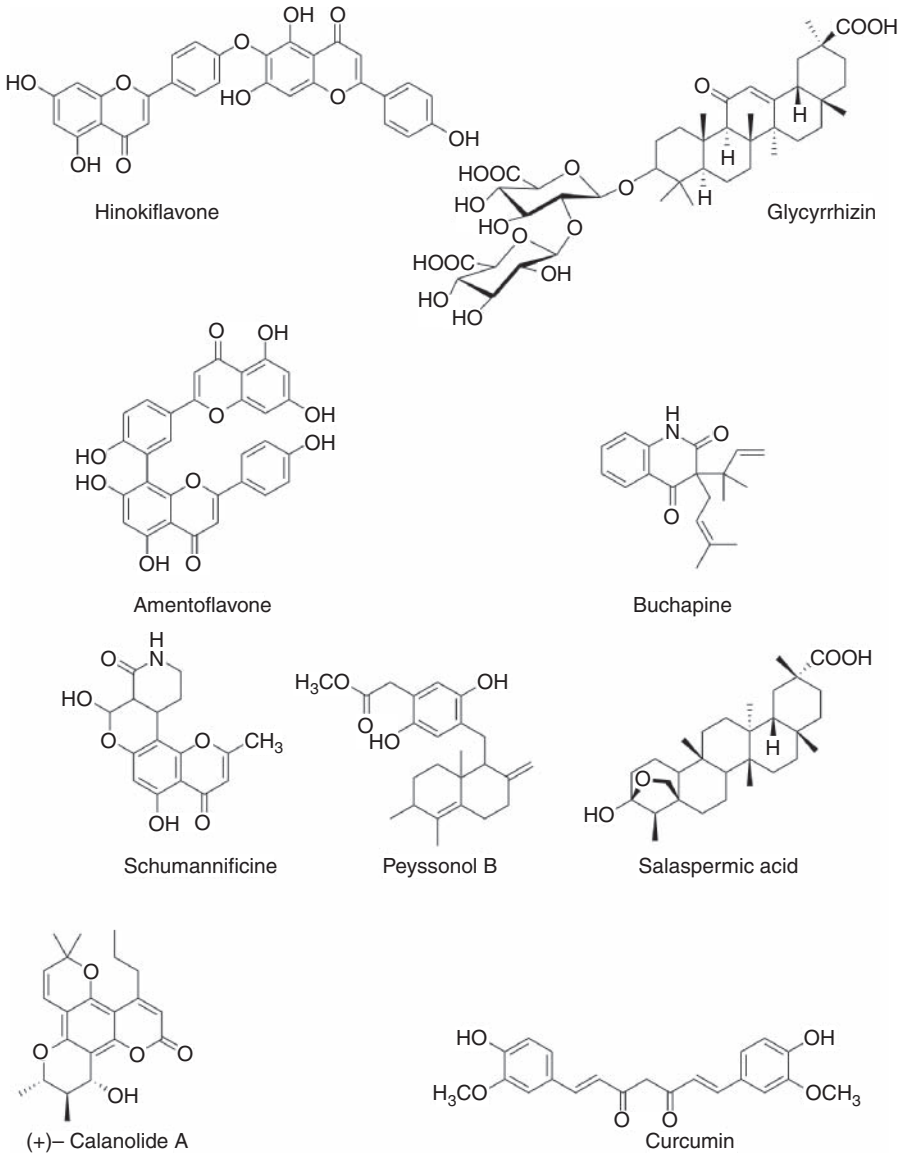

Figure 4.6 Chemical structures of plant-derived compounds with anti-HIV activity.

4.4.2.1 Alkaloids

A number of chromone alkaloids were isolated from the rootbark of *Schumanniophyton magnificum*. Of all the compounds tested, schumannificine exhibited the strongest activity against HIV-1. Potent antiherpes simplex virus (HSV)-1 activity was also observed for a number of its derivatives. It was assumed

that the presence of a piperidine ring and unsubstituted hydroxy groups on the molecules is necessary for the anti-HIV activity, which is considered to be due to irreversible binding to gp120 rather than to inhibition of reverse transcriptase or protease (Houghton *et al.*, 1994).

4.4.2.2 Coumarins

Novel HIV-1 RT inhibitory coumarin derivatives were recently isolated from the tropical rainforest trees, *Calaphyllum lanigerum* and *Calaphyllum inophyllum*. Calanolides A and B, as well as inophyllum B, have been established as non-nucleoside-specific inhibitors of HIV-1 RT. It has been shown that non-nucleoside-specific HIV-1 RT inhibitors act as non-competitive inhibitors of this enzyme. Calanolide A inhibited the in vitro replication of HIV-1 in human lymphoblastoid cells (CEM-SS), but was inactive against HIV-2 and avian myeloblastosis virus (AMV) (Currens *et al.*, 1996). Boyer and coworkers (1993) showed that both calanolide A and B effectively blocked the DNA-dependent DNA polymerase as well as the RNA-dependent DNA-polymerase activity of HIV-1. HIV-1 RT activity was also inactivated by inophyllum B (Taylor *et al.*, 1994). In further studies, calanolide A similarly inhibited promonocytotropic isolates (effective concentration for a 50% reduction in the viral cytopathic effect or viral replication EC_{50} 0.12–0.18 μM) and lymphotrophic isolates (EC_{50} 0.15–0.47 μM) from patients with various stages of HIV-1 disease, as well as drug-resistant strains. In enzyme inhibition assays, calanolide A potently and selectively inhibited HIV-1 RT but not cellular DNA polymerase or HIV-2 RT within the concentration range tested (Currens *et al.*, 1996).

4.4.2.3 Flavonoids

Plant flavonoids are a large group of naturally occurring phenylchromones found in the leaves, stems, flowers and fruits of most of the higher plants. A variety of in vitro and in vivo experiments have shown that selected flavonoids exhibit antiallergic, anti-inflammatory, antiviral and antioxidant activities (Kaul *et al.*, 1985; Cody *et al.*, 1988). Several known flavones were examined for their anti-HIV activity. Chrysine was found to be the most active compound in this series (EC_{50} 5.0 μM). Flavonoids with hydroxy groups at C5 and C7 and with a C2 → C3 double bond were more active than the others. Based on the results obtained, it was postulated that the presence of substituents (hydroxyl and halogen groups) in the B-ring leads to an increased toxicity and/or decreased activity of flavonoids in general (Hu *et al.*, 1994).

Two other flavones, baicalin and isoscutellarein-8-methylether, were isolated from *Scutellaria baicalensis*, a plant used as a traditional Chinese herbal medicine (Li *et al.*, 1993; Nagai *et al.*, 1995). Baicalin affected syncytium formation on CEM-SS monolayer cells, expression of the HIV-1-specific core antigen p24 and reverse transcriptase activity in the HIV-1-infected H9 lymphocytes

(Li *et al.*, 1993). Mahmood *et al.* (1996) tested nine flavonoids, isolated from *Rosa damascena* (Rosaceae), on anti-HIV-1 activity. Kaempferol and its 3-*O*-β-glucopyranoside exhibited the greatest activity against HIV-1 infection of human lymphoblastic (C8166) cells. Kaempferol was effective in reducing maturation of the infectious viral progeny, apparently by selectively inhibiting the viral protease. In contrast, quercetin and two 3-substituted derivatives of kaempferol appeared to inhibit HIV-1 infection by preventing the binding of gp120 to $CD4^+$.

HIV-1 integrase mediates the insertion of viral DNA into host cellular DNA that is essential for viral replication and virion production. Recently, Kim *et al.* (1998) isolated two flavonol glycoside gallate esters with anti-HIV integrase activity from the leaves of *Acer okamotoarum*. Swertifrancheside, a biflavonoid isolated from *Swertia franchetiana*, was found to be a potent inhibitor of the HIV-1 RT (IC_{50} 43 μM). The compound inhibited enzyme activity by binding to the template-primer (Pengsuparp *et al.*, 1995). Eleven biflavonoids and their methyl ethers, isolated from *Rhus succedanea* and *Garcinia multiora*, were evaluated for their anti-HIV-1 RT activity. Robustaflavone and hinokiflavone demonstrated the highest activity against HIV-1 RT; the two compounds displayed similar activity, with IC_{50} values of 65 and 62 μM, respectively (Lin *et al.*, 1997).

4.4.2.4 Lignans

Lignans exhibit a wide range of biological properties, including antifungal, antimicrobial, antiviral and antitumorigenic activities, and inhibition of many enzyme systems. The antiviral activities of lignans have been reviewed by MacRae and co-workers (1989). Gnabre and co-workers (1995, 1996) isolated several lignans from the desert plant, *Larrea tridentata* (Zygophyllaceae), by bioassay-guided chromatography. The most predominant anti-HIV-1 compound was 3′-*O*-methyl-nordihydroguaiaretic acid, denoted as mal.4. Mal.4 was shown to exert its inhibitory activity by interfering with the binding of Sp1 protein to HIV long terminal repeat (LTR) promoter, thus blocking the proviral transcription; HIV Tat (= a potent transcription activator encoded by the HIV-1) transactivation; and suppression of viral replication. Two other lignans, phyllamycin B and retrojusticidin B, isolated from *Phyllanthus myrtifolius* (Euphorbiaceae), have been demonstrated to have a strong inhibitory effect on HIV-1 RT activity but far less inhibitory effect on human DNA polymerase-α activity (Chang *et al.*, 1995). Anolignan A and anolignan B, two dibenzylbutadiene lignans isolated from *Anogeissus acuminata* (Combretaceae), were also identified as active inhibitors of HIV-1 RT activity (Rimando *et al.*, 1994).

4.4.2.5 Tannins

Tannins, such as oligomeric hydrolyzable tannins, complex tannins and other metabolites and condensates, have repeatedly been isolated from medicinal

plants. Most of them have exhibited antiviral activities. It was supposed that tannins, like a number of polyanionic compounds, including polyhydroxy carboxylates derived from phenolic compounds, selectively inhibit HIV replication by interacting with the surface glycoprotein, gp120, to irreversibly prevent the binding of virus to $CD4^+$ receptor. It was also found that reverse transcriptase represents another target for tannins.

It has been shown by several researchers that Chinese herbs represent an important potential source of reverse transcriptase inhibitors. For example, several tannins isolated from Chinese galls exhibit strong inhibitory effects against HIV-1 RT (Takechi *et al.*, 1985; Nishizawa *et al.*, 1989; Nonaka *et al.*, 1990; Okuda *et al.*, 1992; Büechi, 1998). Recently, El-Mekkawy and co-workers (1995) screened methanolic extracts of 41 medicinal plants used in traditional Egyptian medicine for anti-HIV-1 RT activity. The fruit extracts from *Phyllanthus emblica*, *Quercus pedunculata*, *Rumex cyprius*, *Terminalia bellerica*, *Terminalia chebula* and *Terminalia horrida* revealed significant antiviral activity, with an IC_{50} of 50 μg/mL. Through bioassay-guided fractionation of the active extract of *P. emblica*, several tannins, e.g. 1,6-di-*O*-galloyl-β-D-glucose, 1-*O*-galloyl-β-D-glucose, digallic acid and putranjivain A, were isolated as the potent inhibitory substances. The most active compound was putranjivain A, with an IC_{50} of 3.9 μM. The inhibitory mode of action was non-competitive with respect to the substrate but competitive with respect to a template-primer.

4.4.2.6 Triterpenes

Antiviral saponins have been isolated from various plants, e.g. aescine from *Aesculus hippocastanum*, primula saponins from *Primula veris* and *Anagallis arvensis*, saikosaponin A from *Bupleurum falcatum*, theasaponins from *Thea sinencis* and gymnemic acid from *Gymnema sylvestris*. As yet, the mechanisms of inhibition of virus replication by saponins are not understood in detail.

Glycyrrhizin, the main saponin of *Glycyrrhiza glabra*, inhibits the growth of a number of DNA and RNA viruses, including HIV-l, in vitro. It was found that glycyrrhizin interferes with virus adsorption, which was further complemented by an inhibitory effect on protein kinase C. This enzyme seems to be required for the binding of HIV-1 particles to the cellular $CD4^+$ receptors (Ito *et al.*, 1987). 1-β-Hydroxyaleuritolic acid 3-p-hydroxybenzoate, isolated from the roots of *Maprounea africana*, and salaspermic acid, isolated from the roots of the liana *Tripterygium wilfordii* (Celastraceae), were shown to be inhibitors of HIV-1 RT. Salaspermic acid also inhibited HIV-1 replication in H9 lymphocytes. A structure–activity correlation of salaspermic acid with ten related compounds indicated that the acetal linkage of ring A and the carboxyl group in ring E may be required for the anti-HIV-1 activity (Chen *et al.*, 1992b; Pengsuparp *et al.*, 1995).

Celasdin-B, isolated from *Celastrus hindsii*, betulinic acid and platanic acid, both isolated from the leaves of *Syzygium claviforum*, were found to be inhibitors of HIV-1 replication in H9 lymphocytes. Evaluation of anti-HIV

activity with structurally related triterpenoids revealed that the C-3 hydroxy group, the C-17 carboxylic acid group and the C-19 substituents contribute to enhanced anti-HIV-1 activity (Fujioka and Kashiwada, 1994; Kuo and Kuo, 1997).

4.4.3 Essential oils and isolated secondary metabolites with further antiviral properties

4.4.3.1 Essential oils

Essential oils are well known for their high levels of antifungal and antibacterial activity. Otherwise, there is little information concerning the effects of essential oils on viruses or viral infections in either animal or plant systems. Shukla *et al.* (1989) tested the antiviral activity of the essential oils of *Foeniculum vulgare* and *Pimpinella anisum* against potato virus X, tobacco mosaic virus and tobacco ring spot virus on the host, *Chenopodium amaranticolor*. Both essential oils totally inhibited the formation of local lesions at concentrations of 3000 ppm, when the viruses were pretreated with essential oils 30 min before inoculation. Bishop (1995) tested the essential oil of *M. alternifolia* (Myrtaceae) for antiviral activity against tobacco mosaic virus. When applied to *Nicotiana glutinosa* (Solanaceae) plants as a preinoculation spray at 100, 250 and 500 ppm, the essential oil was effective in significantly decreasing lesion numbers for at least 10 days post-inoculation.

Furthermore, there is considerable evidence emerging from in vitro studies and controlled trials of the potential of plant-derived phyto-antiviral agents for the treatment of human viral infections (Reichling *et al.*, 2009). In the past decades, many essential oils were investigated towards their antiviral activity. Most of them were tested against enveloped RNA and DNA viruses, such as herpes simplex virus type 1 and type 2 (DNA viruses), dengue virus type 2 (RNA virus), junin virus (RNA virus), influenca virus (RNA virus), whereas only few essential oils (e.g. oregano oil and clove oil) were also tested against non-enveloped RNA and DNA viruses, such as adenovirus type 3 (DNA virus), poliovirus (RNA virus) and coxsackie-virus B-1 (RNA virus) (see Table 4.12).

The antiviral activity of the essential oils tested could be clearly demonstrated for enveloped viruses of both the DNA and RNA type. On the contrary, the non-enveloped viruses were not attacked by essential oils.

Mode and mechanism of antiviral action To learn more about the effect of essential oils on enveloped viruses, we investigated exemplarily the antiviral activity of anise oil, hyssop oil, thyme oil, dwarfpine oil, citrus oil, manuka oil, ginger oil, camomile oil and sandalwood oil against HSV-1 and HSV-2 in vitro. The replication cycle of herpes simplex virus is characterized by a complex sequence of different steps which offers opportunities to antiviral agents to intervene. In order to determine the mode of action, essential oils

Table 4.12 Antiviral activity of selected essential oils against different enveloped viruses

Plant source	Viruses	IC_{50} (%; ppm)	References
Herpes simplex virus (HSV; DNA virus)			
Aloysia gratissima	HSV-1	65 ppm	Garcia *et al.* (2003)
Artemisia douglasiana	HSV-1	83 ppm	Garcia *et al.* (2003)
Citrus limon	HSV-1	0.0015%	Koch (2005)
Eucalyptus globulus	HSV-1	0.009%	Schnitzler *et al.* (2001)
	HSV-2	0.008%	Schnitzler *et al.* (2001)
Eupatorium patens	HSV-1	125 ppm	Garcia *et al.* (2003)
Houttuynia cordata	HSV-1	0.0013%	Hayashi *et al.* (1995)
Hyssopus officinalis	HSV-1	0.0001%	Koch (2005)
	HSV-2	0.0006%	Koch (2005)
Illicium verum	HSV-1	0.004%	Koch (2005)
	HSV-.2	0.003%	Koch (2005)
Leptospermum scoparium	HSV-1	0.0001%	Reichling *et al.* (2005)
	HSV-2	0.00006%	Reichling *et al.* (2005)
Matricaria recutita	HSV-1	0.00003%	Koch (2005)
	HSV-2	0.00015%	Koch (2005)
Melaleuca alternifolia	HSV-1	0.0009%	Schnitzler *et al.* (2001)
	HSV-2	0.0008%	Schnitzler *et al.* (2001)
Mentha piperita	HSV-1	0.002%	Schuhmacher *et al.* (2003)
	HSV-2	0.0008%	Schuhmacher *et al.* (2003)
Pinus mugo	HSV-1	0.0007%	Koch (2005)
	HSV-2	0.0007%	Koch (2005)
Santalum album	HSV-1	0.0002%	Koch (2005)
	HSV-2	0.0005%	Koch (2005)
Tessaria absinthioides	HSV-1	105 ppm	Garcia *et al.* (2003)
Thymus vulgaris	HSV-1	0.001%	Koch (2005)
	HSV-2	0.0007%	Koch (2005)
Zingiber officinale	HSV-1	0.0002%	Koch (2005)
	HSV-2	0.0001%	Koch (2005)
Dengue virus (DEN; RNA virus)			
Artemisia douglasiana	DEN-2	60 ppm	Garcia *et al.* (2003)
Eupatorium patens	DEN-2	150 ppm	Garcia *et al.* (2003)
Influenza virus (INV; RNA virus)			
Houttuynia cordata	INV	0.0048%	Hayashi *et al.* (1995)
SARS-associated coronar-virus (SARS-CoV; RNA virus)			
Laurus nobilis	SARS-CoV	120 μg/mL	Loizzo *et al.* (2008)
Junin virus (JUNV; RNA virus)			
Heterothalamus alienus	JUNV	44.2 ppm	Duschatzky *et al.* (2005)
Buddleja cordobensis	JUNV	39.0 ppm	Duschatzky *et al.* (2005)
Lippia junelliana	JUNV	20.0 ppm	Garcia *et al.* (2003)
Lippia turbinata	JUNV	14.0 ppm	Garcia *et al.* (2003)
Aloysia gratissima	JUNV	52.0 ppm	Garcia *et al.* (2003)
Heterotheca latifolia	JUNV	90.0 ppm	Garcia *et al.* (2003)
Tessaria absinthioides	JUNV	63.0 ppm	Garcia *et al.* (2003)
Newcastle disease virus (NDV; RNA virus)			
Origanum vulgare	NDV	0.025 %	Siddiqui *et al.* (1996)

IC_{50}: 50% inhibitory concentration. Host cells: Vero cells (African green monkey kidney cells); HeLa cells. Test method: plaque reduction assay; pretreatment of viruses for 1h before cell infection.

were added to host cells and viruses at different stages during viral infection. To identify the stage and target site at which infection might be inhibited,

(a) host cells (African green monkey kidney cells) were pretreated for 1 h with essential oils prior to inoculation with herpesviruses (pretreatment of cells),
(b) herpesviruses were incubated with essential oils for 1 h prior to infection of host cells (pretreatment of virus),
(c) herpesviruses were mixed with essential oils and added to the host cells immediately (adsorption),
(d) host cells were incubated with essential oils after penetration of herpesviruses into the cells (intracellular replication).

Inhibition of HSV replication was measured by a plaque reduction assay. In this assay, the number of plaques (pfu: plaque forming units) of drug-treated cells and viruses were expressed in percent (pfu%) of the untreated control (number of plaques formed by viruses in the absence of essential oil). In all assays, the maximum non-cytotoxic concentrations of the essential oils tested (0.0006–0.01%) were used.

According to our findings, pretreatment of cells with essential oils 1 h prior to virus infection did not reduce the virus plaque formation. It means that essential oils did not affect the adsorption of viruses to cell surface, indicating that the essential oils did not interfere with virus binding by blocking cellular receptors. On the other hand, pretreatment of viruses with essential oils 1 h prior to cell infection caused a significant reduction of plaques of 95–99% (pfu: 5–1%) for HSV-1 and of 70–98% (pfu: 30–2%) for HSV-2, respectively (see Fig. 4.7). Out of the oils tested, only dwarf pine oil and citrus oil reduced plaque formation of about 80% for HSV-1 and HSV-2 when added during adsorption of virus to host cells. All other essential oils exhibited somewhat different antiviral effects with respect to HSV-1 and HSV-2.

In contrast, when essential oils were added to the overlay medium after penetration of viruses into the host cells, only manuka oil significantly reduced plaque formation of HSV-1 of about 40%.

In conclusion, our results indicate that in particular free viruses are very sensitive to essential oils. Both types of herpes simplex virus are inactivated before adsorption or during adsorption to cell surface but not after penetration into cells, the typical mode of action of nucleodside analogues like acyclovir. These findings suggest that essential oils interfere with the virus envelope or masking viral compounds which are necessary for adsorption or entry into host cells. Recently, an electron microscopic examination demonstrated that the envelope of HSV-1 was disrupted when treated with oregano oil and glove oil. Furthermore, eugenol (4-hydroxy-3-methoxy-allyl-benzene), the main component of clove oil, was shown to be a very effective agent against HSV-1 and HSV-2 in vitro. All these findings fit the data of investigations on the antiviral activity of essential oils against enveloped DNA and RNA viruses mentioned above.

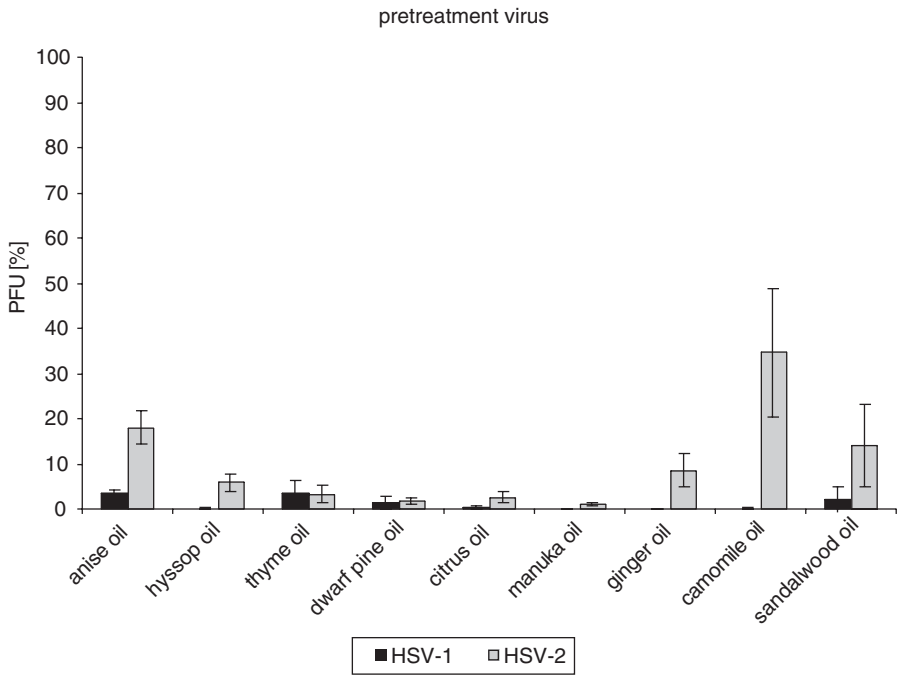

Figure 4.7 Mode of antiviral action of selected essential oils against HSV-1 and HSV-2. PFU, plaque forming unit; data were expressed as percent (% v/v) of untreated control.

4.4.3.2 Alkaloids

Several Amaryllidaceae isoquinoline alkaloids were tested against HSV-1. Alkaloids which may eventually prove to be antiviral agents had a hexahydroindole ring with two functional hydroxyl groups. It was established that the antiviral activity of alkaloids was due to the inhibition of multiplication and not to the direct inactivation of extracellular viruses. The mechanism of the antiviral effect could be explained, in part, as a blocking of viral DNA polymerase activity (Renard-Nozaki *et al.*, 1989) (see Table 4.13).

4.4.3.3 Flavonoids

Naturally occurring flavonoids possess a variable spectrum of antiviral activity against certain RNA and DNA viruses acting to inhibit infectivity and/or replication. In several in vitro test systems, flavanols and flavonols have been shown to be more active than flavones (Lyu *et al.*, 2005). Galangin, a 3,5,7-trihydroxyflavone isolated from *Helichrysum aureonitens* and *Callicarpa japonica*, exhibited anti-HSV-1 activity at concentrations ranging 12–47 μg/mL. In the same concentration range, galangin also significantly inhibited the cytopathic effect of coxsackie virus B type 1 (COXB1). It was assumed that the anti-HSV-1 action resulted in a suppression of viral binding to host cells at an

Table 4.13 Isolated secondary metabolites with antiviral activity

Compounds	Viruses	Host cells/biological targets	Evaluation of antiviral activity	EC_{50}/IC_{50} (μg/mL)	References
Alkaloids					
Acridone-type					
1,3-*O*-Methyl-*N*-methyl-acridone	HSV-2	HEF	pra	4.9	Yamamoto *et al.* (1989)
Acronycine	HSV-2	HEF	pra	3.3	Yamamoto *et al.* (1989)
5-Methoxyacronycine	HSV-2	HEF	pra	5.5	Yamamoto *et al.* (1989)
Dimethoxyacronycine	HSV-2	HEF	pra	6.5	Yamamoto *et al.* (1989)
Atalaphillidine	HSV-2	HEF	pra	0.73	Yamamoto *et al.* (1989)
	HSV-2	HEF	pra	0.82	Yamamoto *et al.* (1989)
N-Methylatalaphilline	HSV-2	HEF	pra	8.4	Yamamoto *et al.* (1989)
Citracridone-I	HSV-2	HEF	pra	1.3	Yamamoto *et al.* (1989)
Citrusinine-I	HSV-2	HEF	pra	0.74	Yamamoto *et al.* (1989)
	HSV-1	HEF	pra	0.56	Yamamoto *et al.* (1989)
	HSV-2	HEF	pra	0.74	Yamamoto *et al.* (1989)
	HCMV	HEF	pra	1.5	Yamamoto *et al.* (1989)

(*Continued*)

Table 4.13 (*Continued*)

Compounds	Viruses	Host cells/biological targets	Evaluation of antiviral activity	EC_{50}/IC_{50} (μg/mL)	References
5-Hydroxy-*N*-methyl-severifoline	HSV-2	HEF	pra	2.0	Yamamoto *et al.* (1989)
Isoquinoline-type					
Deoxypancratistatin	JEV	Vero	mtt	0.48	Gabrielsen *et al.* (1992)
	YFV	Vero	mtt	0.4	Gabrielsen *et al.* (1992)
	DV-4	LLCMK2	pra	0.69	Gabrielsen *et al.* (1992)
	PTV	Vero	mtt	0.66	Gabrielsen *et al.* (1992)
	RVFV	Vero	pra	5.1	Gabrielsen *et al.* (1992)
	SFV	Vero	mtt	1.7	Gabrielsen *et al.* (1992)
cis-Dihydronarciclasine	JEV	Vero	mtt	0.96	Gabrielsen *et al.* (1992)
	YFV	Vero	mtt	1.3	Gabrielsen *et al.* (1992)
	DV-4	LLCMK2	pra	2.5	Gabrielsen *et al.* (1992)
	PTV	Vero	mtt	2.2	Gabrielsen *et al.* (1992)
	RVFV	Vero	pra	1.4	Gabrielsen *et al.* (1992)
trans-Dihydronarciclasine	JEV	Vero	mtt	0.004	Gabrielsen *et al.* (1992)
	YFV	Vero	mtt	0.003	Gabrielsen *et al.* (1992)

	DV-4	LLCMK2	pra	0.015	Gabrielsen *et al.* (1992)
	PTV	Vero	mtt	0.008	Gabrielsen *et al.* (1992)
Isonarciclasine	JEV	Vero	mtt	0.72	Gabrielsen *et al.* (1992)
	YFV	Vero	mtt	0.22	Gabrielsen *et al.* (1992)
	DV-4	LLCMK2	pra	0.27	Gabrielsen *et al.* (1992)
	PTV	Vero	mtt	0.28	Gabrielsen *et al.* (1992)
	RVFV	Vero	pra	3.3	Gabrielsen *et al.* (1992)
Lycorine	JEV	Vero	mtt	0.33	Gabrielsen *et al.* (1992)
	YFV	Vero	mtt	0.28	Gabrielsen *et al.* (1992)
	DV-4	LLCMK2	pra	0.24	Gabrielsen *et al.* (1992)
	PTV	Vero	mtt	0.50	Gabrielsen *et al.* (1992)
	RVFV	Vero	pra	0.93	Gabrielsen *et al.* (1992)
Lycoricidine	JEV	Vero	mtt	0.056	Gabrielsen *et al.* (1992)
	YFV	Vero	mtt	0.053	Gabrielsen *et al.* (1992)

(*Continued*)

Table 4.13 (*Continued*)

Compounds	Viruses	Host cells/biological targets	Evaluation of antiviral activity	EC_{50}/IC_{50} (μg/mL)	References
	DV-4	LLCMK2	pra	0.059	Gabrielsen *et al.* (1992)
	PTV	Vero	mtt	0.042	Gabrielsen *et al.* (1992)
	RVFV	Vero	pra	0.15	Gabrielsen *et al.* (1992)
	SFV	Vero	mtt	0.058	Gabrielsen *et al.* (1992)
Narciclasine	JEV	Vero	mtt	0.008	Gabrielsen *et al.* (1992)
	YFV	Vero	mtt	0.006	Gabrielsen *et al.* (1992)
	DV-4	LLCMK2	pra	0.015	Gabrielsen *et al.* (1992)
	PTV	Vero	mtt	0.0074	Gabrielsen *et al.* (1992)
Pancratistatin	JEV	Vero	mtt	0.022	Gabrielsen *et al.* (1992)
	YFV	Vero	mtt	0.016	Gabrielsen *et al.* (1992)
	DV-4	LLCMK2	pra	0.063	Gabrielsen *et al.* (1992)
	RVFV	Vero	pra	0.16	Gabrielsen *et al.* (1992)
Pretazettine	JEV	Vero	mtt	0.60	Gabrielsen *et al.* (1992)
	YFV	Vero	mtt	0.50	Gabrielsen *et al.* (1992)

	PTV	Vero	mtt	0.61	Gabrielsen *et al.* (1992)
	RVFV	Vero	pra	2.9	Gabrielsen *et al.* (1992)
	SFV	Vero	mtt	0.82	Gabrielsen *et al.* (1992)
Miscellaneous alkaloids					
Hippeastrine	HSV	Vero	pra	25.0 μg (100% plaque inhibition)	Renard-Nozaki *et al.* (1989)
Piperidiono-type					
Rohitukine	HSV-1	Vero	vap	1.6 μM	Houghton *et al.* (1994)
Schummannificine	HSV-1	Vero	vap	0.5 μM	Houghton *et al.* (1994)
N-Methylschumma-nnificine	HSV-1	Vero	vap	0.5 μM	Houghton *et al.* (1994)
Anhydroschummannificine	HSV-1	Vero	vap	0.06 μM	Houghton *et al.* (1994)
N-Methylanhydroschumma-nnificine	HSV-1	Vero	vap	0.5 μM	Houghton *et al.* (1994)
Pyridino-type					
Schummanniophytine	HSV-1	Vero	vap	40.0 μM	Houghton *et al.* (1994)
Isoschummanniophytine	HSV-1	Vero	vap	50.0 μM	Houghton *et al.* (1994)
N-Methylschummanniophytine	HSV-1	Vero	vap	50.0 μM	Houghton *et al.* (1994)
Pyrrolidinoindoline-type					
Hodgkinsine A	VSV	Vero	pra	10.0	Saad *et al.* (1995)
	HSV-1	Vero	pra	30.0	Saad *et al.* (1995)

(*Continued*)

Table 4.13 (*Continued*)

Compounds	Viruses	Host cells/biological targets	Evaluation of antiviral activity	EC_{50}/IC_{50} (μg/mL)	References
Coumarins					
Collinin	HBV	DNA replication		68.3	Chang *et al.* (1997)
Oxynitidine	HBV	DNA replication		200	Chang *et al.* (1997)
Diterpenoids					
Scopadulciol	HSV-1	HeLa	pra	0.016 μM	Hayashi and Hayashi (1996)
Flavonoids					
Flavan-type					
Flavan	HRV-IB	HeLa	pra	0.27 μg per plate	Denyer *et al.* (1994)
4′,6-Dichloroflavan	HRV-1B	HeLa	pra	0.02 μg per plate	Denyer *et al.* (1994)
7-*O*-Galloyltricetiflavan	COX B3	Hep-2	cpe	12.5	Li *et al.* (2006)
	HSV-1	Vero	cpe	30.0	Li *et al.* (2006)
	INFA	MDCK	cpe	15.7	Li *et al.* (2006)
	RSV	Hep-2	cpe	5.0	Li *et al.* (2006)
7,4′-*O*-Di-galloyltricetiflavan	COXB3	Hep-2	cpe	25.0	Li *et al.* (2006)
	HSV-1	Vero	cpe	20.0	Li *et al.* (2006)
	INFA	MDCK	cpe	30.0	Li *et al.* (2006)
	RSV	Hep-2	cpe	10.0	Li *et al.* (2006)
Flavanone-type					
Naringenin	HSV-1	Vero	cpe	1.69	Lyu *et al.* (2005)
Flavone-type					
Isoscutellarein	INFA	sialidase		20.0 μM	Nagai and Miyaichi (1992)
	INFA	MDBK	mtt	16.0 nmol/well	Nagai and Miyaichi (1992)

Isoscutellarein-8-methylether	INFA	sialidase		55.0 μM	Nagai and Miyaichi (1992)
	INFA/B	MDCK	mtt	20.0 μM	Nagai and Miyaichi (1992)
3-Methoxyflavone-type					
Ternatin	ADV	Vero	pra	3.74	Simeos *et al.* (1990)
	HSV-1	Vero	pra	16.46	Simeos *et al.* (1990)
	HSV-2	Vero	pra	5.23–37.4	Simeos *et al.* (1990)
	VSV	Vero	pra	14.21–17.95	Simeos *et al.* (1990)
	POLIO	Vero	pra	1.5	Simeos *et al.* (1990)
Flavonol-type					
Galangin	HSV-1	VK	pra	12–47	Meyer *et al.* (1997)
	COX B1	VK	pra	12–47	Meyer *et al.* (1997)
	ADV 31	PLC/PRF/5	pra	12–47	Meyer *et al.* (1997)
Isoquercitrin	HSV-1	Vero	pra	40.0 μg (inhibition: 100%)	Abou-Karam and Shier (1992)
Quercetin	potato virus X	plant tissue		1.0	French and Towers (1992)
	HSV-1	Vero	cpe	1.69	Lyu *et al.* (2005)
Aescuflavoside	RSV	MDCK	cpe	4.5	Wei *et al.* (2004)
Aescuflavoside A	RSV	MDCK	cpe	6.7	Wei *et al.* (2004)

(*Continued*)

Table 4.13 (*Continued*)

Compounds	Viruses	Host cells/biological targets	Evaluation of antiviral activity	EC_{50}/IC_{50} (μg/mL)	References
Bifavonoid-type					
Ginkgetin	HSV-1	HeLa	pra	0.91	Hayashi *et al.* (1992)
	HSV-1	Vero	pra	0.76	Hayashi *et al.* (1992)
	HSV-2	Vero	pra	0.83	Hayashi *et al.* (1992)
	HCMV	HEL	pra	1.75	Hayashi *et al.* (1992)
Isoflavone-type					
Genistein	HSV-1	Vero	cpe	1.35	Lyu *et al.* (2005)
Lignans					
Lactone-type					
4′-Demethylpodophyllotoxin	HSV-1	CV-1	pra	0.04	San Feliciano *et al.* (1993)
	VSV	BHK	pra	0.1	San Feliciano *et al.* (1993)
Deoxypodophyllotoxin	HSV-1	CV-1	pra	0.01	San Feliciano *et al.* (1993)
	VSV	BHK	pra	< 0.01	San Feliciano *et al.* (1993)
Deoxypicropodophyllotoxin	HSV-1	CV-1	pra	0.8	San Feliciano *et al.* (1993)
	VSV	BHK	pra	0.08	San Feliciano *et al.* (1993)
Diphyllin	VSV	HEL	eptt	0.25	Asano *et al.* (1996)
	SINV	3T3-L1	pra	1.0	MacRae *et al.* (1989)

Diphyllin apioside	VSV	HEL	eptt	0.25	Asano *et al.* (1996)
Diphyllin apioside-acetate	VSV	HEL	eptt	0.13	Asano *et al.* (1996)
Epipodophyllotoxin acetate	HSV-1	CV-1	pra	0.2	San Feliciano *et al.* (1993)
	VSV	BHK	pra	0.1	San Feliciano *et al.* (1993)
Epipicropodophyllotoxin acetate	HSV	CV-1	pra	0.8	San Feliciano *et al.* (1993)
Epipicropodophyllotoxin	VSV	BHK	pra	2.0	San Feliciano *et al.* (1993)
Justicidin A	VSV	HEL	eptt	0.13	Asano *et al.* (1996)
Justicidin B	VSV	HEL	eptt	0.06	Asano *et al.* (1996)
Justicidin C	VSV	HEL	eptt	16.0	Asano *et al.* (1996)
Justicidin D	VSV	HEL	eptt	16.0	Asano *et al.* (1996)
Justicidin B	SINV	3T3-L1	pra	0.01	MacRae *et al.* (1989)
Justicidinoside A	VSV	HEL	eptt	16.0	Asano *et al.* (1996)
Justicidinoside B	VSV	HEL	eptt	125	Asano *et al.* (1996)
Justicidinoside C	VSV	HEL	eptt	125	Asano *et al.* (1996)
α-Peltatine	MCMV	3T3-L1	pra	0.01	MacRae *et al.* (1989)

(*Continued*)

Table 4.13 (*Continued*)

Compounds	Viruses	Host cells/biological targets	Evaluation of antiviral activity	EC_{50}/IC_{50} (μg/mL)	References
β-Peltatine A methylether	HSV-1	CV-1	pra	0.01	San Feliciano *et al.* (1993)
β-Peltatine A methylether	VSV	BHK	pra	0.01	San Feliciano *et al.* (1993)
Podophyllotoxin	MCMV	3T3-L1	pra	0.01	MacRae *et al.* (1989)
Podophyllotoxione	HSV-1	CV-1	pra	0.4	San Feliciano *et al.* (1993)
Podophyllotoxin	VSV	BHK	pra	1.0	San Feliciano *et al.* (1993)
Non-lactone-type					
Acetyljunaphthoic acid	HSV-1	CV-1	pra	< 20.0	San Feliciano *et al.* (1993)
	VSV	BHK	pra	> 40.0	San Feliciano *et al.* (1993)
Methylacetyljunaphthoate	HSV-1	CV-1	pra	20.0	San Feliciano *et al.* (1993)
	VSV	BHK	pra	20.0	San Feliciano *et al.* (1993)
Methyljunaphthoate	HSV-1	CV-1	pra	< 20.0	San Feliciano *et al.* (1993)
	VSV	BHK	pra	10.0	San Feliciano *et al.* (1993)
Rhinacanthin E	INFA	MDCK	pra	7.4	Kernan *et al.* (1997)
Rhinacanthin F	INFA	MDCK	pra	3.1	Kernan *et al.* (1997)

Naphthodianthrones					
Hypericin	HSV-1	BSC-1	pra	20	Tang *et al.* (1990)
	INFA	MDCK	pra	> 100	Tang *et al.* (1990)
	ADV	HeLa	pra	> 100	Tang *et al.* (1990)
	POLIO 1	BSC-1	pra	> 100	Tang *et al.* (1990)
	Mo-MuLV	SC-1	XC cell assay	6.0	Tang *et al.* (1990)
Phenolic compounds					
Salicin	PMV	Vero	eptt	25.0	Van Hoof *et al.* (1989)
Salireposide	PMV	Vero	eptt	25.0	Van Hoof *et al.* (1989)
	SFVL10	Vero	eptt	50.0	Van Hoof *et al.* (1989)
Woodorien	HSV-1	Vero	pra	100 (plaque formation: 16%)	Xu *et al.* (1993)
Sesquiterpenes					
Arvoside B	VSV	CER	pra	14.0	De Tommasi *et al.* (1990)
β-Bisabolene	HRV-1B	HeLa	pra	14.4 μg/plate	Denyer *et al.* (1994)
Arcurcumene	HRV-1B	HeLa	pra	20.4 μg/plate	Denyer *et al.* (1994)
Epicubebol glycoside	VSV	CER	pra	36.0	De Tommasi *et al.* (1990)
	HRV-1B	HeLa	pra	25.0	De Tommasi *et al.* (1990)
β-Sesquiphellandrene	HRV-1B	HeLa	pra	0.90 μg/plate	Denyer *et al.* (1994)

(*Continued*)

Table 4.13 (*Continued*)

Compounds	Viruses	Host cells/biological targets	Evaluation of antiviral activity	EC_{50}/IC_{50} (μg/mL)	References
α-Zingiberene	HRV-1B	HeLa	pra	1.9 μg/plate	Denyer *et al.* (1994)
Tannins					
Casuaricitin	HSV-1	CV-1	pra	0.044	Fukuchi *et al.* (1989)
Coriariin A	HSV-1	CV-1	pra	0.038	Fukuchi *et al.* (1989)
Cornusiin A	HSV-1	CV-1	pra	0.039	Fukuchi *et al.* (1989)
Geraniin	HSV-1	CV-1	pra	0.093	Fukuchi *et al.* (1989)
Oenothein B	HSV-1	CV-1	pra	0.036	Fukuchi *et al.* (1989)
Penta-galloylglucose	HSV-1	CV-1	pra	0.047	Fukuchi *et al.* (1989)
Rugosin D	HSV-1	CV-1	pra	0.034	Fukuchi *et al.* (1989)
Tellimagrandin I	HSV-1	CV-1	pra	0.036	Fukuchi *et al.* (1989)
4,8-Tetramer of epicatechin gallate	HSV-1	CV-1	pra	0.14	Fukuchi *et al.* (1989)
Tannic acid	HSV-1	CV-1	pra	0.034	Fukuchi *et al.* (1989)
	HSV-1	CV-1	pra	0.15	Fukuchi *et al.* (1989)
	HSV-2	CV-1	pra	0.15	Fukuchi *et al.* (1989)
	HSV-1	Veto	pra	0.086	Fukuchi *et al.* (1989)
	HSV-1	Vero	pra	0.043	Fukuchi *et al.* (1989)

	HSV-2	Vero	pra	0.041	Fukuchi *et al.* (1989)
	HSV-1	A-549	pra	0.061	Fukuchi *et al.* (1989)
Triterpenes					
Oleanane-type					
Glycyrrhizin	VZV	HEF	Immunoperoxidase method	710 μM	Baba and Shigeta (1987)
Hydroxyoleanonic lactone	HSV-1	Vero	pra	5.0	Poehland *et al.* (1987)
	HSV-2	Vero	pra	5.0	Poehland *et al.* (1987)
Soyasaponin II	HSV-1	HeLa	pra	54 μM	Hayashi *et al.* (1997)
	HCMV	HEL	pra	104 μM	Hayashi *et al.* (1997)
	POLIO 1	Vero	pra	> 1000 μM	Hayashi *et al.* (1997)
	INF	MDCK	pra	88 μM	Hayashi *et al.* (1997)
Ursane-type					
Ursonic acid	HSV-1	Vero	pra	2.5	Poehland *et al.* (1987)
	HSV-2	Vero	pra	8.0	Poehland *et al.* (1987)
Quinovic acid -3β-*O*-(β-Fucopyranosyl)-4-β-D-glucopyranosid	VSV	CER	pra	20.0	Aquino *et al.* (1989)
-3β-*O*-(β-Quinovopyranosyl)-(28→1)-β-glucopyranosyl ester	VSV	CER	pra	22.4	Aquino *et al.* (1989)

(*Continued*)

Table 4.13 (*Continued*)

Compounds	Viruses	Host cells/biological targets	Evaluation of antiviral activity	EC_{50}/IC_{50} (μg/mL)	References
-3β-*O*-(β-Fucopyranosyl)-(28→1)-β-glucopyranosyl ester	VSV	CER	pra	31.6	Aquino *et al.* (1989)
-3β-*O*-(β-Glucopyranosyl)-(28→1)-β-glucopyranosyl ester	VSV	CER	pra	31.0	Aquino *et al.* (1989)
-3β-*O*-Glucopyranoside	VSV	CER	pra	33.1	Aquino *et al.* (1989)
-3β-*O*-(β-Glucopyranosyl)-(27→1)-β-glucopyranosyl ester	VSV	CER	pra	70.8	Aquino *et al.* (1989)
-3β-*O*-(β-Glucopyranosyl)-(28→1)-β-glucopyranosyl ester	VSV	CER	pra	33.1	Aquino *et al.* (1989)
Dammarane-type					
Dammaradienol	HSV-1	Vero	pra	2.5	Poehland *et al.* (1987)
	HSV-2	Vero	pra	3.0	Poehland *et al.* (1987)
Dammarenediol-II	HSV-1	Vero	pra	7.0	Poehland *et al.* (1987)
	HSV-2	Vero	pra	7.0	Poehland *et al.* (1987)

Dammarenolic acid	HSV-1	Vero	pra	3.0	Poehland *et al.* (1987)
	HSV-2	Vero	pra	2.0	Poehland *et al.* (1987)
Hydroxydammarenone	HSV-1	Vero	pra	2.0	Poehland *et al.* (1987)
	HSV-2	Vero	pra	5.0	Poehland *et al.* (1987)
Hopanane-type					
Hydroxyhopanone	HSV-1	Vero	pra	7.0	Poehland *et al.* (1987)
	HSV-2	Vero	pra	5.0	Poehland *et al.* (1987)
Miscellaneous triterpenes					
Eichlerianic acid	HSV-1	Vero	pra	7.0	Poehland *et al.* (1987)
	HSV-2	Vero	pra	8.0	Poehland *et al.* (1987)
Shoreic acid	HSV-1	Vero	pra	7.0	Poehland *et al.* (1987)
	HSV-2	Vero	pra	8.0	Poehland *et al.* (1987)

EC_{50}/IC_{50}, effective/inhibition concentration for a 50% reduction in the viral cytopathic effect or in viral replication. Cpe, cytopathic reduction assay; pra, plaque reduction assay; mtt, colorimetric method to determine viable cells; vap, viral antigen production in cells; eptt, endpoint titration technique. For abbreviations of the viruses and host all systems reviewed, see Tables 4.9 and 4.10.

early stage of replication (Hayashi *et al.*, 1997; Meyer *et al.*, 1997). The effect of isoscutellarein-8-methylether was investigated on the single-cycle replication of mouse-adapted influenza A and B virus (INFA and INFB) in Madin–Darby canine kidney (MDCK) cells. The compound suppressed the replication of these viruses 6–12 h after incubation, in a dose-dependent manner, by 50% at 20 μM and 90% at 40 μM. In contrast, the agent had only a slight effect on the haemagglutination and RNA-dependent RNA polymerase activities of these viruses in vitro. The same compound completely prevented the proliferation of mouse-adapted INFA in mouse lung by intranasal (0.5 mg/kg body weight) and intraperitoneal (4.0 mg/kg body weight) administration, and it was more potent than the known anti-influenza virus substance, amantadine (Nagai *et al.*, 1995). Isoscutellarein, extracted from the leaves of the same plant, showed significant anti-influenza virus activity, similar to that of isoscutellarein-8-methylether. The agent inhibited the replication of INFA in Madin–Darby bovine kidney (MDBK) cells, with an IC_{50} value of 16 μM; and it non-competitively blocked (IC_{50} 20.0 μM) the hydrolysis of sodium pnitrophenyl-*N*-acetyl-α-D-neuraminate by influenza virus sialidase (Nagai and Miyaichi, 1992).

Ginkgetin, a biflavone isolated from *Cephalotaxus drupacea*, caused dose-dependent inhibition of the replication of HSV-1, HSV-2 and human cytomegalovirus (HCMV). Adsorption of HSV-1 to host cells and virus penetration into cells were unaffected by this agent. On the other hand, ginkgetin suppressed viral protein synthesis and exerted strong inhibition of transcription of immediate-early genes (Hayashi *et al.*, 1992) (see Fig. 4.8).

4.4.3.4 Lignans

San Feliciano and co-workers (1993) isolated 19 cyclolignans from *Juniperus sabina* (Cupressaceae). These compounds exhibited antiviral activity against HSV-1 and vesicular stomatitis virus (VSV); the antiviral activity depended on structural variations. The *trans* and *cis* configurations of tetralinelactones were far more active than those of naphthalene and non-lactonic cyclolignan classes.

4.4.3.5 Miscellaneous phenolic compounds

A polyphenolic complex (PC), isolated from *Geranium sanguineum* (Geraniaceae), inhibited the reproduction of INFA and INFB in vitro and in ovo. When influenza viruses were treated with 1 mg/mL of PC, their haemagglutination, neuraminidase and infective activities were reduced completely. Moreover, the PC protected white mice in an experimental influenza infection (Serkedjieva and Manolova, 1992).

Curcumin, a typical yellow pigment of *Curcuma longa*, is widely used as a spice and food colouring (curry). In the past, curcumin exhibited a variety of pharmacological effects, such as antitumour, anti-inflammatory and anti-infectious activity. Recently, Mazumder and co-workers (1995) showed that the agent also inhibits purified HIV-1 integrase in vitro.

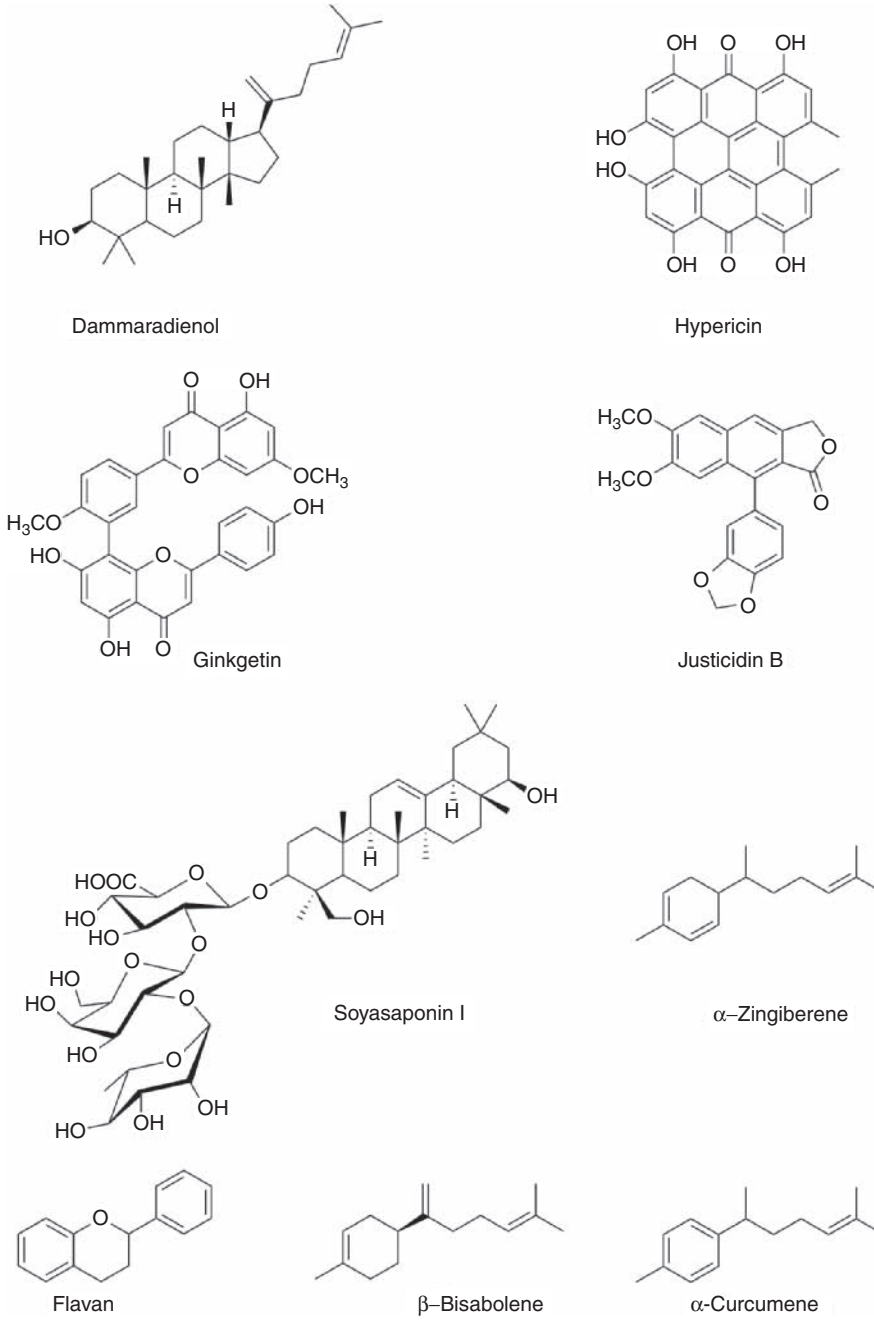

Figure 4.8 Chemical structures of several plant-derived compounds with antiviral properties.

4.4.3.6 Thiosulfinates

Extracts of *Allium sativum* (Alliaceae) have been used traditionally to treat a number of infectious diseases caused by bacteria, fungi, protozoa and viruses. Recently, Weber and co-workers (1992) isolated diallyl thiosulfinate (allicin), allyl methyl thiosulfinate, methyl allyl sulfinate, ajoene, alliin, deoxyalliin, diallyl disulfide and diallyl trisulfide from garlic cloves. The compounds were tested against HSV-1, HSV-2, parainfluenza virus type 3 (Para-3), vaccinia virus (VV), VSV and human rhinovirus type 2B (HRV-2B) (host cells: Vero cells, HeLa cells; evaluation of antiviral activity: plaque reduction assay). At the highest concentration tested (1.000 mg/mL), the infectivity of all viruses was substantially reduced by a fresh garlic extract. The thiosulfinates appeared to be the active components; the predominant agent was allicin. At a concentration of 25 μg/mL, VSV (reduction of virus titre: $1.3\log_{10}$), HSV-1 (reduction of virus titre: $0.5\log_{10}$) and Para-3 (reduction of virus titre: $0.2\log_{10}$) were sensitive to allicin. The experimental results suggest that fresh garlic extracts, as well as allicin, have direct virucidal activity but no intracellular antiviral properties.

4.4.4 Mode of antiviral action

The replication cycle of a virus is characterized by a cascade of coordinately regulated events such as adsorption of virus to host cell membrane, penetration of virus into host cell, uncoating of nucleic acid, replication of virus nucleic acid and virus proteins inside the host cell, assembly of virus nucleic acid and virus proteins to complete viral particles (virions) and last but not least releasing viral particles from host cells by budding or lysis (see Fig. 4.9).

Phyto-antiviral agents are known to inhibit different viral enzymes such as integrase, nucleic acid polymerase or reverse transcriptase. In addition, these compounds are also able to prevent virus adsorption to host cells by

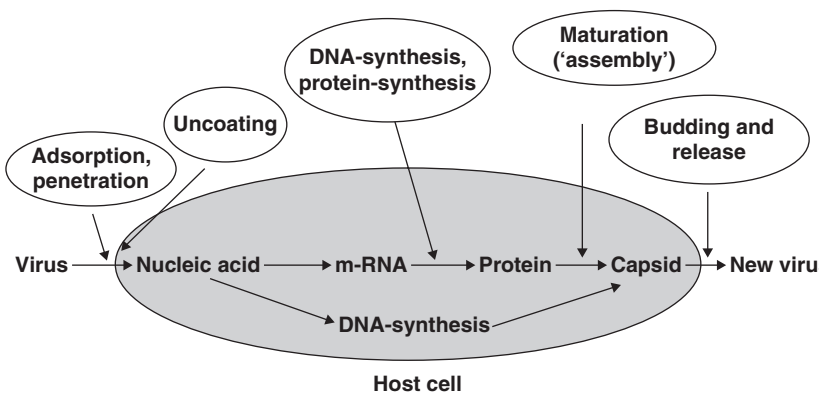

Figure 4.9 Different stages of virus infection cycle. These stages are potent targets for intervention of secondary plant metabolites.

blocking virus ligands necessary for the attachment to host cells or vice versa by blocking host cell receptors. The different targets and mode of actions are summarized in Table 4.14.

4.5 Conclusions

During the course of evolution, plants have developed effective defence strategies to protect themselves from phytopathogenic microbes and herbivores in their environment. Disease resistance in plants depends on the activation of coordinated, multicomponent defence mechanisms. A single mechanism or compound with a single metabolic site of action is thought to be unsatisfactory because of the rapid development of resistant strains of pathogens to one selected substance. One mechanism for disease resistance in plants is their ability to accumulate low-molecular-weight antimicrobial substances (phytoalexins) as a result of infection. In the last decade, many new phytoalexins with antibacterial and antifungal properties have been described. All these compounds belong to the secondary metabolites, including coumarins, isoflavonoids, other phenolic compounds, polyacetylenes and sesquiterpenes. Other components of the disease resistance complex include the presence of preformed antimicrobial agents and physical barriers, lignification, suberization and the formation of callose.

The importance of the plant kingdom as a source of new antimicrobial substances is illustrated by the present review on plant-derived antibacterial, antifungal and antiviral agents. During the last two decades, hundreds of different new secondary metabolites with antimicrobial activity have been isolated, e.g. alkaloids, coumarins, flavonoids, lignans, quinones, miscellaneous phenolic compounds, miscellaneous terpenes and tannins. Unfortunately, little is known about the mode of action for most of the natural antibacterial and antifungal agents. In contrast to plant-derived antibacterial and antifungal substances, several secondary metabolites with antiviral properties have exhibited competitive in vitro and in vivo activities with those found for synthetic antiviral drugs. It has been shown that plant-derived antiviral secondary metabolites interfere with many viral targets, ranging from adsorption of the virus to the host cell via the inhibition of virus-specific enzymes (e.g. reverse transcriptase, protease) to release of the virus from the cells.

From the results so far achieved, it is anticipated that bioactive plant-derived secondary metabolites will be used as leads to synthesize new and more active antimicrobial agents as well as substances with new pharmacological effects by repeated structural modification. It is expected that structurally modified natural products will exhibit increased potency, selectivity, duration of action and bioavailability and reduced toxicity. The terpenoid alkaloid, paclitaxel, isolated from the bark of *Taxus brevifolia*, provides an example of this new strategy; paclitaxel and some of its derivatives show an anticancerous activity against ovarian and mammary carcinomas.

Table 4.14 Compilation of important viral targets attacked by selected phyto-antiviral agents (see also Tables 4.11 and 4.13)

Viral targets	Virus	Selected compounds	Selected plants	Refrences
Viral enzymes				
Reverse transcriptase, inhibition (RT)	HIV-1	*Naphthoquinones*: hypericin	*Hypericum perforatum*	Matthée *et al.* (1999)
RT	HIV-1	*Naphthoquinones*: michellamines A–C	*Ancistrocladus korupensis*	Matthée *et al.* (1999)
RT	HIV-	*Coumarins*: calanolides A/B; inophyllum B	*Calophyllum lanigerum*, *Calaphyllum inophyllum*	Matthée *et al.* (1999)
RT	HIV-1	*Tannins*: shephagenins A/B; caffeoylquinate-type; galloylquinate-type; putranjivain	*Shepherdia argentea*, *Phyllantus emblica*	Matthée *et al.* (1999)
RT	HIV-1	*Phloroglucinols*: mallotojaponin; mallotochromene	*Mallotus japonicus*	Matthée *et al.* (1999)
RT	HIV-1	*Alkaloids*: benzophene anthridine-type; isoquinoline-type; quinoline-type	*Cephaelis ipecacuanha*, *Euodia roxburghiana*	Matthée *et al.* (1999)
RT	HIV-1	*Flavonoids*: flavanone-type; flavone-type; flavonol-type; isoflavone-type	*Swertia franchetiana*, *Garcinia multiora*, *Rhus succedanea*	Matthée *et al.* (1999), Lin *et al.* (1997)
RT	HIV-1	*Lignans*: gomisin; phyllamycin B; retrojusticidin B	*Schisandra chinensis*, *Phyllanthus myrtifolius*	Matthée *et al.* (1999), Chang *et al.* (1995)

RT	HIV-1	*Triterpenes*: nigranoic acid; betulinic acid; platanic acid	*Schisandra sphaerandra*, *Syzigium claviflorum*	Matthée *et al.* (1999)
DNA-polymerase, inhibition	HIV	*Cumarins*: Calanolide A/B	*Calophyllum lanigerum*, *C. inophyllum*	Taylor *et al.* (1994)
DNA-polymerase, inhibition	HSV-1; HSV-2; JEP; PTV	*Alkaloids*: Piperidone-type; piperidione-type; acridone-type; quinoline-type	Amaryllidaceae	Renard-Nozaki *et al.* (1989)
RNA-polymerase, inhibition	INFA; HSV-1; HSV-2	*Polyphenols*	*Geranium sanguineum*	Jassim and Naji (2003)
Integrase, inhibition	HIV-1	*Lignans*: arctigenin; trachelogenin	*Ipomoea cairica*	Matthée *et al.* (1999)
Integrase, inhibition	HIV-1	*Flavonoids*: flavone-type; flavonol-type	*Acer okamotoarum*	Kim *et al.* (1998)
Protease, inhibition	HIV-1	*Triterpenes*: hopanane-type; ursolic acid; maslinic acid	*Geum japonicum*	Jassim and Naji (2003)
Viral DNA synthesis, inhibition	HSV-1	*Tannins*: oligomeric caffeic acids; eugenin	*Geum japonicum*	Jassim and Naji (2003)
Viral DNA synthesis, inhibition	HSV-1	Yatein; samarangenin	*Chamaecyparis obtusa*, *Limonium sinensis*	Kuo *et al.* (2002, 2006)
Viral RNA-synthesis, inhibition	POLIO 1	*Flavonoid*: 3-methylkaempferol	*Psiadia dentata*	Robin *et al.* (2001)
Virus replication				
Inhibition	HIV-1; VSV, INFA	Phyllamycin B, retrojusticin B; justicin A–D	*P. myrtifolius*	Chang *et al.* (1995)
Inhibition	HSV-1; HSV-2; VSV; INFA	*Anthraquinones*: aloeemodin	*Aloe barbadensis*, *Rhamnus frangula*	Jassim and Naji (2003)

(*Continued*)

Table 4.14 (*Continued*)

Viral targets	Virus	Selected compounds	Selected plants	Refrences
Inhibition	HSV-1; HSV-2; RSV; INFA, COXB3	*Flavonoids*: Flavane-type; flavone-type; flavonol-type; isoflavone-type	*Helichrysum aureonitens*, *Callicarpa japonica*, *Pithecellobium clypearia*	Meyer *et al.* (1997), Nagai *et al.* (1995), Lyu *et al.* (2005), Li *et al.* (2006)
Inhibition	INFA; INFB	Polyphenolic complex	*G. sanguineum*	Serkedjieva and Manolova (1992)
Inhibition	HSV-1; HSV-2; Para-3; VV; VSV; HRV-2B	*Thiosulfinates*: allicin; ajoene, alliin; deoxyalliin; diallyl disulfide	*Allium sativum*	Weber *et al.* (1992)
Inhibition of immediate–early (α-)gene expression	HSV-1	*Lignans*: Yatein	*Chamaecyparis obtusa*	Kuo *et al.* (2006)
Host cell surface				
Adsorption/attachment, inhibition	HSV-1; HSV-2; INFA; HCMV	*Triterpenes*: Oleanane-type; ursane-type; damaran-type; hopanane-type; oligomeric caffeic acids	*Glycyrrhiza glabra*, *Tripterygium wilfordii*, *Syzygium claviforum*, *Celastrus hindsii*	Ito *et al.* (1987), Peng-suparp *et al.* (1995), Fujioka and Kashi-wada (1994), Kuo and Kuo (1997)
Adsorption/attachment, inhibition	ADV; COXB1; HRV-1B; HSV-1; INFA	*Flavonoids*: flavanone -type; flavone-type; flavonol-type; isoflavone-type	*H. aureonitens*, *C. japonica*	Meyer *et al.* (1997), Nagai *et al.* (1995), Nagai and Miyaichi (1992)

Interaction with the glycoprotein gp120	HIV-1	*Tannins*: Caffeoylquinate-type; galloylshikimate-type; galloylquinate-type; gallotannins		Matthée *et al.* (1999)
Interaction with the glycoprotein gp120	HIV-1	Piperidone-type; piperidione-type; acridone-type; quinoline-type	*Schumanniophyton magnificum*	Houghton *et al.* (1994)
Interaction with the glycoprotein gp120	HIV-1	*Flavonoids*: Flavone-type; flavonol-type	*Rosa damascena*	Mahmood *et al.* (1996)
Binding to p24	HIV-1	*Flavonoids*: baicalin	*Scutellaria baicalensis*	Li *et al.* (1993)
Virus release	HSV-1	Hypericin	*Hypericum perforatum*	Matthée *et al.* (1999)

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Chapter 5

NEW MEDICAL APPLICATIONS OF PLANT SECONDARY METABOLITES

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Abstract: In the last years, only a limited number of structurally new plant secondary metabolites have entered the scene to attract high medical interest. In contrast, the development of new techniques in the fields of biochemistry, cell biology and molecular biology, e.g. new assay techniques, which are also applicable in the field of natural products chemistry, allowed the finding of several new insights into the molecular mode of action and the pharmacological characterization of known plant secondary metabolites. Accordingly, the pharmacological or clinical characterization of several known plant-derived compounds like the taxanes, artemisinin derivatives or boswellic acids was significantly broadened. Furthermore, new and important medical applications or pharmacological mechanisms of known compounds were found like the antiviral activity of betulinic acid derivatives or the inhibition of tubulin polymerization by combretastatin A-4. Recently, a strong interest has evolved in natural products exhibiting chemopreventive activity. Consequently, the role of several plant polyphenols, like curcumin and xanthohumol, as protective and pharmacologically active, dietary constituents has become an increasingly important area of research. Besides the investigation of single compounds, there is still a growing interest in the application of standardized extracts, complex phytochemical mixtures with a well-defined content of the bioactive constituents. Therefore, new insights concerning the application of extracts from *Ginkgo biloba* and *Hypericum perforatum* are also discussed.

Keywords: polyphenols; chemoprevention; anticancer; taxanes; camptothecins; combretastatin A-4; betulinic acid; antiviral; artemisinin; curcumin; xanthohumol; boswellic acids; anti-inflammatory; antidepressant; *Hypericum perforatum*; ginkgo

5.1 Introduction

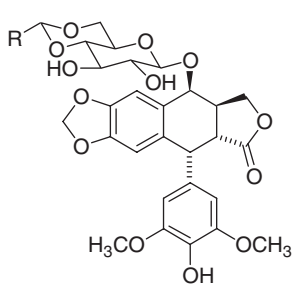
Plants continue to be a rich and valuable source of new compounds with potent pharmacological activity. Whereas only a few plant-derived secondary metabolites have been directly used as drugs, many pharmacologically active compounds have served as leading models for semisynthetic and synthetic drugs. Additionally, there is a growing interest in the application of standardized extracts, complex phytochemical mixtures with a well-defined content of the bioactive constituents. Emphasizing the therapeutic importance of natural compounds, various books and articles have been published in the past concerning their medical aspects (Butler, 2005; Newmann and Cragg, 2007; Potterat and Hamburger, 2008). The present chapter provides an overview of plant-derived compounds that have attracted medical and pharmacological interest in the last 10 years.

5.2 Compounds with anticancer and chemopreventive activity

A strong interest has been maintained in natural products exhibiting antitumour activity. Besides the development and improvement of drugs for the treatment of various human tumours, research has also been focused on the chemopreventive activity of natural products. For cancer treatment, important molecular targets are the eukaryotic DNA topoisomerases, the microtubuli apparatus and the different enzymes responsible for the accelerated cell cycle in cancer cells. For in vitro investigations on chemopreventive effects, modulation of carcinogen metabolism by inhibition of different cytochrome P enzymes (e.g. Cyp1A; Miranda *et al.*, 2000; Gerhäuser *et al.*, 2002; for literature, see Moon *et al.*, 2006) or induction of the phase II detoxification enzymes like quinone reductase activity (Dietz *et al.*, 2005) is used and, for example, comprehensively addressed for xanthohumol. Further targets of compounds with chemopreventive activity are pro-inflammatory enzymes like COX-2 (Bertagnolli, 2007) or transcription factor activator proteins (Matthews *et al.*, 2007) and in this field increasing impact for natural products research can be expected. As further chemopreventive targets for hormone-dependent cancer, the enzyme aromatase (Ta and Walle, 2007) and the oestrogen receptors (Sarkar *et al.*, 2006) have been investigated. Besides direct inhibition of the above-mentioned enzymes/receptors, reduction of gene expression is also addressed as a possible contribution to chemopreventive activity (Janakiram *et al.*, 2008). Interestingly, in the field of chemoprevention, the activity of dietary polyphenols like curcumin, xanthohumol, epigallocatechin-3-*O*-gallate or cocoa proanthocyanidins still plays a dominating role. Besides the characterization of the well-established antioxidant and radical scavenger activity of polyphenols, emerging literature suggests that chemopreventive

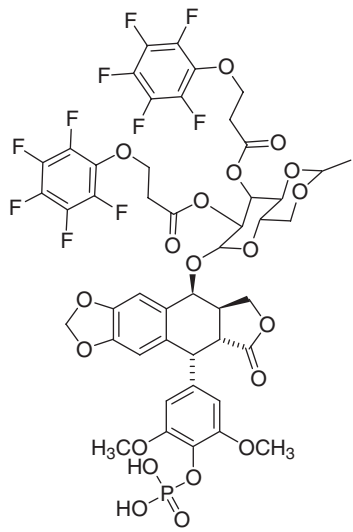
activity may also be ascribed to their ability to modulate components of cell signalling pathways (for literature, see Ramos, 2007; Khan *et al.*, 2008) and thus to multifunctional effects (Lu *et al.*, 2006). It should be noticed that the antioxidative effects is possible not only due to the direct reaction with or inhibition of enzymes, but also due to electron transfer (or H-atom transfer) from polyphenols to ROS-induced radical sites on the DNA (Anderson *et al.*, 2001). In vivo tests comprises induction of cancer or aberrant crypt foci in mice or rats with different inducers like benzo(a)pyrene (Banerjee *et al.*, 2006), 7,12-dimethylbenz[a]anthracene (Kumaraguruparan *et al.*, 2007) or azoxymethane (Lamy *et al.*, 2007). For overview on molecular targets and animal models, see Kwon *et al.*, 2007.

Two types of DNA topoisomerase have been described in the eukaryotic cell. They play an essential role in the transcription, replication and repair of DNA. Type II topoisomerase enables the supercoiling of DNA by catalyzing double-strand breaks. It has been demonstrated that the antitumour activity of some well-known anticancer drugs, the epipodophyllotoxins, etoposide (Vepesid®) and teniposide (Vumon®) (Fig. 5.1), is due to the inhibition of topoisomerase II (Liu, 1989; Pommier and Kohn, 1989). Both compounds are semisynthetic derivatives of the lignan podophyllotoxin, a constituent of the mayapple, *Podophyllum peltatum* (L.) (Berberidaceae), also known as American mandrake. Etoposide, in particular, is since decades in extensive clinical use against various cancer types, e.g. small-cell lung cancer, non-small-cell



R = -CH_3 Etoposide (**1**)

R =  Teniposide (**2**)



Tafluposide (**3**)

Figure 5.1 Structures of the epipodophyllotoxins, etoposide (**1**) and teniposide (**2**), and F 11782 tafluposide (**3**).

lung cancer, ovarian cancer and breast cancer. Both are preferentially used in combination regimen with other anticancer drugs, such as cisplatin, carboplatin and cyclophosphamide. On overview about toxicity profiles, pharmacokinetics and mechanisms of action can be found in Hartmann and Lipp, 2006. A relatively new etoposide derivative with interesting activity is tafluposide (F 11782) (Fig. 5.1). It is a dual inhibitor of topoisomerases I and II which impairs the binding of the enzyme to DNA, but does not stabilize the cleavage complex (Etiévant *et al.*, 2000; Kluza *et al.*, 2006).

Another target of anticancer drugs is topoisomerase I. This enzyme allows the relaxation of supercoiled DNA by catalyzing breaks of one DNA strand. The intermediates generated, enzyme-linked DNA breaks called the 'cleavable complex', form gates that can be passed by one DNA strand. At the end of the strain passage reaction, topoisomerases religate the DNA without loss of bases or change in the DNA sequence. It has been shown that topoisomerase I is the main cellular target of camptothecin (Fig. 5.2) and its semisynthetic derivatives, e.g. topotecan (hycamptamine = 9-dimethylaminomethyl-10-hydroxycamptothecin), 9-aminocamptothecin, belotecan (CKD-602 = 7-[2-(N-isopropylamino)ethyl]camptothecin), exatecan (DX-8951f, fully synthetic), irinotecan (CPT-11 = 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin) and 9-nitrocamptothecin (rubitecan) (Fig. 5.2). Design, synthesis and development of various camptothecin derivatives are summarized in Liew and Yang (2008).

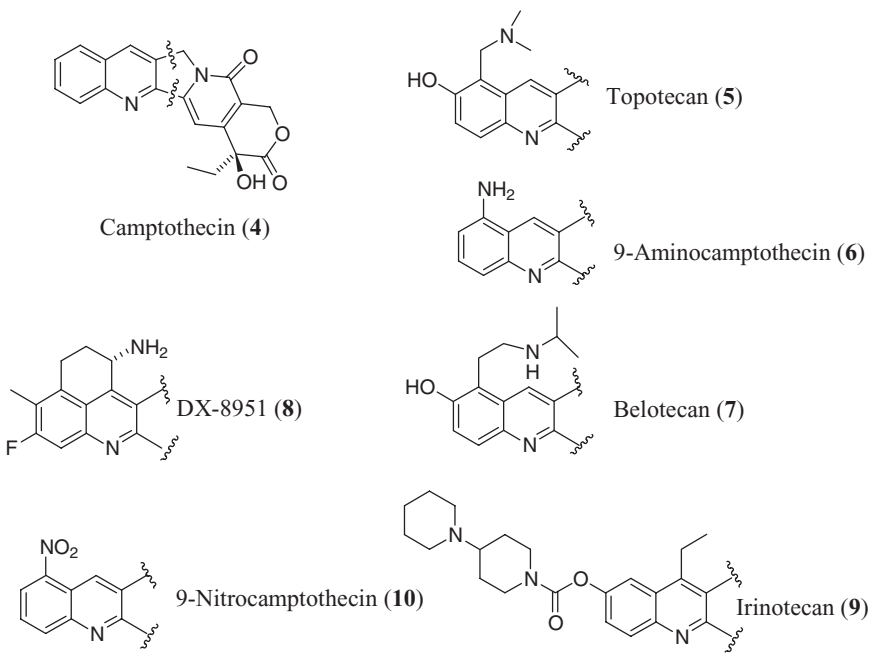


Figure 5.2 Structures of camptothecin (**4**) and its semisynthetic derivatives.

The antileukaemic activity of camptothecin, first isolated from *Camptotheca acuminata* DECNE. (Nyssaceae), was demonstrated in various in vitro models in the late 1960s (Wall *et al.*, 1966; for overview, see Wall and Wani, 1995). Because of its extreme insolubility in water, clinical trials were performed with the readily soluble sodium salt (Gottlieb and Luce, 1972; Muggia *et al.*, 1972), not knowing that this compound was only one-tenth as active as camptothecin (Wani *et al.*, 1980). The trials were halted because of disappointing response rates and severe side effects (Moertel *et al.*, 1972). Since it was found that camptothecins possess topoisomerase I inhibitory properties, several more water-soluble semisynthetic analogues (Fig. 5.2) have been synthesized and evaluated for their anticancer activity. Hsiang and co-workers (1985) showed that camptothecin-induced DNA breaks are mediated by blocking topoisomerase I-cleavable complexes and inhibiting the religation of the topoisomerase I reaction (Svejstrup *et al.*, 1991). The correlation between inhibition of purified topoisomerase I by camptothecin and antitumour activity has been clearly demonstrated (Hsiang *et al.*, 1989). Racemic camptothecins were separated into the corresponding 20-(*S*) and 20-(*R*) analogues (Wani *et al.*, 1987) and it was shown that only the 20-(*S*)-camptothecins are active in topoisomerase I inhibition and in in vivo assays (Jaxel *et al.*, 1989; Giovanella *et al.*, 1991). It is already known that the camptothecins bind to the DNA-topoisomerase-I-complex and not on free DNA or free enzyme, but the complex cellular consequences of treatment with camptothecins on the molecular levels including target/compound interactions, primary and secondary mechanisms of cell killing and problems concerning the development of camptothecin resistance is still under investigation (Liu *et al.*, 2000). It has been assumed that reasons for resistance phenomena could be inadequate accumulation in the tumour, resistance-conferring alterations in topoisomerase I or alterations in the cellular response to the topoisomerase I/camptothecin interaction (Rasheed and Rubin, 2003). An excellent recent overview of extracellular and intracellular interactions of camptothecins has been given by Beretta and Zunino, 2007. Nowadays, the camptothecins are also used as lead structures for the development of improved topisomerase I inhibitors (e.g. the homocamptothecins) with metabolically more stable lactone features (Teicher, 2008).

In aqueous solution, camptothecins occur in equilibrium of two forms, the α-hydroxylactone and the α-hydroxycarboxyl form (see Fig. 5.3). The α-hydroxylactone moiety is considered to be essential for topoisomerase I

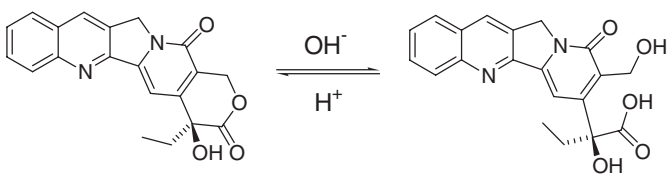
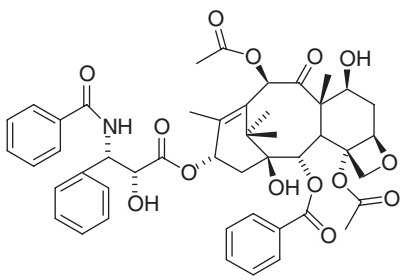


Figure 5.3 Equilibrium between the hydroxylactone and hydroxycarboxyl of camptothecins.

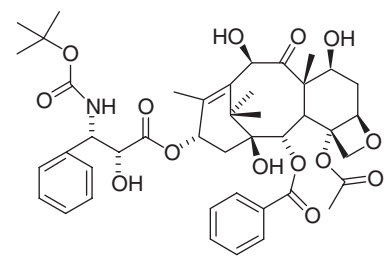
activity, whereas α-hydroxycarboxyl forms are less active antitumour agents (Giovanella *et al.*, 1991). The carboxylate form can reverse to the lactone at acidic pH and it has been concluded that the cellular pharmacokinetics and the antitumour activity of the camptothecins are influenced by the pH of the target tissue (Teicher *et al.*, 1993; Pommier, 1996; Gabr *et al.*, 1997). It has been hypothesized that the limited recycling of the sodium salt to camptothecin under physiological conditions was the reason for its disappointing results in the original trials.

A wide range of clinical and scientific data has been published on the subject of camptothecins. Numerous recent studies have shown the in vitro activity of camptothecin derivatives in different cell systems or xenograft models and antitumour activity against colorectal-, gastric-, cervical-, small-cell lung cancer and various other malignancies in clinical trials (for literature, see Garcia-Carbonero and Supko, 2002; Legarza and Yang, 2005). Combination with other antitumour agents, e.g. cisplatin or oxaliplatin, has produced promising results (O'Reilly and Ilson, 2001; Wasserman *et al.*, 2001). As with many antitumour agents, the main toxic side effects of camptothecins were evident in tissues with high cell turnover, and led to haematological toxicity. Further adverse events are nausea, vomiting and diarrhoea (Rougier and Bugat, 1996). In view of their broad spectrum of antitumour activity, the camptothecins will be valuable components for future cancer treatment. Up to now, topotecan (Hycamtin®) is indicated for the second-line treatment of adult patients with metastatic ovarian carcinoma or recurrent small-cell lung cancer and irinotecan (Campto®) is indicated alone or in combination for the treatment of colon and rectal cancer. Expansion of the therapeutic indications is in progress.

Taxane-derived compounds were isolated for the first time in the 1960s. However, the approval for marketing of paclitaxel (Taxol®) (Fig. 5.4) occurred in December 1992. Excellent reports on the discovery, development, plant sources, synthesis and semisynthesis, preclinical and clinical studies of



Paclitaxel (**11**)



Docetaxel (**12**)

Figure 5.4 Structure of paclitaxel (Taxol®) (**11**) and the new semisynthetic derivative, docetaxel (Taxotere®) (**12**).

paclitaxel (Fig. 5.4) until 1994 have been summarized by Suffness (1995). The present chapter, therefore, discusses this period very briefly and will focus on the clinical results of the last years and on the semisynthetic derivative, docetaxel (Taxotere®) (Fig. 5.4).

Paclitaxel is a diterpenoid with an alkaloidal side chain. It was first isolated from *Taxus brevifolia* NUTT. (Taxaceae) following discovery of the cytotoxicity of the compound to human epidermoid carcinoma cells (KB cells) and its toxicity in leukaemia cell systems (Wani *et al.*, 1971). Prior to the isolation of paclitaxel (Fig. 5.4), there was no report on the antitumour activity of natural taxane derivatives. Subsequent studies with various other tumour models have shown only low activity. But since it was noted that paclitaxel possessed very high activity in the B16 melanoma assay (Suffness and Wall, 1995), the compound fulfilled the United States National Cancer Institute (US NCI) criteria to become a candidate for development. Clinical phase I and phase II trials were conducted over the period from 1983 to 1986. In the later stage of the development of Taxol®, its use was limited by the lack of sufficient supplies of the therapeutic agent. The restricted number of *T. brevifolia* trees, the small yield of bark per tree, their slow growth rate and the low content of paclitaxel (Fig. 5.4) in the bark (approximately 0.02%) raised concerns about the reliability of this source (Croom, 1995).

Today, paclitaxel and docetaxel can be obtained on a larger scale by semisynthesis from 10-deacetylbaccatin III, a precursor that is extracted from the needles of *Taxus baccata* (L.), the European yew, and other *Taxus* species. Some years ago, the industrial production of paclitaxel was changed to recovery from plant cell cultures of *Taxus* cells cultivated in fermenters (Industrial Forum, 2002). However, clinical interest received a significant boost by the announcement of its remarkable efficacy against ovarian cancer (McGuire *et al.*, 1989; Einzig *et al.*, 1992). Subsequently, paclitaxel has been studied for activity against other solid tumours. Since the first report on the activity of paclitaxel in breast cancer was published in 1991 (Holmes *et al.*, 1991), many papers have reported on encouraging response rates of patients with breast cancer after the application of paclitaxel (for literature, see Saloustros *et al.*, 2008). Clinical activity has also been observed in various other types of cancer and the number of trials with paclitaxel alone or in combination with other drugs is legion. Recent investigations have emphasized the increase of response rates, improvement of the given formulations (Scripture *et al.*, 2005), the extension of therapy on other tumour types and reduction of severe side effects by combining taxanes with other anticancer drugs, such as epirubicin, topotecan, etoposide and others (for literature, see Chu *et al.*, 2005; Markman, 2008).

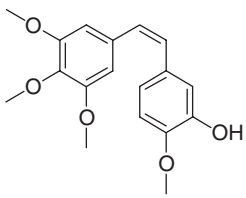
The development of docetaxel (Taxotere®) (Fig. 5.4) was enforced because of its better solubility and higher potency compared to paclitaxel (in in vitro assays inhibiting cold- or calcium-induced depolymerization of tubulin, it is twice as potent as paclitaxel; Guéritte-Voegelein *et al.*, 1991). The clear differences between paclitaxel and docetaxel on the molecular level, a wider

cell cycle activity and an apoptotic effect mediated by bcl-2 phosphorylation has been discussed for docetaxel, can possibly explain the different clinical behaviour of both compounds (Gligorov and Lotz, 2004). Phase I clinical trials were started in 1990 (e.g. Extra *et al.*, 1993). In several subsequent clinical trials, it was shown that docetaxel possesses activity against, for example, ovarian, breast, head, neck cancer and non-small-cell lung and prostate cancer (e.g. Ansari *et al*. 2008; for literature, see Katsumata, 2003; Montero *et al.*, 2005; Saloustros and Georgoulias, 2008). Side effects during treatment with paclitaxel and docetaxel are especially peripheral neuropathy (Argyriou *et al*., 2008) and, for example, neutropenia, myelosuppression, hypersensitivity reactions and alopecia (Fumoleau, 1997). Paclitaxel is already registered in several countries for the treatment (first- or second-line therapy) of ovarian and breast cancer as well as Kaposi sarcoma, and docetaxel (alone or in combination) for the (first- or second-line therapy) treatment of metastatic breast cancer, metastatic non-small-cell lung, metastatic prostate and metastatic gastric cancer. The main mechanisms involved in the resistance to the taxanes paclitaxel and docetaxel are most likely associated with the generation of multidrug transporters and the alteration of the tubulin/microtubule system (for overview, see Galletti *et al.*, 2007).

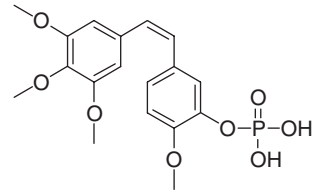
The importance of paclitaxel and docetaxel (Fig. 5.4) is due not only to their broad anticancer activity and the development of several further taxane derivatives from the lead paclitaxel (Butler, 2005), but also to their special mode of action. Whereas other classic spindle poisons, such as colchicine, bind to soluble tubulin and inhibit its polymerization (Berg *et al*., 2002), both of these compounds stabilize microtubules and inhibit depolymerization back to tubulin (Schiff *et al.*, 1979); therefore, a new candidate for the treatment of cancer has become available. In the meantime, more natural compounds with a similar mode of action, especially the macrocyclic epothilones (Altmann *et al*., 2007; Donovan and Vahdat, 2008) isolated from the myxobacterium *Sorangium cellulosum*, but also diterpenoids, eleutherobin or sarcodictyin, isolated from corals, have reached clinical or preclinical trials and are possible drug candidates for the future (Singh *et al*., 2008).

A newly developed plant-derived compound potently inhibiting polymerization of tubulin is the disodium salt of combretastatin A-4 phosphate, a better water-soluble derivative of combretastatin A-4 (Fig. 5.5). This compound was isolated from the bark of *Combretum caffrum* (ECKL. and ZEYH.) KUNTZE (Combretaceae) a plant native in South Africa. Combretastatin A-4 showed binding to the colchicine binding side of tubulin, as well as cytotoxic and antiangiogenic activity (Cirla and Mann, 2003). In the past years, it was investigated in several in vivo models as well as in phase I and phase II studies especially in combination with other anticancer drugs like carboplatin und paclitaxel (for literature, see Chaplin *et al*., 2006; Hinnen and Eskens, 2007).

In addition to alkaloids, polyphenols, especially flavonoids, display a remarkable array of biochemical and pharmacological activity (for review, see



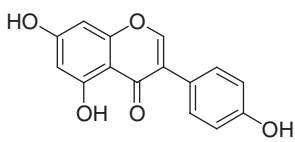
Combretastatin A-4 (**13**)



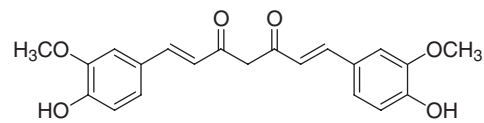
Combretastatin A-4 phosphate (**14**)

Figure 5.5 Structure of combretastatin A-4 (**13**) and combretastatin A-4 phosphate (**14**).

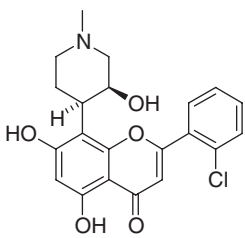
Harborne, 1994; Gomes *et al*., 2008). With regard to their anticancer activity, the growth inhibitory effects of various polyphenols, especially quercetin derivatives and genistein (Fig. 5.6) on several malignant tumour cell lines, e.g. gastric, colon and breast cancer cells (for review, see Harborne, 1994; Ravindranath *et al*., 2004; Kandaswami *et al*., 2005) and other inhibitory effects on carcinogenesis have already been described (for review, see Yang *et al*., 2001). There is growing interest in compounds with chemopreventive properties, since they may reduce the incidence of cancer in human populations. The daily intake in the human diet of plant-derived compounds that have such activities contributes markedly to human health. The pharmacological significance and nutrition benefit of polyphenols, prominent components in various fruits, vegetables and spices, as well as in tea, beer, cocoa and wine, is



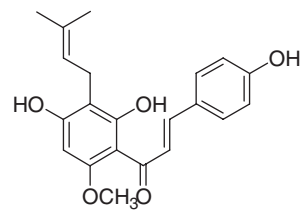
Genistein (**15**)



Curcumin (**16**)



Flavopiridol (**17**)



Xanthohumol (**18**)

Figure 5.6 Structures of genistein (**15**), curcumin (**16**), the semisynthetic flavone, flavopiridol (**17**), which is closely related to (+)-*cis*-5,7-dihydroxy-2-methyl-8-[4-(3-hydroxyl-1-methyl)-piperidinyl]-4H-1-benzopyran-4-one, and the chalcone xanthohumol (**18**).

the subject of intensive discussion (Ramos, 2007, 2008; Syed *et al*., 2008). Of increasing importance are especially the investigations on the metabolism of the polyphenols after oral intake and the pharmacological activity of the resulting phase I and phase II metabolites. The pharmacological activity of polyphenol glucuronides and sulphates will be a promising research field in future.

Genistein (Fig. 5.6), e.g. from *Glycine max* SIES. et ZUCC. emend. BENTH. (Fabaceae) and curcumin (Fig. 5.6) from *Curcuma xanthorrhiza* ROXB. or *Curcuma longa* (L.) (Zingiberaceae) are under pharmacological and clinical investigation since several years. Recently, xanthohumol (Fig. 5.6) from *Humulus lupulus* L. (Cannabaceae) was also the subject of intensive research. Genistein and curcumin underwent intensive chemoprevention trials by the US NCI (Kelloff *et al.*, 1996a, 1996b) after showing strong in vitro inhibition of enzymes related to the genesis of cancer, e.g. protein kinase C (Lin *et al.*, 1997) or ornithine decarboxylase (White *et al.*, 1998), and preventive activity in breast, colonic cancer and leukaemic cell lines, as well as in animal models (Adlercreutz and Mazur, 1997; Raynal *et al.*, 2008). In addition to its estrogenic and antiestrogenic effects (e.g. Kostelac *et al*., 2003; Chrzan and Bradford, 2007), genistein is a specific inhibitor of tyrosine protein kinase and interferes with epidermal growth factor (EGF), $pp60^{v\text{-}src}$ and $pp110^{gag\text{-}fes}$, (Akiyama *et al.*, 1987; Akiyama and Ogawara, 1991), which may be the basis of its antitumour potency. Recent research clarified that the effect of genistein is most likely a sum of various interactions within the molecular level including also the inhibition of activation of NF-kappaB/Akt dependent signalling pathways (for review, see Banerjee *et al*., 2008).

Curcumin (Fig. 5.6) exhibited strong inhibitory activity against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced generation of superoxide radical anions via inhibition of protein kinase C (Nakamura *et al.*, 1998) and radical scavenger activity against NO radicals (Sreejayan and Rao, 1997). It is also a strong inhibitor of phosphorylase kinase (Reddy and Aggarwal, 1994). Other observations include induction of apoptosis via different mechanisms (Balasubramanian and Eckert, 2007; Shankar and Srivastava, 2007), suppression of the activation of transcription factors, such as nuclear factor (NF)-kappa B and activating protein 1 (AP–1) (Pendurthi *et al.*, 1997), enhancement of antibody response (South *et al.*, 1997), inhibition of cyclooxygenase and lipoxygenase (Ammon *et al.*, 1993), inhibition of multidrug resistance-linked drug transporters (Shukla *et al.*, 2009). Thus, it is most likely that the biological effects of curcumin are a result of its interaction with multiple molecular targets (Thangapazham *et al*., 2006; Hatcher *et al*., 2008). The chemopreventive effects of curcumin after topical or oral application in mice models have also been reported (Huang *et al.*, 1997; Shankar *et al*., 2008; for review, see Kunnumakkara *et al*., 2008) and clinical as well as preclinical studies on curcumin suggest a remarkable potency for the use in prevention and therapy of cancer (Aggarwal *et al*., 2003).

Hop (*H. lupulus* L., Cannabaceae) cones contain many structurally related prenylated chalcones with xanthohumol (Fig. 5.6) being the most abundant

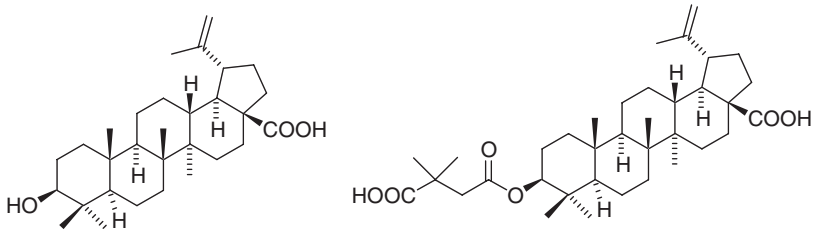


Figure 5.7 Structures of betulinic acid (**19**) and bevirimat (**20**).

one. Due to the good availability of xanthohumol which can be isolated from hop cones or synthesized in good yields (Vogel *et al*., 2008), its pharmacological characterization advanced significantly and it has been shown to exhibit an interesting spectrum of pharmacological effects. In addition to antiproliferative activity against different cancer cell lines (Miranda *et al.*, 1999; Delmulle *et al*., 2006), it also exhibited apoptotic (Colgate *et al*., 2007) and chemopreventive activity due to protective effects against carcinogens or procarcinogens (for literature, see Section 5.2). Several in vitro studies substantiated effects on enzymes (Monteiro *et al*., 2007) and transcription factors (Albini *et al.*, 2006) involved in the genesis of cancer and in vivo growth inhibition of a vascular tumour (Albini *et al.*, 2006) and inhibition of angiogenesis in xenografts (Monteiro *et al.*, 2008) has been reported.

Flavopiridol (Fig. 5.6), a semisynthetic flavone closely related to (+)-*cis*–5,7-dihydroxy-2-methyl-8-[4-(3-hydroxy-1-methyl)-piperidinyl]-4H-1-benzopyran-4-one (Fig. 5.7), which was originally isolated from the stem bark of *Dysoxylum binectariferum* (Meliaceae) (Naik *et al*., 1988), is currently undergoing phase I and II clinical trials as a potential antineoplastic agent. Flavopiridol is a potent inhibitor of members of the cyclin-dependent kinase (CDK) family, e.g. CDK1, CDK2 and CDK4, which are important enzymes in the regulation of the cell cycle (Worland *et al.*, 1993; Losiewicz *et al.*, 1994; Carlson *et al.*, 1996). At low concentrations (0.1–0.4 μmol), flavopiridol binds to the adenosine triphosphate (ATP)-binding site of CDK1 and CDK2, resulting in competitive CDK inhibition with respect to ATP and, therefore, blocking cell cycle progression in the G1 and G2 phase (Carlson *et al*., 1996). Further inhibitory effects on other enzymes, such as protein kinase C, have also been observed but only at higher concentrations (concentration required to produce half the maximum inhibition [IC_{50}] = 6 μM) (Losiewicz *et al.*, 1994). Very recent research on the apoptotic activity of flavopiridol confirms the importance of its interaction with cellular pathways besides the CDKs (Takada *et al*., 2008). The antitumour activity of flavopiridol has been demonstrated in vitro in different cycling and non-cycling cancer cell lines, including prostate, breast, lung and leukaemia cells (e.g. Bible and Kaufmann, 1996; Mayer *et al*.,

2005). In addition, it was active in a wide range of human tumour xenografts in vivo (Drees *et al.*, 1997; Arguello *et al.* 1998; Wirger *et al.*, 2005), and several phase I studies in combination with other anticancer drugs (e.g. Fornier *et al.*, 2007) as well as phase II studies are still running (e.g. Karp *et al.*, 2007). Flavopiridol appears to be an interesting synergistic or additive partner of other agents, such as 5-fluorouracil and cisplatin but, on the other hand, it was shown that combination with cell cycle phase-specific agents, such as paclitaxel (Fig. 5.4) or topotecan (Fig. 5.2), led to a sequence-dependent cytotoxicity in vitro. When cells were treated with paclitaxel and flavopiridol simultaneously or in the flavopiridol–paclitaxel sequence, strong antagonism was observed. When paclitaxel preceded flavopiridol, the cytotoxicity was greater than additive (Bible and Kaufmann, 1997). Augmenting effects were also observed between topoisomerase I inhibitors (like topotecan) and flavopiridol (Motwani *et al.*, 2001).

5.3 Antiviral compounds

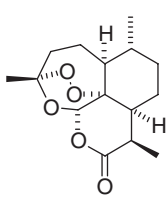
The successful treatment of viral diseases, especially AIDS and different types of hepatitis, is a maintaining challenge and there is still strong interest in new antiviral drugs. Many compounds of plant origin with inhibitory effects against several viruses in vitro have been identified (e.g. De Clercq, 2000; Curini *et al.*, 2006). However, in most cases, the activity observed was not sufficient for therapeutic application or problems achieving therapeutic concentrations due to limited bioavailability or possible side effects exist. This is, for example, true for the α-glucosidase I inhibitors castanospermine and 6-butyrylcastanospermine which has been reported to inhibit HIV replication and HIV-mediated syncytium formation in vitro (Asano, 2003).

Recently, the utilization of triterpenoids as anti-HIV agents has attracted strengthened attention. Especially betulinic acid derivatives with side-chain modifications at positions C-3 and C-28 are reported to inhibit HIV replication at nanomolar concentrations. Although sharing the same betulinic core, these compounds represents two different and unique modes of molecular mechanisms. 3-*O*-(3′,3′-dimethylsuccinyl)-betulinic acid (DSB, also named bevirimat or PA 457, Fig. 5.7) was found to interfere with HIV CA(capsid)/SP(spacer peptid)1 processing during HIV-1 maturation (Li *et al.*, 2003; for literature, see Yu *et al.*, 2005). Bevirimat partially blocks the cleavage of CA/SP1, a crucial region in HIV-1 morphogenesis resulting in the production of non-infectious HIV-1 particles. Oral administration of bevirimat to HIV-1-infected SCID-hu Thy/Liv mice reduced viral RNA by more than two log units and protected immature and mature T cells from virus-mediated depletion. It was active up to 3 days after inoculation with both WT HIV-1 and an AZT-resistant HIV-1 clinical isolate in plasma concentrations also achievable in humans after oral dosing (Stoddart *et al.*, 2007). Furthermore, a phase I and II study on the safety, virological effects as well as on

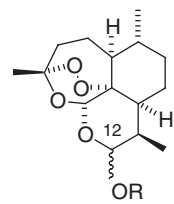
the pharmakodynamic and pharmacokinetic of bevirimat was successfully done in HIV-infected human adults (Smith *et al*., 2007). The betulinic acid derivative IC9564 inhibited HIV-1 envelope-mediated membrane fusion by targeting the HIV-1 envelope glycoproteins (Holz-Smith *et al*., 2001). Several other betulinic acid derivatives with high in vitro activity against HIV-1 had been synthesized (Huang *et al*., 2006) and further triterpenoids like moronic acid are under investigation (Yu *et al*., 2006)

5.4 Antimalarial drugs

The potent antimalarial compound, artemisinin (quinghaosu) (Fig. 5.8), has been isolated from *Artemisia annua* (L.) (Asteraceae), a plant used in traditional Chinese medicine for malarial therapy (Trigg, 1989). Among the three species of the genus *Artemisia* containing artemisinin (*A. annua*, *A. lanceolata* and *A. apiacea*), *A. annua* appears to be the most interesting one (Covello, 2008) containing approximately 0.1–0.2% artemisinin in the wild type. The cultivation of *A. annua* centred in Asia, but a significant amount is also produced in Africa (Kindermans *et al*., 2007). The production of artemisinin via tissue culture techniques or chemical synthesis is currently not economical (for review, see Covello, 2008). Artemisinin and its semisynthetic derivatives, e.g. artemether and sodium artesunate (Fig. 5.8), have been under intensive pharmacological, toxicological and clinical investigation since the mid-1970s. Most clinical studies of artemisinin, artemether and artesunate outside of China, concentrate in areas with endemic incidence of multiresistent malaria. Due to the short half-life and fast elimination of artemisinin derivatives, the combination therapy (artemisinin combination therapy [ACT]) with an antimalarial drug exhibiting long efficacy (for example mefloquine and lumefantrine) is clearly superior to mono therapy (German and Aweeka, 2008). Previous investigations on the state-of-the-art ACT are summarized, for



Artemisinin (**21**)



$R = CH_3$	Artemether	(**22**)
$R = CH_2CH_3$	Arteether	(**23**)
$R = CO(CH_2)_2COONa$	Artesunate, Na+	(**24**)

Figure 5.8 Structure of the potent antimalarial compound, artemisinin (**21**), of its semisynthetic lactol derivatives, artemether (**22**, 12β), arteether (**23**, 12β) and sodium artensuate (**24**, 12α).

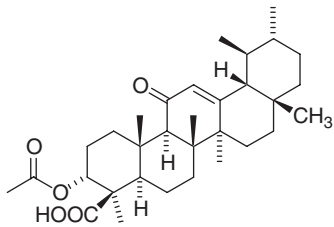
example, in Nosten and White (2007). Besides the well-established combination of artemisinin and lumefantrine, investigations on other effective combination regimen like artesunate-mefloquine, artemether-lumefantrine and dihydroartemisinin-piperaquine have also been done (e.g. Hutagalung *et al*. 2005; Ashley *et al.*, 2006; for review, see Nosten and White, 2007). Various studies are nowadays conducted to find the optimal regimen and optimal galenic formulations with high efficacy, low side effects and low recrudescence rates for the therapy of children (e.g. Abdulla *et al*., 2008).

In spite of the extensive clinical data available for artemisinin and its derivatives (Fig. 5.8), their mode of action is not fully understood and still under intensive discussion. The activity of artemisinin depends on the 1,2,4-trioxane structure containing the characteristic endoperoxide bridge, which is proposed to be activated by cellular ferrous iron forming free radical metabolites (C radicals) during oxidative processes and resulting in a damage of critical cellular macromolecules (Posner *et al.*, 1994). It has been published that reduced haeme is one important source of cellular iron for the production of these radicals leading vice versa to an alkylation of haeme itself (Meshnick *et al*., 1996). This would be, together with a selective uptake of artemisinin by *Plasmodium falciparum*-infected red blood cells (Kamchonwongpaisan *et al*., 1997), a plausible explanation for the high specific activity of artemisinin against the parasite due to the high content of haeme in the digestive vacuole (for review, see Meshnick, 2002). Consistently, damage on the morphology of the digestive vacuole membrane is one of the earliest effects of artemisinin (Del Pilar Crespo *et al.*, 2008). Surprisingly, recent in vitro results contradict the responsibility of C-centred-radicals for the antimalarial activity due the fact that a relationship between the propensity towards the formation of radical marker products and the antimalarial activity was not observable (Haynes *et al*., 2007). A recently proposed target of artemisinin is the sarco/endoplasmic reticulum Ca^{2+}ATPase (SERCA) homologue in *P. falciparum*, PfATP6 (Eckstein-Ludwig *et al*., 2003), but the clinical relevance of PfATP6 inhibition remains unclear.

Because the seco-cadinan skeleton of artemisinin and its derivatives is not essential for the antimalarial activity, these compounds were used as template for the synthesis of several more simple 1,2,4 trioxolanes with comparable or higher antimalarial activity and improved pharmaceutical characteristics, the so-called second generation of endoperoxides (Vennerstrom *et al*., 2004; Posner *et al*., 2008).

5.5 Anti-inflammatory drugs

Extracts of the gum resin of *Boswellia serrata* ROXB. (*Burser*aceae) have been used in traditional ayurvedic medicine as an antiphlogistic remedy. It has been shown that an alcoholic extract of the gum displayed marked anti-inflammatory activity in carrageenan- and dextran-induced oedema in mice



3-*O*-acetyl-11-keto-ß-boswellic acid (**25**)

Figure 5.9 Structure of 3-*O*-acetyl-11-keto-β-boswellic acid (AKBA) (**25**).

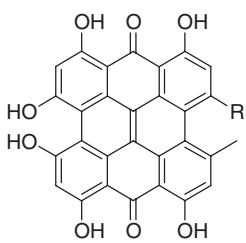
and rats (Singh and Atal, 1986), and boswellic acids have been identified as the main anti-inflammatory active principle. Subsequently, anti-inflammatory activity was observed in papaya latex-induced rat paw inflammation (Gupta *et al.*, 1992), antiarthritic activity in bovine serum album-induced arthritis in rabbits (Sharma *et al.*, 1989). Investigation of the mechanism revealed that some of the boswellic acids are strong inhibitors of leukotriene B_4 synthesis via the 5-lipoxygenase pathway in neutrophils.

The highest inhibitory activity against 5-lipoxygenase was measured for acetyl-11-keto-β-boswellic acid (AKBA) (Fig. 5.9) ($IC_{50} = 1.5$ μM) (Safayhi *et al.*, 1992). It was shown that AKBA is a direct, non-competitive and non-redox-type inhibitor of 5-lipoxygenase, acting at a selective site for pentacyclic triterpenes that is different from the arachidonate binding site (Safayhi *et al.*, 1992, 1995). Studies on the structural requirements for binding and inhibitory activity led to the assumption that the pentacyclic triterpene ring is crucial for a selective binding, whereas the 11-keto group in combination with a hydrophilic group substituted at C-4 is essential for the 5-lipoxygenase inhibitory activity (Sailer *et al.*, 1996a, b). Early studies of its anti-inflammatory activity showed a lack of activity on cyclooxygenase-I and 12-lipoxygenase in human platelets as well as on peroxidation of arachidonic acid by Fe–ascorbate (Safayhi *et al.*, 1992). In contrast, recent investigations pointed to a more complex mode of action and revealed an interference of boswellic acids with platelet-type 12-lipoxygenase (Pöckel *et al.*, 2006) as well as with cyclooxygenase 1 in intact human platelets (AKBA $IC_{50} = 6$ μM; Siemoneit *et al.*, 2008). Additionally, potent inhibition of human leukocyte elastase by AKBA was demonstrated (Safayhi *et al.*, 1997). Further results showed an inhibition of the activation of the transcription factor NFκB (Cuaz-Pérolin *et al.*, 2008) by modification of the NFκB/IκB complex via the inhibition of the IκB/kinase complex (Syrovets *et al.*, 2005). It can be concluded that the effects of the boswellic acids on the molecular level are multicausal (Pöckel and Werz, 2006). Although studies with extracts of *B. serrata* gum resin have produced promising results in patients with chronic polyarthritis (Etzel, 1996), collagenous colitis (Madisch *et al.*, 2007) and osteoarthritis of the knee (Kimmatkar *et al.*, 2003; and with an AKBA-enriched extract; Sengupta *et al.*, 2008), a final assessment

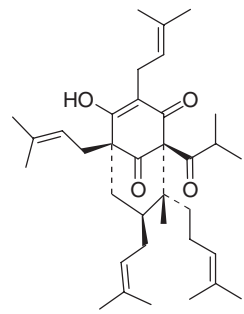
of the therapeutic potential of boswellic acids remains open, until results of state-of-the-art clinical trials with a greater number of patients, representative data on its pharmacokinetics and the side effects that occur are available (for literature on clinical studies, see Ernst, 2008). Current research on the boswellic acids also focused on their apoptotic and cytotoxic activity against various cancer cell lines (e.g. Hostanska *et al*., 2002; Liu *et al*., 2002; Büchele *et al*., 2006).

5.6 Antidepressant drugs

Preparations with standardized extracts of St. John's wort, *Hypericum perforatum* (L.) (Guttifereae), are well established for the treatment of depressive disorders of mild-to-medium severity. Various placebo-controlled clinical trials have confirmed its antidepressant potency, which is comparable to synthetic antidepressants, such as imipramine or sertraline (Gastpar *et al*., 2005; for literature, see Schulz, 2002). Side effects are usually less pronounced and less severe compared to standard antidepressants (for overview, see Schulz, 2006). Nevertheless, several investigations focused on drug interactions caused by the application of *Hypericum* extracts (Will-Shahab *et al*., 2008; for literature, see Di *et al*., 2008). On the molecular level, these interactions are mainly attributed to the inhibition of enzymes belonging to the cytochrome P450 group (for literature, see Mannel, 2004). Despite its widespread usage, the exact molecular mechanism of the antidepressant action of extracts of St. John's wort is not fully understood. A significant inhibitory effect on the neuronal uptake of several neurotransmitters not only of serotonin, noradrenaline and dopamine, but also of GABA and L-glutamate has been observed. It can be assumed that the phloroglucinol derivative hyperforin (Fig. 5.10) is mainly responsible for this broad-spectrum effect (Chatterjee *et al*., 1998; Wonnemann *et al*., 2001), exhibiting its activity by an elevation of the intracellular Na^+



R = CH_3 Hypericin (**26**)
R = CH_2OH Pseudohypericin (**27**)



Hyperforin (**28**)

Figure 5.10 Structure of the antidepressant compounds hypericin (**26**) and pseudohypericin (**27**), and of the phloroglucinol derivative, hyperforin (**28**).

concentration, probably due to activation of sodium conductive pathways (Müller *et al*., 2001).

Besides hyperforin, extracts of St. John's wort typically contain hypericin and pseudohypericin derivatives (Fig. 5.10) as well as flavonoids as potential therapeutically active components. A forced swimming test with rats indicated that hypericin and pseudohypericin can contribute to the antidepressant activity of *Hypericum* extracts. Antagonism of the effect by sulpirid indicated that the dopaminergic system is involved (Butterweck *et al*., 1998). Hypericin and *Hypericum* extracts have also been reported to reduce mRNA levels in brain areas involved in HPA (hypothalamic-pituitary-adrenal) axis control in rats (Butterweck *et al*., 2001). Furthermore, long-term administration of St. John's wort and hypericin can modify levels of neurotransmitters like serotonin (Butterweck *et al*., 2002a). It has been also discussed that a reduction of the HPA axis function by flavonoids can contribute to the antidepressant effect (Butterweck *et al*., 2008), but it is difficult to assess the clinical relevance of these new findings.

Another constituent of St. John's wort, amentoflavone (I3′,II8-biapigenin), significantly inhibited binding at serotonin, dopamine, opiate and benzodiazepine receptors in vitro (Butterweck *et al*., 2002b). Therefore, it is likely that the antidepressant activity of *Hypericum* extracts in vivo is not due to a single group of constituents and to affinity to one receptor but to several pharmacologically active constituents that are present in the extract.

5.7 Anti-ischaemic drugs

Employing standardized extracts of *Ginkgo biloba* (L.) leaves with a defined content of ginkgo-flavone glycosides and terpene lactones (24% ginkgo-flavone glycosides and 6% terpene lactones; EGb 761) is accepted in the treatment of various peripheral or cerebral circulatory disorders (Schweizer and Hautmann, 1999; for literature, see Horsch and Walther, 2004). Since the 1970s, many pharmacological in vitro and in vivo studies were performed on *Ginkgo* extracts, mainly on EGb 761, and also on its single constituents, e.g. the ginkgolides and bilobalide (Fig. 5.11): e.g. antioxidant and free radical

Ginkgolide B (**29**) Bilobalide (**30**)

Figure 5.11 Structure of the terpene lactones, ginkgolide B (**29**) and bilobalide (**30**).

scavenging activity; protection of hypoxia; effects on haemorheology and platelet aggregation; protection against hypoxia and ischaemia, regulation signalling pathways, gene transcription and cellular metabolism. These studies have been reviewed by Oberpichler-Schwenk and Krieglstein (1992), Ahlemeyer and Krieglstein (1998) and Smith and Luo, 2004.

Due to the dramatically increasing number of elderly people suffering from Alzheimer's disease and different other forms of dementia and the limited arsenal of applicable drugs, the investigations on possible positive effects for these patients dominate the latest clinical studies and pharmacological investigations on *G. biloba* extracts. The outcome is up to now very controversial. Whereas the results of several in vitro and in vivo models support a possible activity (for literature, see Ahlemeyer and Krieglstein, 2003), a broad clinical trial investigating the efficacy of *G. biloba* in people suffering from Alzheimer's disease showed insignificant differences between placebo and the used application schedule of *G. biloba* extract (DeKosky *et al.*, 2008). Accordingly, an earlier clinical trial on elderly people with dementia and age-associated memory impairment could not substantiate significant beneficial effects with the used regimen (Van Dongen *et al.*, 2003).

5.8 Immunostimulatory drugs

Extracts of mistletoe, *Viscum album* (L.), have frequently been applied in adjuvant cancer therapy (e.g. Bock *et al.*, 2004; Augustin *et al.*, 2005). In recent years, major effects of the extracts have been attributed to mistletoe lectin I, viscumin (ML I), which was demonstrated to be an immunomodulatory agent (for literature, see Elluru *et al.*, 2006). Standardized aqueous mistletoe extracts with a constant lectin content are now on the market. However, their clinical efficacy is still a matter of controversy. Mistletoe lectin I is a double chain (type 2) RIP with a molecular weight of 60 kDa. The ribosome-inactivating properties are due to the A-chain, whereas the B-chain represents the lectin part and binds specifically to glycosylated cell surface proteins containing terminal galactose residues (Franz, 1986; Endo *et al.*, 1988; Soler *et al.*, 1998).

Mistletoe extracts exert cytotoxicity and induce apoptosis (programmed cell death) in several cell types (e.g. Choi *et al.*, 2004; Cebovic *et al.*, 2008). In addition, inhibitory effects on tumour angiogenesis and metastasis of haematogenous and non-haematogenous tumour cells in mice have been observed (Yoon *et al.*, 1995). Stimulation of the cellular defence system has also been shown in several studies. ML I and mistletoe extracts enhanced the activity of natural killer cells and T-lymphocytes (Heiny *et al.*, 1998), as well as the levels and the activity of lymphocytes, peritoneal macrophages and monocytes in vivo and in vitro (Stein and Berg, 1996; Stein *et al.*, 1998; Büssing *et al.*, 2008). The production of cytokines, tumour necrosis factor-α (TNF-α) (Ribereau-Gayon *et al.*, 1996; Lyu and Park, 2007) and the activation

of human polymorphonuclear leukocytes was observed in vitro (Braun *et al.*, 1995).

5.9 Conclusions

Our knowledge of the molecular basis of diseases has dramatically increased in the last years. As a result, more specific bioassays based on receptors and enzymes have been developed and high-throughput screening led to even more interesting compounds from plant origins. It is clear that plants continue to provide us with new drugs and leading structures. Low molecular weight compounds, peptides and proteins with influence on specific cell functions will play an important role in the development of new drugs in the future because a plethora of biologically active molecules with new and unprecedented carbon skeletons are necessary as lead molecules for drug discovery.

Further rapid progress can also be expected in the field of combinatorial biosynthesis. Creation of novel gene combinations or hybrid genes may produce novel secondary metabolites, due to the effect of a new enzyme with new enzymatic properties on a metabolic pathway. Therefore, it may be possible to create new 'natural products', which have novel or more potent biological activities. However, the screening of plant extracts is hampered by the problem of non-specific effects and false-positive or -negative results because of matrix compounds, such as lipids, tannins and chlorophylls (Beutler *et al.*, 1995). Therefore, automatic purification procedures are still desirable.

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Chapter 6

PRODUCTION OF NATURAL PRODUCTS BY PLANT CELL AND ORGAN CULTURES

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Abstract Natural products have been used as medicines, food additives or in technical applications by humans since thousands of years. Due to various reasons, a sufficient supply of the plant raw material has become increasingly difficult in recent years. Since more than 30 years, laboratories worldwide are trying to produce natural products for commercial application with plant cell and organ cultures. The commercial success of this research is still very limited, due to too low product yields resulting into production costs, which are not acceptable. Among organ cultures, root and hairy root cultures are the most promising for the production of secondary metabolites in good yields. This chapter describes the methods used to overcome these intrinsic problems in product yields and the hopes which are set on the new developments to transfer the appropriate plant genes into microorganisms, which may become more applicable for large scale production of plant natural products than plant cells themselves. First results in this field, e.g. the synthesis of benzylisoquinoline alkaloids in recombinant yeast cells, are already very promising.

Keywords: plant cell cultures; organ cultures; hairy roots; plant natural products; elicitation; biosynthesis; RNAi; formation of natural products in recombinant microorganisms

6.1 Introduction

In the first edition of this book, Walton *et al.* (1999) reported that Mitsui Petrochemicals used cell cultures for production of shikonin and purpurin, Nitto Denko produced ginseng cell mass in rather large quantities as food additive. Paclitaxel production was still in the pipeline. In the meantime, paclitaxel and derivatives thereof are produced with yew cell cultures by

Table 6.1 Industrial production of secondary compounds by plant cell cultures

Product	Species	Company	Reference
Shikonin	*Lithospermum erythrorhizon*	Mitsui Petrochemical Ind. Ltd.	Fujita *et al.* (1982)
Ginsenosides	*Panax ginseng*	Nitto Denko Corp.	Ushiyama (1991)
Purpurin	*Rubia akane*	Mitsui Petrochemical Ind. Ltd.	Personal communication
Paclitaxel	*Taxus spec*.	Phyton Biotech GmbH	www.phytonbiotech.com/

Phyton Biotech GmbH at Ahrensburg (Germany) for Bristol-Myers-Squibb using bioreactors with a working volume of up to 50 m^3 and a capacity of 880 000 litres per year (www.phytonbiotech.com/index.htm) (Table 6.1).

Plant natural products have great importance, e.g. as food additives or in drug discovery (Ji *et al.*, 2009). Since long time, due to different reasons, there is great interest to produce natural products by plant cell cultures. Despite intensive work in many laboratories all over the world, still the same difficulties as years before prevent a broad application of cell culture systems for plant natural product formation: the product yields are **too low** that a commercial application is economically feasible.

Can we still expect manifold application of plant cell culture methods for natural product formation? The answer is '**yes**'. Not in all cases, however, such plant cells themselves will be cultivated in large bioreactors for natural product formation. In the meantime, plant cell culture methods are integrated in a multitude of fundamental and applied research techniques which one does not realize at a first glance. These techniques are used not only for plant propagation and plant breeding, but also in isolation procedures for enzymes, genes, transcription factors as wells as identification of transporters, regulation networks and many others. In future, transformed microorganisms may be used for plant natural products formation as well.

In recent years, a great number of valuable reviews covering the whole field (Bourgaud *et al.*, 2001; Zhong, 2001; Rao and Ravishankar, 2002; Verpoorte *et al.*, 2002; Vanisree *et al.*, 2004; Zhang *et al.*, 2004; Canter *et al.*, 2005; Fumagali *et al.*, 2008; McCoy and O'Connor, 2008; Pfeifer, 2008; Smetanska, 2008; Kolewe *et al.*, 2008; Sourrouille *et al.*, 2009) or special aspects of natural product accumulation by plant cells were published (Brincat *et al.*, 2002; Chattopadhyay *et al.*, 2002; Straathof *et al.*, 2002; Zhang *et al.*, 2002; Fuss, 2003; Müller, 2004; Wink *et al.*, 2005: Zhou and Wu, 2006; Frense, 2007; Memelink and Gantet, 2007; Roytrakul and Verpoorte, 2007; Matkowski, 2008; Reddy *et al.*, 2008; Chemier *et al.*, 2009; Exposito *et al.*, 2009). Therefore, here only some newer developments are described in more details.

6.2 Production of natural products by cell and organ cultures

The situation is still similar to 1999: many natural compounds of great medicinal importance, even such complex structures like paclitaxel, are produced by undifferentiated cell cultures. However, others, e.g. morphine, vinblastine or many terpenoids, e.g. cardenolides, are not found in unorganized cell cultures. Shoot organ cultures of *Artemisia annua* contain small amounts of the antimalarian agent artemisinin, *Catharanthus roseus* shoot culture accumulate small amounts of the dimeric indole alkaloid vinblastine after regeneration of roots. On the other hand, root organ cultures, especially transformed root cultures (called hairy roots), turned out to be excellent systems to study root-specific secondary metabolism (Wink *et al.*, 2005).

In such cases, where cell suspensions, just like root and shoot cultures, accumulate at least small amounts of the compounds of interest, one is able to improve the product yields using the 'traditional methods', which will include selection of high producing plants for initiating the cultures of choice. From the best cultures, high producing cell lines are isolated; the media are optimized further with respect to growth and productivity (cf., e.g. Kolewe *et al.*, 2008). Despite all these efforts, however, the yields were still too low for practical application.

6.3 Elicitation

Nowadays, it is well accepted that secondary plant products serve plants as part of a defence system. In 1982, Wolters and Eilert noticed for the first time that the acridone alkaloid content increased in rue callus cultures which were cocultivated with fungi. Through the years, fungal cell wall components, microbial phytotoxins, but also UV-light, various heavy metals or ultrasound treatment turned out to be able to enhance secondary product accumulation in plants. Intensive investigations by many laboratories about the signal cascade of this process resulted in the identification of jasmonic acid (JA) and its derivatives as important *elicitors* within this complex process (for review, see e.g. Wasternack and Hause, 2002; Namdeo, 2007). JA was used in a multitude of cell and organ culture systems to increase the product yields. In 1994, Weiler and co-workers described the phytotoxin coronatine as a very potent elicitor. Lauchli and Boland (2002) synthesized various indanoyl amino acid conjugates, from which coronalone turned out to be the most effective substitute of JA. Berim *et al.* (2005) reported a tenfold increase of 6-methoxypodophyllotoxin content (up to about 2.7% of dry weight) in suspension cultures of *Linum nodiflorum* by treatment with coronatine. Most remarkable is that an additional lignan, 5′-demethoxy, 6-methoxypodophyllotoxin, is increased from 0.10 to more than 5% of dry weight. Fuhrmann and Fuss

in our laboratory (unpublished) found a high increase of podophyllotoxin and 6-methoxypodophyllotoxin accumulation in cultures of *Linum album* by coronatine treatment as well. More detailed examples of elicitation in different cell culture systems using various methods can be found, e.g., in Kolewe *et al.*, 2008; the importance of nitric oxide within the signalling network is described by Xu (2007).

A promising novel approach was used by Chaudhuri and co-workers (2009). These authors introduced a synthetic gene under the control of the 35S CaMV promoter with the help of *Agrobacterium rhizogenes* into hairy roots of *Convolvulus sepium*, *Withania somniferum* and *Tylophora tanakae* and into plants of *Convolvulus arvensis* and *Arabidopsis thaliana*. This gene encodes the fungal elicitor protein cryptogein (*crypt*) which is secreted by the oomycete *Phytophthora cryptogea* and elicitates defence responses in tobacco. The exact mechanism of resistance is not clear. At least, in tobacco, induction of secondary products is involved (Chaudhuri *et al.*, 2009). The authors isolated and cultivated hairy roots containing the crypt-construct. In comparison with appropriate controls, growth of *C. sepium* and *W. somniferum* hairy roots was stimulated, as was the content of calystegins in *Calystegia*, not, however, the withaferin content in *Withania*. The difference between growth of control and roots of *Tylophora* carrying the crypt-construct was significant but not so evident as in *Withania* and *C. sepium.* The content of tylophorine was not increased. The authors were able to regenerate plants from hairy roots of *C. arvensis* and *A. thaliana* carrying the crypt-construct. In roots and shoots of *C. arvensis*, a small increase (35%) of calystegin content was observed. However, in *Arabidopsis*, it was not clear whether there was an influence on flavonoid accumulation, which was investigated by the authors, as well.

6.4 Increase/decrease of product yields by genetic manipulation

Due to great progress in molecular biological methods, our knowledge of gene regulation in plants, especially of genes involved in secondary product accumulation, made tremendous progress in the last 10 years. This was accompanied by a more detailed knowledge in the enzymology of the biosynthesis of the main classes of natural products which resulted in the identification of the cDNAs and the appropriate genes, respectively, enabling studies about the role of transcription factors (Grotewold *et al.*, 1994; Memelink *et al.*, 2000; Memelink and Gantet, 2007) as well as of the function of the promoter and/or enhancer regions of the genes. Finally, this resulted in the understanding that the diversity of natural product accumulation in plant cells is regulated in a very complex **network** (Goossens *et al.*, 2003a; Jorgensen *et al.*, 2005; Morreel *et al.*, 2006; Böttcher *et al.*, 2008; Jiao *et al.*, 2009).

Agrobacterium tumefaciens and *A. rhizogenes*, are most often used for introduction of genes into plants. Differentiated plant material, like shoots, leaves

or roots, are needed. Another disadvantage is the fact that both species can only be used with dicotyledonous and a limited number of monocotyledonous plants. If one wants to transform callus or species from other subdivisions with *Agrobacteria*, then protoplasts can be tried or gene transfer by particle bombardment has to be used.

Secondary products of different chemical structures are produced and accumulated in roots of many plant species. So-called hairy roots, which are established by transformation with *A. rhizogenes* are known to grow relatively fast, are genetically stable (often in contrast to callus) and produce in most cases the compounds found in the roots of the differentiated plant (which is very often in contrast to callus as well). Therefore, the use of transformed root cultures can be very helpful in studying secondary product formation on an enzymological as well molecular level. It is not necessary to initiate suspension cultures from the transformed plant by a special hormone treatment, which may be responsible for additional unknown effects on product accumulation. Further, single seedlings of the plant species under investigation can be micropropagated in suspension or in solid medium in order to use plant material of the same and reproducible genotype. Not in all but in many cases, differentiated plants can be regenerated from the hairy roots which leads to new genotypes (Allen *et al.*, 2008), if additional specific genes encoding steps in secondary metabolism were introduced into the hairy roots.

6.5 Biosynthetic pathways delineation using RNA-interference

Allen and co-workers (2008) achieved an overexpression of alkaloids of about 40% by introducing an additional gene encoding the salutaridinol-7-*O*-acetyltransferase (SalAT) via the hairy root technique in opium poppy. A suppression of SalAT was achieved by the RNA-interface (RNAi) technique, leading to the accumulation of salutaridine, which is not detectable in control plants, up to about 23% of total alkaloid content.

Bayindir and co-workers (2008) applied the RNAi technique to decide between two possible biochemical pathways of product formation in plants. Lignans are dimers of phenylpropanoids, which are found widespread in the plant kingdom. Many of them have interesting biological activities after additional chemical derivatization, e.g. podophyllotoxin, some of them are used in medicine. Others, e.g. sesamin or secoisolariciresinol diglucoside, are supposed to be of health promoting quality and are used as food additives. (−)-Hinokinin is a potent anti-human hepatitis B virus agent and has high anti-inflammatory and analgesic activities. According to Medola and co-worker (2007), (−)-hinokinin has the potential to become a new drug. Hinokinin is found in cell cultures of *Linum corymbulosum*, for practical use its yields are still too low. Further improvements of productivity

are hampered by our limited knowledge of its biosynthesis. According to Bayindir and co-workers, two different pathways from pinoresinol to (−)-hinokinin could be possible, that via lariciresinol/secoisolariciresinol or via sesamin (Fig. 6.1). Using RNAi, the authors proved that the pathway via lariciresinol/secoisolariciresinol is active in *L. corymbulosum*. RNAi is a method to silence the specific expression of certain genes. The authors transformed shoots of *L. corymbulosum* with *A. rhizogenes* containing binary vectors with intron-spliced 'hairpin' RNA constructs in order to silence the gene for the pinoresinol-lariciresinol reductase (PLR) in *L. corymbulosum*. Reduction of PLR-activity as well as reduction of PLR mRNA resulted in reduction of hinokinin accumulation in the resulting hairy roots as compared with appropriate controls.

The genus *Linum* is an interesting subject to study the biosynthesis of the lignans podophyllotoxin (PTOX) and its derivative 6-methoxypodophyllotoxin (MPTOX), especially on a molecular level. Cell cultures of the Iranian species *Linum album* and *Linum persicum* accumulate PTOX up to about 1% dry weight together with small amounts of its derivative MPTOX. In hairy roots, the MPTOX concentration reaches up to 5% of dry weight, together with traces of PTOX (Wink *et al.*, 2005). Such yields, however, are still too low for commercial use. Application of genetic engineering did not yet result in overcoming these low yields (see Fig. 6.2).

Several groups are trying to improve product yields in cell cultures of plants synthesizing alkaloids which are important from a medicinal point of view like paclitaxel, camptothecin, scopolamine/hyoscyamine, morphine and their derivatives. As mentioned before, the work on paclitaxel was successful in the way that it can be produced commercially. In all other cases, substantial progress was made, but it was not sufficient to achieve commercial application. In the case of tropane alkaloids, the biosynthesis is known almost completely and several steps can be manipulated by molecular biological techniques. But even here one is still unable to overcome the obstacle that hyoscyamine/scopolamine is not formed in unorganized cell cultures.

The same is true for the benzylisoquinoline alkaloids: berberine or sanguinarine are synthesized by cell cultures of different plant species. Optimization by traditional methods like selection of optimal cell lines and increase of production by medium optimization is possible. Many of the genes involved in biosynthesis were cloned. Unfortunately, the medicinal importance of these compounds is much lower than that of morphine. Morphine, however, is not accumulated by unorganized cell suspension cultures.

6.6 Mass cultivation of plant cell cultures

Despite great progress and the establishment of special bioreactors for root and shoot organ cultures (cf., e.g. Takayama and Akita, 1994; Wildi *et al.*, 2003), a scale up of such organ cultures up to volumes of several thousand

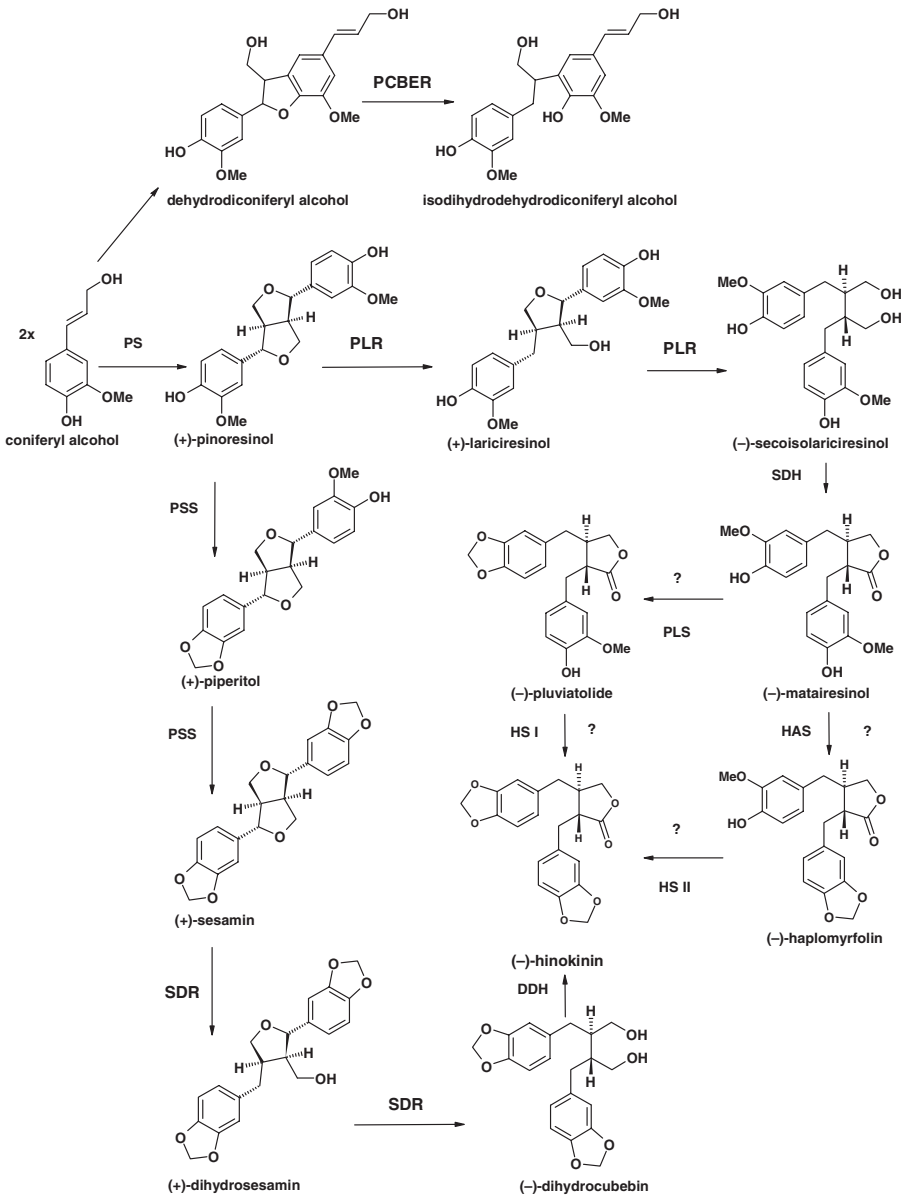

Figure 6.1 Possible biosynthetic pathways from coniferyl alcohol via sesamin or via secoisolariciresinol to hinokinin in cell cultures of *Linum strictum* ssp. *corymbulosum*. Enzymes known from other plant species: PS: pinoresinol synthase; PLR: pinoresinol-lariciresinol reductase; SDH: secoisolariciresinol dehydrogenase; PSS: piperitol-sesamin synthase. Theoretic steps: SDR: sesamin-dihydrosesamin reductase; PLS: pluviatoid synthase HS I/HS II: hinokinin synthase (from Bayindir *et al.*, 2008).

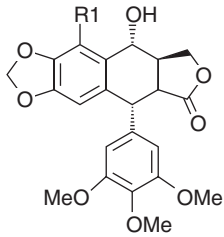

Figure 6.2 The lignans podophyllotoxin (R1 = H) and 6-methoxypodophyllotoxin (R1 = OMe).

litres seems to be difficult. The alternative, to force excretion of the secondary compounds into the medium, has been shown for several examples, but the real breakthrough is still waited for. More knowledge about transporters involved in natural product transport and/or secretion are needed (Goossens *et al.*, 2003b; Roytrakul and Verpoorte, 2007).

The principal problems concerning mass cultivation of cell suspension cultures can be regarded as solved, as demonstrated by the successful cultivation of *Taxus* cells in bioreactors of 50 000 litres by Phyton Biotech at Ahrensburg. In the 1970s, shear sensitivity of plant cells was somewhat overestimated (Wagner and Vogelmann, 1977). There are differences between cell lines. We now know that stirred tank reactors equipped with a stirrer of low shear force may give even better results than airlift reactors (e.g. Spieler *et al.*, 1985). Of importance is an optimal oxygen supply for most cell cultures to ensure optimal productivity (Garden, 2003).

More details about mass cultivation of plant cells can be found in Sajc *et al.*, (2000), Zhong (2001), Linden *et al.* (2001), Choi *et al.* (2001), Chattopadhyay *et al.* (2002) and Eibl and Eibl (2008).

6.7 Production of recombinant proteins by plants and plant cell cultures

Since several years, research groups all over the world study the possibility to use plants cultivated in the field or glass houses as well as suspension cultures in large scale bioreactors for the production of recombinant proteins, which can be used in medicinal treatment of human or animals. The main reason for the interest in such a production system is that many human or animal pathogens will not grow in plants so that there is no risk of a dangerous contamination with, e.g., vira.

Detailed data can be found in recent reviews (e.g. Fischer *et al.*, 2000, 2007; James and Lee, 2001; Schillberg *et al.*, 2003; Nilesh and Timko, 2004; Liénard *et al.*, 2007; Huang *et al.*, 2009). First commercially used systems are expected in near future. Therefore, a regulatory framework dealing with pharmaceuticals

from genetically modified plants and plant cells was established as well (Spök *et al.*, 2008).

6.8 Production of plant natural products in microbes

Commercial production of primary as well as of secondary compounds with microbes of different systematic origin is very common in biotechnology. Until recently, production of plant secondary products in microorganisms was regarded as completely impossible. The tremendous progress in molecular biological methods in recent years enabled a change in the field.

6.8.1 Terpenoids

Ajikumar and co-workers (2008) summarize the progress in the terpenoid field. Whereas single genes of many biosynthetic steps are expressed in *Escherichia coli* or yeast since longer time, nowadays several genes can be expressed in one microorganism. Five sequential paclitaxel genes were expressed in yeast leading to the formation of taxadiene (1 mg/L) and up to 25 μg/L taxadiene-5α-ol (DeJong *et al.*, 2006). Lindahl and co-workers (2006) introduced the gene for amorpha-4,11-diene synthase into *Saccharomyces cerevisiae*, which accumulated up to 600 μg/L amorpha-4,11-diene. Ro and co-workers (2006) expressed three genes involved in artemisinin metabolism into yeast (amorphadiene synthase, amorphadiene oxidase and a cytochrome P450 reductase, which in concert diverts carbon flux from farnesyl diphosphate to artemisinic acid). These authors succeeded in producing up to 100 mg/L of the precursor artemisinic acid for production of artemisinin, the important new antimalarian medicine (Ro *et al.*, 2006, 2008).

6.8.2 Flavonoids

Successful production of up to 700 mg/L flavanons and 113 mg/L anthocyanins in *E. coli* is reported by Leonard and co-workers (2008).

6.8.3 Isoquinoline alkaloids

The groups of Sato (Minami *et al.*, 2008) and of Smolke (Hawkins and Smolke, 2008) investigated isoquinoline alkaloid formation in transgenic *E. coli* and *S. cerevisiae*. The Japanese group studied especially the first part of the biosynthetic pathway from tyrosine to scoulerine. As it turned out to be difficult to establish the early steps like in plants from tyrosine to dopamine and 4-hydroxyphenylacetaldehyde which then is condensed to (*S*)-norcoclaurine by norcoclaurinesynthase (NCS), they simplified this part. They introduced a monoamine oxidase (MAO), an NCS, a norcoclaurine 6-*O*-methyltransferase (6OMT), coclaurine-*N*-methyltransferase (CNMT) and

a 3′-hydroxy-*N*-methylcoclaurine-4′-*O*-methyltransferase (4′OMT) into *E. coli* and achieved to synthesize reticuline from dopamine used as substrate. An important point was that this group had two isoforms of NCS in hand, only one of which was expressed at high level in *E. coli.* In the second step of their studies on aporphine-type alkaloid biosynthesis, they introduced a recently identified P450 enzyme (CYP80G2, corytuberine synthase; Ikezawa *et al.*, 2008) together with a *Coptis japonica* CNMT and the berberine bridge enzyme (BBE), respectively, into yeast. After growing the yeast cells in medium, which contained reticuline excreted from *E. coli* cells, magniflorine and scoulerine, respectively, were found. Final yields were 55 mg/L (*S*)-reticuline, 7.2 mg/L magniflorine and 8.3 mg/L scoulerine, respectively.

Hawkins and Smolke (2008) started with (*R*,*S*)-norlaudanosoline, which is commercially available. By introduction of a 6OMT, a CNMT and a 4′OMT into yeast, these cells transformed the substrate to (*R*,*S*)-reticuline. Additional expression of three enzymes (NCS, 6OMT, CNMT) from *Thalictrum flavum*, *Papaver somniferum* and *A. thaliana* resulted in the synthesis of (*S*)-scoulerine, (*S*)-tetrahydrocolumbamine and (*S*)-tetrahydroberberine from (*S*)-reticuline. Expression of a human P450 enzyme together with the appropriate reductase resulted into formation of salutaridine from reticuline, showing a novel activity of this enzyme.

6.8.4 Monoterpene indole alkaloids

Runguphan and O'Connor (2009) studied the accumulation of monoterpene indole alkaloids in *C. roseus*. They introduced a gene coding for a strictosidine synthase with an altered substrate specificity. A dream of plant biologists was fulfilled: the cell cultures accepted not only the natural substrates (secologanin and tryptamine) but additionally some tryptamine derivatives leading to a broader product spectrum. For recent reviews, see also Chemler and Koffas (2008) and Leonard *et al.* (2009).

6.9 Perspectives

The success in paclitaxel production on a commercial scale by *Taxus* cell cultures has demonstrated that biotechnological production of natural products by plant cells can be achieved. However, it is still very limited, despite more than 30 years of intensive work in many laboratories. The principal drawbacks are low product yields. Many interesting compounds are not produced in suspension cultures but only in root or shoot organs, e.g. hyscyamine and digoxin, respectively, which means that a certain degree of differentiation is necessary for the expression of the appropriate biosynthetic pathway. We do not yet know the appropriate elicitors and transcription factors just like the post-biosynthetic events involved in secondary metabolism to overcome this problem. Additionally, in most cases, the natural compounds are present in

Table 6.2 Some high producing cell cultures

		Yield (% dry weight)	
Product	**Plant**	**Cell culture**	**Differentiated plant**
Ajmalicine	*Catharanthus roseus*	1.0	0.3
Anthraquinones	*Morinda citrifolia*	18	2.2
Berberine	*Coptis japonica*	13	2.0
Ginsenosides	*Panax ginseng*	27	4.5
Nicotine	*Nicotiana tabacum*	3.4	2.0
Rosmarinic acid	*Coleus blumei*	27	3
Shikonin	*Lithospermum erythrorhizon*	20	1.5

Source: Data from Smetanska (2008)

the differentiated plants only in small amounts. The question will be whether this depends on low production rates, on substantial degradation during the production, and/or whether there is sufficient place to store the secondary products in the cells? We know examples that cell cultures can accumulate essentially higher amounts of natural products than the differentiated plant as shown in Table 6.2.

But is this a general phenomenon? Productivity of microorganism was improved substantially due to the excretion of the products by the cells into the medium, which provides a much larger 'sink' for product accumulation. In most cases, however, plant cells store the compounds within the cells; excretion is the exception. This demands research to find transporter molecules which could direct the final product into the extracellular space. Our knowledge about such transporters is still very scarce. On the other hand, plant cell cultures of some species are known to excrete degrading enzymes into the medium (e.g. *Coleus*; Stepan-Sarkissian *et al.*, 1993) which can degrade the excreted products.

On the other hand, although in many cases the product yields in the cultivated plants are small, these plants are used for commercial isolation of the compounds. The product yields in cell culture systems have to be substantially higher than in the differentiated plant that a biotechnological production process can compete with the traditional methods. Of course, this may change with increasing difficulties to supply enough raw material from differentiated plants for extraction.

Another difficulty should not be underestimated. Besides other criteria, the production system of natural compounds used in medicine has to be approved by the relevant Food and Drug Administration (FDA). Until now, it was always tried to exchange traditional production processes of natural product procurement by cell culture systems. In such a case, the new production system has to be approved by the FDA which, of course, entails additional expenses.

The recent progress in molecular biology promises a multitude of possible new approaches to develop biotechnological production of plant natural products. Most important is the progress in our knowledge concerning the biosynthetic pathways and the regulatory control mechanisms, although there still is a great lack in understanding the biosynthetic network in the plant. The data on incorporation of plant genes into *E. coli* and *S. cerevisiae* give hope that in future it may become possible to incorporate whole biosynthetic pathways for production of complicated alkaloids, terpenoids or other compounds. One has, however, still to be realistic: until now, only few genes were incorporated into microorganisms, to incorporate some 20 different genes and to express them in a regulated way to produce such compounds like morphine, hyoscyamine or podophyllotoxine is some greater challenge. At the moment, this is still impossible because not all the genes necessary are yet available. Still, the product yields of the transformed microorganisms have to be increased; it has to be proven that over the years transformed microorganisms are stable with respect to productivity, a difficulty which is known for plant cell cultures too. On the other hand, not all genes from all pathways may be expressed optimally in all microorganisms. This is demonstrated by the few examples described. Minami and co-workers (2008) as well as Hawkins and Smolke (2008) describe that, for example, the different genes of the isoquinoline pathways in different plant species may lead to very different enzyme activities within the final product biosynthesis. Connected to this problem is the absence of the correct subcellular compartmentation within the cell.

Finally, the products produced by microorganisms need the approval by FDA as well.

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