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Chapter 3

INSECT DETOXIFICATION AND SEQUESTRATION STRATEGIES

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Abstract: Plants defend themselves from insect herbivores with a vast array of chemical defences, yet insects have evolved several mechanisms for detoxifying these and even sequestering them for their own use. Gene duplication within the large cytochrome P450 gene family plays diverse roles in detoxification, employing fine-tuning of substrate specificity, transcriptional control and insensitivity to plant-derived P450 inhibitors. Multiple mechanisms exist for avoiding activation of glucosinolates and cyanogenic glucosides, and for sequestering or even synthesizing the latter. Housekeeping enzymes have been re-directed to novel detoxicative functions. Often, the primary molecular target of the plant toxin has mutated to a less sensitive form, and multiple parallel evolutionary responses of this type can be seen in communities of herbivores consuming plants posing the same chemical challenges. These and other mechanisms have enabled insects to continue to exploit plants for food throughout the long history of their co-evolutionary struggle.

Keywords: Plant-insect co-evolution, cytochrome P450, UDP-glycosyl transferase, flavin-dependent monooxygenase, cyanogenic glucoside, glucosinolate, pyrrolizidine alkaloid, iridoid glucoside, cardenolide

3.1 Introduction

Plants defend themselves from insect herbivores with a vast array of chemical defences, which can be constitutively produced or induced by herbivory. These defences include toxins, feeding deterrents, and volatile signals to attract parasites and predators of the herbivores. Generally speaking, the defences employed by a particular plant species are effective against the vast majority of species of potential herbivores that might encounter it, yet one or

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a few specialist insect species can usually be found to have evolved the countermeasures required to overcome all of these defences and to make a living by exploiting the plant as a food source.

Most herbivorous insect species are specialized in this way, subsisting on a small number of related host plants or one or two plant families that employ the same types of chemical defences, and reliably detoxifying them. When facing an array of compounds that is uniform and predictable, the insect can deploy its countermeasures constitutively and may even use these compounds as oviposition and feeding stimulants. This specialization may further extend to the co-option of the plant chemical defences by sequestration – the selective uptake, transport, modification, storage and deployment of plant secondary chemicals for the insect's own defence.

A minority of herbivores are generalists to a greater or lesser degree, feeding on a wider variety of plants and possessing the ability to deal with diverse chemical defences. Since no single countermeasure is equally effective against all toxins, generalists must not only possess a diversity of detoxicative strategies, but must also have the ability to detect when they are required and to deploy them when appropriate.

This review considers some of the biochemical mechanisms of detoxification and sequestration that are utilized by generalist and specialist insect herbivores.

3.2 Diverse roles of insect cytochromes P450

Cytochrome P450 enzymes encoded by the CYP gene superfamily comprise a large and ubiquitous class of heme-thiolate proteins, found in all organisms, that collectively catalyze a huge range of reactions, many of which play an important role in detoxification of xenobiotics. The enzyme holds a heme group with an iron atom at its centre and changes in the oxidation state of this iron atom are essential for the reaction cycle, in which one atom of molecular oxygen is transferred to a substrate and the other reduced to water. The degree of completion of this cycle can vary considerably and, depending on the enzyme and substrate, a large variety of reactions can result, including aliphatic or aromatic hydroxylation, epoxidation, dealkylation, oxidative deamination, oxidative desulphuration, *N*-hydroxylation and sulphoxidation.

The membrane-associated P450s require a supply of electrons, which are transferred from NADPH or NADH by membrane-associated redox partners, including NADPH cytochrome P450 reductase. These features pose special challenges for *in vitro* studies of P450 enzyme activity, whether in membrane fractions from the organism of interest or by heterologous expression. The biochemistry and molecular biology of P450s is an enormous subject, and the literature relevant to insects has been extensively reviewed (Feyereisen, 2011, 2012).

P450s play many roles in insects, including synthesis of hormones and pheromones, fatty acid metabolism and detoxification of xenobiotic compounds such as host plant toxins and insecticides. Many of the detoxifying type are inducible by xenobiotics, i.e. transcription rates are normally regulated at a low level but increase in response to the appearance of a foreign or endogenous compound. Although this may function adaptively to detoxify harmful compounds from the environment, the inducers are not necessarily the same molecules as the toxins that need to be eliminated.

The induction process is best understood in mammals, as exemplified by the role of the aryl hydrocarbon receptor (AHR) in the upregulation of CYP1A1 by polycyclic aromatic hydrocarbons such as benzo(α)pyrene. A protein complex containing AHR and molecular chaperones resides in the cytoplasm. On binding to a suitable inducer, AHR forms a heterodimer with the related ARNT (aryl hydrocarbon receptor nuclear translocator) protein, which moves to the nucleus, binds to DNA at specific enhancer sequences (XRE-AhR) near the promoters of CYP1A1 and other genes, and interacts with transcription factors and other coactivators to promote transcription. Homologues of these proteins exist in insects, but their roles in the regulation of insect P450s are poorly understood.

An evolutionary scenario for the evolution of a novel detoxicative function using P450s could operate as follows: The genomes of all insects already contain several P450 genes, with diverse metabolic capabilities. Some may exhibit activity, however slight, against the novel toxin. A regulatory mutation occurs, by addition of an enhancer or removal of a repressor, that constitutively up-regulates this P450 (insertion of a transposable element in the promoter region is one mechanism known to have increased P450 expression in cases of insecticide resistance). Improved survivorship due to partially effective detoxification provides the opportunity for new mutations that increase catalytic efficiency. Gene duplication may occur, further increasing the expression level and providing the opportunity for one of the new copies to diverge in function while the other retains the ancestral role. Regulatory elements are modulated to allow for inducibility and tissue-specific expression. Further selection will occur on catalytic ability to circumvent plant-derived inhibitors of P450s. The result is a novel P450 that rapidly detoxifies a specific toxin on demand, when and where needed – an adaptationist's dream! Remarkably, many aspects of this scenario can be seen in the evolution of resistance to insecticides and host plant-derived toxins.

3.2.1 Furanocoumarin detoxification by *Papilio* spp. and others

Furanocoumarins have a basic structure consisting of a furan ring fused with coumarin. Depending on the orientation of the furan, they are classified as linear (e.g. xanothotoxin) or angular (e.g. angelicin) (Figure 3.1a). Their highly aromatic nature makes them susceptible to photoactivation; when exposed to ultraviolet (UV) light, the linear furanocoumarins cross-link

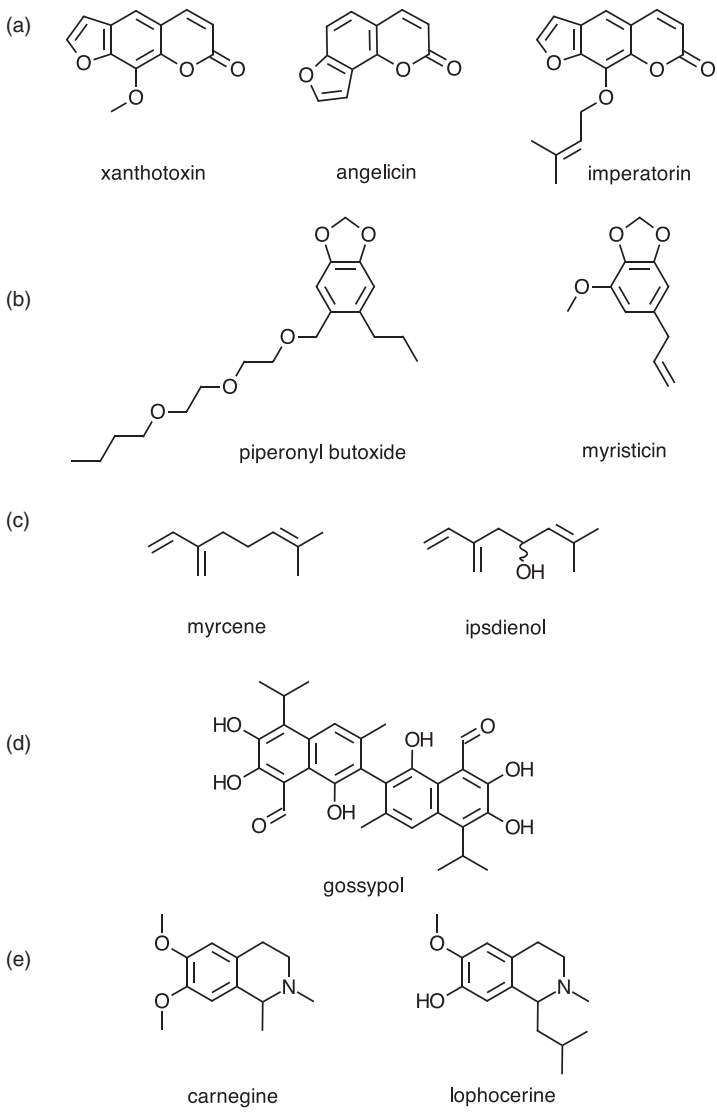


Figure 3.1 Compounds detoxified by P450s in insects. (a) Furanocoumarins: xanthotoxin, angelicin, imperatorin. (b) P450-inhibiting Methylenedioxyphenyl compounds: piperonyl butoxide, myristicin. (c) Myrcene, substrate of CYP9T2, and its product ipsdienol. (d) Gossypol. (e) Cactus alkaloids: carnegine, lophocerine.

nucleic acids, causing mutations in DNA and interfering with transcription. Furanocoumarins are found in a wide range of plants, with the greatest diversity in the Apiaceae.

The gene for CYP6B1, the first described member of the CYP6B subfamily, was isolated from *P. polyxenes*, which specializes on Rutaceae and Apiaceae

and encounters and tolerates concentrations of up to 1% of furanocoumarins in the diet. When expressed with a baculovirus expression system in Sf9 cells, along with housefly P450 reductase, CYP6B1 metabolizes the linear furanocoumarins xanthotoxin and psoralen efficiently, and the angular furanocoumarin angelicin less so (Wen *et al.*, 2003).

Comparative molecular modelling and site-directed mutagenesis identified residues in the substrate recognition sites SRS1 and SRS6 responsible for catalysis and substrate specificity of CYP6B1 for xanthotoxin (Chen *et al.*, 2002). CYP6B3, a paralogous enzyme from the same species, is induced by a wider range of linear and angular furanocoumarins (Hung *et al.*, 1995). CYP6B3 metabolizes xanothotoxin and angelicin less efficiently than CYP6B1 but the furanochromones visnagin and khellin more efficiently (Wen *et al.*, 2006a). Several alleles of each gene were identified and the pattern of sequence variation indicates strong purifying selection for CYP6B1 and somewhat weaker selection for CYP6B3. Thus, gene duplication, followed by subfunctionalization, has enabled *P. polyxenes* to efficiently detoxify the most common furanocoumarin it encounters, while retaining the ability to detoxify other classes of chemicals (e.g. furanochromones).

Homologous P450s from generalist *Papilio* species have also been identified and expressed. CYP6B4, CYP6B17, and CYP6B21 from *P. glaucus*, which occasionally feeds on furanocoumarin-containing plants, and CYP6B25 and CYPB26 from *P. canadensis*, which never does, were compared with CYP6B1 on a range of linear and angular furanocoumarins (Li *et al.*, 2003). CYP6B1 was more efficient in metabolizing linear furanocoumarins than the *P. glaucus* enzymes, which were more efficient than the *P. canadensis* enzymes. In turn, the enzymes from the generalist species have a broader substrate range than CYP6B1. These patterns are in accordance with the encounter frequency of these compounds in the diet of these species.

Sequence comparisons with CYP6B genes of another generalist lepidopteran, *Helicoverpa zea*, enabled reconstruction of the hypothetical sequences ancestral to the CYP6B subfamily, to the *Papilio* branch, and to the *P. glaucus/P. canadensis* sub-branch (Figure 3.2). Inferred substitution patterns along the branches of the phylogenetic tree, along with molecular modelling of the hypothetical sequences, suggested that the ancestor of the *Papilio* branch could efficiently metabolize linear furanocoumarins due to a narrowing of the catalytic pocket, but that this activity was reduced in the generalists' enzymes due to the substitution for other residues at two sites occupied by phenylalanines in the *Papilio* ancestor (Li *et al.*, 2003).

Comparison of CYP6B1 from *P. polyxenes* with CYP6B8 from the broad generalist *H. zea* revealed several interesting contrasts (Li *et al.*, 2004). CYP6B8 metabolized xanthotoxin, but with a 30-fold lower metabolic clearance rate compared to CYP6B1. However, CYP6B8 also metabolized a much wider range of chemically diverse substrates, including quercetin, flavone, chlorogenic acid, indole-3-carbinol and rutin. Molecular modelling of both structures showed that CYP6B8 has a more flexible overall folding, a more

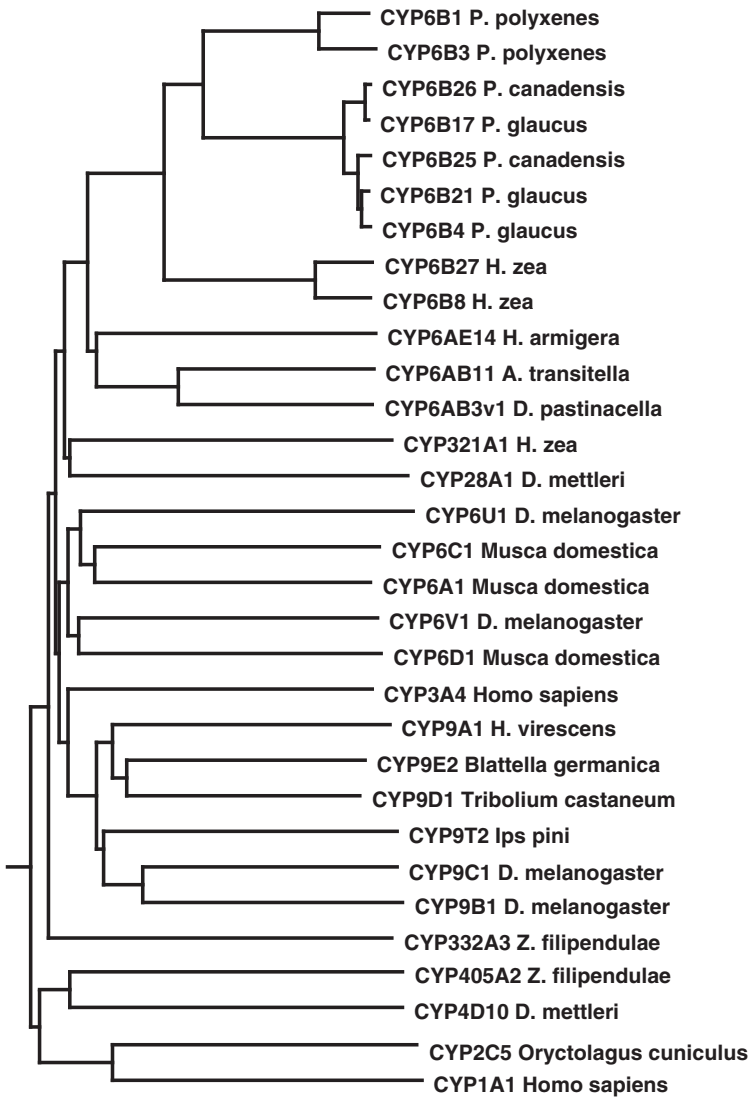


Figure 3.2 Neighbour-joining phylogenetic tree of cytochrome P450 sequences discussed in the text.

elastic catalytic pocket and an additional substrate access channel, compared to CYP6B1. Thus, in this comparison, a trade-off has occurred between efficiency and breadth, which is probably representative of the evolutionary changes in the CYP6B subfamily widely distributed in Lepidoptera.

CYP6B1 in *P. polyxenes* is expressed constitutively at a high level and is further induced by dietary xanthotoxin. Analysis of the promoter region upstream of the coding sequence has revealed DNA sequences called

xenobiotic response elements (XRE) that mediate this expression pattern. By expressing various deletion and substitution constructs of the CYP6B1 promoter using the CAT reporter system in Sf9 cells, an 18-bp region upstream of the transcriptional start site termed XRE-xan that was responsible for both constitutive and inducible expression of the reporter construct was defined (Petersen *et al.*, 2003). This region overlapped with sequences identical to an ecdysone response element and an antioxidant response element characterized from other organisms, and the entire region was termed EcRE/ARE/XRE-xan. Mutations localized to the EcRE decreased expression levels but did not affect inducibility; mutation of a four-base region common to the elements completely abolished basal and inducible expression (Petersen *et al.*, 2003).

An additional element found in the CYP6B1 promoter is similar to the aryl hydrocarbon response elements (XRE-AhR) that regulate the induction of mammalian P450s and other detoxicative genes upon binding to inducers such as benzo[α]pyrene. This compound was also found to induce expression from the CYP6B1 promoter, contributing to the induction by xanthotoxin (Brown *et al.*, 2005). Mutation of the XRE-AhR element decreased the induction by benzo[α]pyrene. Co-expression of *Drosophila* homologues of AhR and ARNT, however, did not produce results similar to mammals (Brown *et al.*, 2005). Whether there are functional analogs to AhR/ARNT in *Papilio* and the identity of the transcription factors mediating the XRE-xan response are unknown.

P. glaucus encounters furanocoumarins in only some of its host plants. In this species, CYP6B4 is not expressed constitutively but is induced up to 300-fold by these compounds in its diet. Sequence analysis of the CYP6B4 promoter has revealed many similarities to CYP6B1; there is a region with 60% identity to the EcRE/ARE/XRE-xan motif, an XRE-AhR sequence, and several separate EcRE and ARE elements (McDonnell *et al.*, 2004).

Deletion analysis of the CYP6B4 promoter driving the CAT reporter gene in Sf9 cells showed that this promoter is also inducible by xanthotoxin, albeit at a lower level than CYP6B1, and that the EcRE/ARE/XRE-xan similar region is necessary for the strongest response, although other upstream and downstream elements are also involved. Similar to CYP6B1, the CYP6B4 promoter is also inducible by benzo[α]pyrene in an additive manner with xanothotoxin, which the authors interpret as evidence for a common receptor for both compounds (McDonnell *et al.*, 2004). Thus, adjustment of both the catalytic efficiency and substrate specificity of the enzyme detoxifying furanocoumarins, as well as the constitutive and inducible levels of its expression, reflects the different encounter rates and selective pressure of this class of toxins on these two *Papilio* species.

Given an efficient detoxification mechanism in a specialist herbivore, one would expect that chemical inhibition of this mechanism would be selected for in the host plant. Several phytochemicals can inhibit P450s; indeed, furanocoumarins can irreversibly inhibit the P450s of mammals and susceptible

insects by being activated by, and subsequently binding to, the enzyme (Neal & Wu, 1994).

CYP6B1 was expressed in Sf9 cells to test the susceptibility of its ability to metabolize xanthotoxin to various inhibitors (Wen *et al.*, 2006b). Xanthotoxin itself did not inhibit CYP6B1 by suicide inactivation. Other furanocoumarins inhibited xanthotoxin metabolism by competing for the active site and being metabolized at a slower rate than xanthotoxin. Angular furanocoumarins, although intrinsically less phototoxic than linear furanocoumarins, due to less severe DNA inactivation, potentiated overall toxicity by occupying the active site of CYP6B1 for longer and, therefore, reducing the rate of xanthotoxin metabolism.

Methylenedioxyphenyl (MDP) compounds, including piperonyl butoxide (PBO), are well-known inhibitors of P450 enzymes (see Figure 3.1b). PBO did not inhibit CYP6B1, but myristicin, which occurs in some host plants of *P. polyxenes*, did do so. PBO did decrease total furanocoumarin metabolism, evidently inhibiting other P450s not yet identified. Thus, the overall effectiveness of P450-mediated detoxification depends in a complex manner on the mixture of compounds present in the food plant.

Other insects regularly encountering furanocoumarins have developed similarly effective detoxification strategies. The parsnip webworm, *Depressaria pastinacella*, feeds on fruits of *Pastinaca* and *Heracleum* species in the Apiaceae, which are defended by concentrations of furanocoumarins up to ten times higher than the leaves consumed by *P. polyxenes*. Overall, P450-catalyzed detoxification activity of furanocoumarins is also correspondingly ten times higher (Berenbaum, 1990). One P450 enzyme from *D. pastinacella*, CYP6AB3v1, is the most specific P450 characterized to date, metabolizing only imperatorin and no other furanocoumarins tested (Mao *et al.*, 2006).

A naturally occurring allelic variant of this enzyme, CYP6AB3v2, differing in five amino acid residues, metabolizes imperatorin up to five times faster, showing that intraspecific variation in the herbivore can significantly affect host adaptation (Mao *et al.*, 2007a). This variant also metabolizes the P450 inhibitor myristicin by putatively converting it to an epoxide, which has a high affinity for the active site and thereby blocks access by other substrates (Mao *et al.*, 2008). CYP6AB11, an enzyme in the same subfamily from the generalist lepidopteran *Amyelois transitella*, which may encounter furanocoumarins in its fig and citrus hosts, is also specialized for imperatorin metabolism, and it slowly turns over PBO as well (Niu *et al.*, 2011).

3.2.2 Monoterpene detoxification and pheromone biosynthesis in pine bark beetles

Adults of pine bark beetles (Coleoptera, Curculionidae: Scolytinae) attack conifer trees by boring through the bark to feed on phloem and to construct interior galleries, where egg-laying and development of the progeny to adulthood occurs. The tree responds by producing a purging flow of

defensive resins containing toxic mono-, sesqui- and diterpenoids. Bark beetles can detoxify host resin monoterpenes by hydroxylating them to alcohols. They also release monoterpene alcohols as aggregation pheromones to recruit enough conspecifics to overcome the tree's defences. It was previously thought that these aggregation pheromones were detoxification products originating exclusively from host defence compounds. Although this may have been a step in their evolution, and although some tree-derived compounds are processed into semiochemicals, the majority of aggregation pheromones seem to be synthesized *de novo* by the beetles (Blomquist *et al.*, 2010).

Myrcene may be present in the beetle due to ingestion, but it is also present due to *de novo* synthesis, even when the beetle feeds on non-myrcene producing trees. CYP9T2 of *Ips pini* catalyzes the hydroxylation of myrcene in the midgut to ipsdienol (Figure 3.1c), which is released as an aggregation pheromone in the faeces. When expressed in Sf9 cells, along with housefly NADH reductase, CYP9T2 produces a mixture of 81% of the R–(–) and 19% of the S–(+) enantiomers of ipsdienol (Sandstrom *et al.*, 2006). However, the pheromone released is 95% R–(–) enantiomer. It is believed that some of the S–(+) ipsdienol is converted to ipsdienone, which is selectively reduced to R–(–) by an ipsdienol dehydrogenase (Blomquist *et al.*, 2010).

3.2.3 Gossypol and CYP6AE14 in *Helicoverpa armigera*

The polyphenolic binaphthyl dialdehyde compound gossypol (Figure 3.1d) is encountered by *Helicoverpa armigera* feeding on cotton, one of more than 150 food plants used by this highly polyphagous species. Expression of the P450 enzyme CYP6AE14 is upregulated in the midgut upon ingestion of gossypol (Mao *et al.*, 2007b), along with several other P450s, carboxylesterases, and UDP glycosyltransferases (Celorio-Mancera *et al.*, 2011; and see Chapter 6). Although direct evidence that CYP6AE14 detoxifies gossypol is lacking, larvae feeding on artificial diet or non-cotton plant material supplemented with gossypol have decreased growth and increased mortality when CYP6AE14 expression is suppressed by feeding double-stranded RNA (dsRNA) targeting the gene. Transgenic *Arabidopsis thaliana* leaves painted with gossypol retarded *H. armigera* growth more when expressing a dsRNA construct targeting CYP6AE14 than when expressing a control dsRNA construct directed against the green fluorescent protein (Mao *et al.*, 2007b). Cotton itself has been transformed with the CYP6AE14 dsRNA construct, which stunts growth and reduces boll damage by *H. armigera* larvae feeding on it (Mao *et al.*, 2011).

3.2.4 Cactophilic *Drosophila* and alkaloid detoxification

Some species of *Drosophila* in the Sonoran desert utilize rotting cactus as an oviposition substrate and larval food source. P450s have been implicated in the detoxification of cactus secondary compounds, such as the

tetrahydroisoquinoline alkaloid carnegine found in saguaro and lophocerine in senita (Figure 3.1e). Microsomes of *Drosophila mettleri* larvae metabolize carnegine, and this is further stimulated by phenobarbital, an inducer of many P450s (Frank *et al.*, 1997). Several P450 genes were isolated from *D. mettleri*; CYP28A1 was found to be induced by senita cactus tissue (Danielson *et al.*, 1997), and CYP4D10 by saguaro cactus tissue supplemented with saguaro alkaloids (Danielson *et al.*, 1998). A population genetic study of these two genes in *D. mettleri* found evidence of positive selection for CYP28A1 but not CYP4D10; there was significant genetic differentiation in the CYP28A1 genes in populations collected from saguaro vs. senita in one locality (Bono *et al.*, 2008). Direct evidence of the ability of these or other *Drosophila* P450s to detoxify cactus alkaloids is still lacking.

3.3 Cyanogenic glucosides

Cyanogenic glucosides are β-glucosides of α-hydroxynitriles derived from amino acids. They are stored in the vacuoles of plant cells and, when disrupted by herbivory or other mechanical damage, they come into contact with plant cellular β-glucosidases, which cleave off the glucose, yielding an α-hydroxynitrile. This is further decomposed into an aldehyde or ketone and hydrogen cyanide by plant α-hydroxynitrilases (Figure 3.3a). Cyanide is highly toxic, because it binds to the iron of cytochrome c oxidase in the mitochondrial respiratory pathway of eukaryotes, blocking the aerobic production of ATP. Thus, cyanogenic glucosides and the enzymes that activate them make up a binary antiherbivory defence system that is immediately activated when needed. The herbivore's own digestive β-glucosidases may also be sufficient to activate them, as the decomposition of the α-hydroxynitrile occurs spontaneously in the alkaline conditions present in the midgut of most lepidopteran larvae. Sub-lethal concentrations of both breakdown products may also act as feeding deterrents due to their bitter taste.

Cyanogenic glucosides have been found in more than 2,650 plant species in 130 different families, including ferns, gymnosperms and angiosperms, suggesting that the ability to synthesize them evolved at least 300 million years ago and that their role in plant-herbivore interactions is quite ancient (Bak *et al.*, 2006). Valine, leucine, isoleucine, phenylalanine, tyrosine, or the nonprotein amino acid 2-(2'cyclopentenyl) glycine may serve as precursors.

The first committed step in their biosynthesis is the conversion of the amino acid to an aldoxime. In flowering plants, this is catalyzed by a single cytochrome, P450, in the CYP79 family. This remarkable multifunctional enzyme performs two successive *N*-hydroxylations, a dehydration, a decarboxylation and isomerization to produce the *Z*-aldoxime. Phylogenetic analysis of CYP79 sequences from plants shows that the family predates the divergence of monocots and dicots (Bak *et al.*, 2006), suggesting that the common ancestor was likely cyanogenic, and that noncyanogenic flowering plants

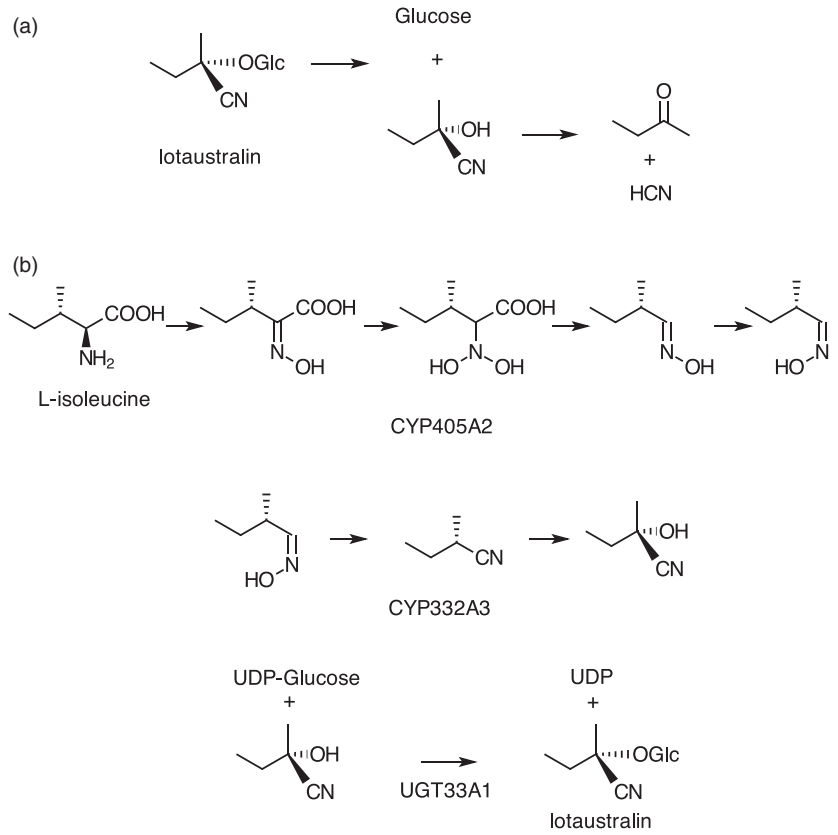


Figure 3.3 Cyanogenic glucosides. (a) Activation of lotaustralin. (b) Biosynthesis of lotaustralin in *Zygaena filipendulae*.

have lost the trait secondarily. In addition to defence against herbivores, cyanogenic glucosides such as dhurrin may also serve as storage reservoirs for reduced nitrogen, as a pathway for recovering the nitrogen from dhurrin without releasing cyanide has been found in sorghum (Jenrich *et al.*, 2007).

Cyanogenic glucosides are also found in millipedes and centipedes, and within the insects Lepidoptera, Coleoptera, and Hemiptera, which synthesize and/or sequester them for their own defence against predators. Millipedes and centipedes evolved before the first appearance of cyanogenic glucosides in plants and are not dependent on sequestration. Some insects feeding on cyanogens can sequester them, while others produce them even when feeding on noncyanogenic plants, or produce a different spectrum of compounds as found in their cyanogenic hosts, demonstrating the ability to synthesize them *de novo*.

It has been suggested that Lepidoptera evolved the ability to synthesize aliphatic cyanogenic glucosides before plants did and, having already

developed efficient mechanisms for their transport and detoxification, they secondarily evolved the ability to sequester them from plants in order to save the energy required for their biosynthesis (Zagrobelny *et al.*, 2008). Species of the *sara-sapho* clade of the tropical butterfly *Heliconius* exhibit an inverse correlation between sequestration and *de novo* synthesis; species that synthesized less of their own aliphatic cyanogens sequestered more simple monoglycoside cyclopentenyl (SMC) cyanogens from *Passiflora* host plants (Engler-Chaouat & Gilbert, 2007).

Insects possess two widespread biochemical mechanisms for detoxification of hydrogen cyanide. The enzyme rhodanese (thiosulphate: cyanide sulphurtransferase) converts hydrogen cyanide into thiocyanate by the transfer of a sulphur atom from thiosulphate. Rhodanese activity is common in insects, but it is not restricted to those consuming cyanogenic plants and may be primarily responsible for other functions (Beesley *et al.*, 1985), including donating sulphur to create iron-sulphur clusters used as prosthetic group in a variety of metalloproteins (Pagani *et al.*, 1984).

The enzyme β-cyanoalanine synthase combines cyanide with cysteine or serine to form β-cyanoalanine. The enzymatic activity and/or the product have been detected in a wide spectrum of Lepidoptera, including those that do not contain cyanogenic glucosides and therefore utilize it mainly for detoxification of host plant compounds. The crucifer specialist *Pieris rapae* was found to be very tolerant of cyanide, as growth and development were unaffected by feeding on transgenic *Arabidopsis thaliana* plants engineered to produce high levels of the cyanogenic glucoside dhurrin (Stauber *et al.*, 2012). Labelled cyanide was incorporated into both β-cyanoalanine and thiocyanate, and both β-cyanoalanine synthase and rhodanese activities were detected in gut tissue. As well as conferring protection against cyanogenic plants, these enzyme activities may also be useful in coping with plants producing benzylglucosinolates, as cyanide is produced as a result of their detoxification (see below).

Heliconius sara sequesters the SMC compound epivolkenin from its host plant *Passiflora auriculata* and converts some of it to sarauriculatin, which retains the glucose moiety but has the nitrile group substituted by a thiol (Engler *et al.*, 2000). The nature of the biochemical reaction and the fate of the nitrile are unknown, but this might represent a novel mechanism for obtaining some nitrogen from the host plant while avoiding the release of cyanide.

Among Lepidoptera that sequester cyanogenic glucosides, the best studied are in the genus *Zygaena*. Larvae of the burnet moth *Z. filipendulae* feed on *Lotus corniculatus* and sequester linamarin and lotaustralin, preferring plants with higher concentrations of these cyanogenic glucosides (Zagrobelny & Møller, 2011). These are stored in so-called cuticular cavities and released in the form of viscous droplets when the larva contracts its body segments on being confronted by a predator. The ratio of these two compounds is regulated by the larvae over a narrower range than present in the host plant, by a combination of *de novo* synthesis of the rarer component and turnover

of the more common one. The total content drops after pupation, and it has been suggested that some of the HCN produced during brief periods during pupation is trapped in β-cyanoalanine and channelled into chitin synthesis (Zagrobelny and Møller, 2011). Adults use the cyanogenic glucosides for their own protection and females provision their eggs with them. They are transferred from the male to the female during mating, and females prefer males with higher concentrations.

Zygaena species are biochemically well equipped for the activation, detoxification, and synthesis of cyanogenic glucosides. Larvae harbour a β-glucosidase in the hemolymph with high activity against linamarin and lotaustralin (Zagrobelny *et al.*, 2004). The hemolymph also contains an α-hydroxylnitrilase. Several tissues have β-cyanoalanine synthase activity, but rhodanese activity has not been found.

The enzymes responsible for biosynthesis have recently been identified (Jensen *et al.*, 2011), and they perform the same reactions that occur in plants. Starting with valine or isoleucine, a single P450 enzyme, CYP405A2, catalyzes the same five transformations as performed by CYP79A1 in plants. CYP332A3 then accomplishes both the dehydration and C-hydroxylation steps, as done by CYP71E1 in plants. Finally, the UDP-glycosyltransferase (UGT) UGT33A1 transfers the glucose (Figure 3.3b).

These *Zygaena* P450s are phylogenetically related to other insect P450s and not those of plants, and likewise for the *Zygaena* UGT (Ahn *et al.*, 2012), establishing that the entire biosynthetic pathway for synthesis of cyanogenic glucosides has developed independently in insects and plants – a remarkable example of convergent evolution (Jensen *et al.*, 2011).

3.4 Glucosinolates

The glucosinolate-myrosinase system is another key innovation defending the plant from biotic agents that disrupt the integrity of its cells. The first component comprises glucosinolates, which are β-thioglucoside *N*-hydroxysulphates with a side chain originating from one of eight amino acids. This side chain forms the basis of classification according to chemical structure, and more than 120 different variants are known.

The initial steps in modifying the amino acid are *N*-hydroxylation and decarboxylation to yield an aldoxime. As these are the same as the initial reactions in the biosynthesis of cyanogenic glucosides, and the enzymes responsible belong to the same CYP79 family, it has been suggested that plant defence by glucosinolates evolved from cyanogenic ancestors (Halkier & Gershenzon, 2006). Glucosinolates have been found in 16 different plant families, the best studied by far being the Brassicaceae (Fahey *et al.*, 2001). Each plant species synthesizes a unique combination, which is stored in specialized cells near the phloem.

The second component of the system is the enzyme myrosinase, a thioglucosidase which is stored in vacuoles of different, non-glucosinolate-containing cells. When cell walls are broken by chewing herbivores or other mechanical damage, the two components come into contact and the myrosinase hydrolyzes the thioglucosidic bond, releasing the glucose. The resulting aglycones are unstable and spontaneously undergo a Lossen rearrangement to form highly toxic isothiocyanates, a process called the 'mustard-oil bomb' (see Chapter 7). Depending on the nature of the glucosinolate and the presence of additional proteins from the plant, alternative, less toxic breakdown products can also be formed: nitriles, epithionitriles or thiocyanates (Figure 3.4).

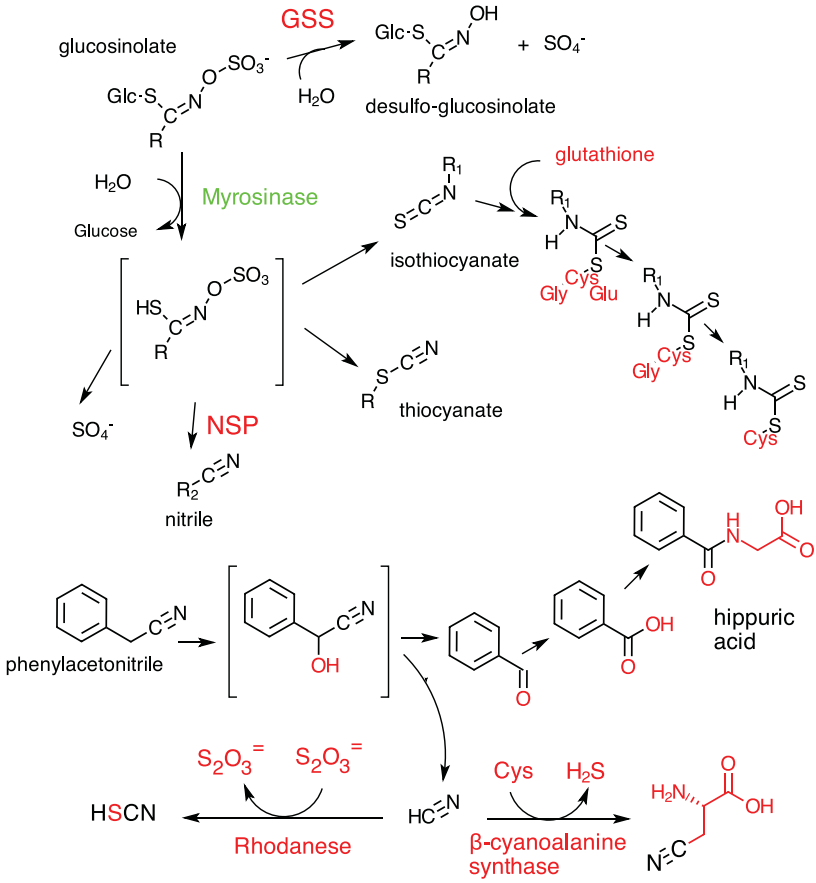


Figure 3.4 Glucosinolates: Activation and detoxification pathways in insects, and metabolism of phenylacetonitrile in *Pieris rapae*. Enzymes and compounds in red are provided by the insect – Glucosinolate sulphatase by *Plutella xylostella*, Nitrile-specifying protein by *Pieris* spp. (*For colour details please see colour plate section.*)

Despite this potent activated defence, several insect groups feed on Brassicaceae and have developed various ways to deal with the mustard-oil bomb. Specialists defuse it or perform the equivalent of a controlled detonation; generalists attempt to mitigate the effects of the explosion. A recent comprehensive review (Winde & Wittstock, 2011) describes many of these solutions.

The first to be elucidated at the molecular level was the counterstrategy of the diamondback moth *Plutella xylostella*, the most significant insect pest of crucifer crops worldwide. Larvae of this species secrete large amounts of a highly active glucosinolate sulphatase (GSS) into the lumen of the midgut, where it cleaves off the sulphate group of intact glucosinolates (Ratzka *et al.*, 2002; see Figure 3.4). The resulting desulphoglucosinolate cannot be activated by the myrosinase and is excreted in the faeces. As ingested plant material enters the gut, there is competition between the ingested myrosinase and the GSS for access to the intact glucosinolates, and the high abundance and activity of the GSS are sufficient to 'defuse' most of them, regardless of chemical type. Thus, the impressive chemical diversity of glucosinolates in Brassicaceae is all for naught, as the diamondback moth can overcome all of them. This remarkably effective counterstrategy resulted from a gene duplication of a pre-existing intracellular arylsulphatase widespread in insects, followed by sequence divergence and acquisition of a signal peptide, targeting the protein for secretion into the midgut lumen (Ratzka *et al.*, 2002). As the diamondback moth rarely feeds on non-cruciferous plants, the GSS is expressed constitutively in the larval midgut at a high level.

In contrast, inducible glucosinolate sulphatase activity is found in the desert locust *Schistocerca gregaria*, which only occasionally feeds on Brassicaceae (Falk & Gershenzon, 2007).

The alternative 'controlled detonation' strategy is employed by another crucifer specialist, larvae of the cabbage butterfly genus *Pieris*. The midgut lumen of larvae contains large amounts of another secreted protein, called the nitrile-specifying protein (NSP). This interacts with the unstable aglycones produced by the myrosinase, converting them to nitriles instead of isothiocyanates (Wittstock *et al.*, 2004; see Figure 3.4). Most of these less toxic nitriles are then excreted in the faeces.

NSP possesses three similar binding domains and is completely unrelated to plant-derived proteins (such as the epithiospecifier protein of *A. thaliana*) that also favour production of nitriles. It is evolutionarily derived instead by two rounds of domain duplication from a secreted single-domain protein (SDMA) of unknown function, found in the midguts of all insects examined (Fischer *et al.*, 2008). A further gene duplication in the pierid lineage produced NSP and another three-domain protein (MA) that lacks nitrile-specifying activity. A mosquito SDMA is induced by a blood meal (Shao *et al.*, 2005) and another SDMA-domain-duplicated protein in cockroaches is induced by feeding (Gore & Schal, 2005); thus, the existence of these proteins in the midgut was a pre-adaptation for the evolution of NSP.

Based on a phylogeny of Brassica-feeding pierids and their legume-feeding ancestors, the estimated appearance of NSP activity is about 80 million years ago, about ten million years after the estimated appearance of the glucosinolate-myrosinase system in the Brassicales (Wheat *et al.*, 2007). Derived pierid lineages that have shifted from Brassicaceae to other plant families have lost NSP activity. The evolutionary innovation of NSP appears to have facilitated species diversification among brassica-feeding pierids, providing an example of the co-evolutionary process hypothesized by Ehrlich & Raven (1964) (see Chapter 11).

Although *Pieris rapae* excretes aliphatic nitriles in the faeces, most of the phenylacetonitrile derived from the aromatic benzylglucosinolate is further metabolized to hippuric acid by a mechanism that releases HCN, thus converting the mustard oil bomb into a 'cyanide bomb' (Stauber *et al.*, 2012; see Figure 3.4). This cyanide is efficiently detoxified by the larvae, as mentioned above. The authors suggest that, paralleling the evolution of cyanogenic and glucosinolate defences in the plants, the glucosinolate-detoxifying pierid lineage evolved from an ancestor well adapted to detoxify its cyanogenic host plant, and it retained this ability because of the continued need to detoxify cyanide resulting from one of the by-products of its key adaptation NSP (Stauber *et al.*, 2012).

Some generalist herbivores have mechanisms for disposing of the isothiocyanates once they are formed, as shown by experiments where ^{14}C-labelled 4- methylsulphinylbutyl glucosinolate was taken up by detached *A. thaliana* leaves, which were then fed to insects (Schramm *et al.*, 2012). Of the total labelled compound ingested, larvae of the generalist *Spodoptera littoralis* excreted 81% in the faeces, 60% in the form of the corresponding isothiocyanate and 10% of the corresponding nitrile. The remaining 11% was in the form of glutathione (CysGlyGlu-) conjugates or CysGly- and Cys- conjugates formed by stepwise cleavage of glutamate and glycine from the attached glutathione (Figure 3.4). These conjugates were also found in the faeces of four other generalists consuming a variety of different glucosinolates (Schramm *et al.*, 2012).

Larvae of the sawfly genus *Athalia* feed on glucosinolate-containing plants and sequester high concentrations of intact glucosinolates in their hemolymph (Opitz & Mueller, 2009). When these sawflies are attacked by a predator, the integument is easily broken in places and droplets of hemolymph appear ('reflex bleeding'). The glucosinolates may deter the predator from further consuming the prey. As sawfly larvae are chewing herbivores, host plant myrosinases would be expected to cleave the glucosinolates, but the mechanism by which the larvae circumvent this is unknown.

Intact glucosinolates are rapidly taken up from the midgut; if larvae are transferred to a plant with a different mixture of aromatic and aliphatic glucosinolates, this new mixture replaces the old one in the hemolymph within 24 hours. When larvae are fed radiolabelled glucosinolates, 80% of the label is excreted in the faeces within 24 hours, indicating a high turnover rate of

sequestered glucosinolates (Müller & Wittstock, 2005). Some of the ingested benzylglucosinolate is desulphated (from the *N*-hydroxysulphate group) and resulphated (onto the glucose) before being excreted. Sulphatation of the glucose of the intact glucosinolate was also observed, and this was suggested to block cleavage by the myrosinase (Opitz *et al.*, 2011).

Plants transport intact glucosinolates in their phloem, and some sucking insects are able to take this up without disrupting the cells containing the myrosinase. The harlequin bug *Murgantia histrionica* accumulates a glucosinolate profile in its tissues that mirrors that of the host plant, but with somewhat less turnover than *Athalia*; six days after shifting to a different plant, glucosinolates from the first plant can still be detected (Aliabadi *et al.*, 2002).

The specialist cabbage aphid *Brevicoryne brassicae* takes up allylglucosinolate from black mustard; some is excreted in the honeydew and some stored in the hemolymph (Kazana *et al.*, 2007). Remarkably, this aphid also makes its own myrosinase, which is stored in intramuscular crystalline inclusions (Bridges *et al.*, 2002). When an aphid is masticated by a ladybird beetle, allylisothiocyanate is released – hence the name 'walking mustard oil bomb' (Kazana *et al.*, 2007). This attack leads to the death of the aphid, but this sacrifice may deter further predation on her parthenogenetically-produced, and thus genetically identical, aphid sisters on the same plant.

3.5 *O*-glucosides and leaf beetles

Leaf beetles in the subtribe Chrysomelina synthesize and release irritating volatile compounds as an anti-predator defence strategy. Larvae possess a series of nine pairs of dorsal glands that synthesize and dispense a wide array of these compounds, which arise from *de novo* synthesis, sequestration from the host plant and subsequent modification, or both.

Phylogenetic analysis based on mitochondrial DNA sequences has been performed on the Chrysomelina for comparison with defensive chemistry and host plant associations (Termonia *et al.*, 2001). According to this analysis, *de novo* synthesis of iridoid monoterpenes is the ancestral state. Species feeding on hosts in the Salicaceae, Betulaceae, Brassicaceae, Polygonaceae, or Sterculiaceae synthesize these iridoids without depending on precursors sequestered from the host.

A shift to sequestration of the glucoside salicin from hosts in the Salicaceae to produce salicylaldehyde has been derived independently twice, once producing a large offshoot clade and once more recently producing the isolated instance of *Phratora vitellinae* within the clade of iridoid producers. Many of these species, including the latter, rely almost exclusively on salicylaldehyde in the defensive glands. Within the larger derived clade sits the even more recently derived *interrupta* group, pursuing a mixed strategy of using host-derived salicylaldehyde and, in addition, synthesizing a mixture of butyric acids *de novo*, which are esterified with the alcohols from plant-derived

alcohol glucosides. Some species in the *interrupta* group have shifted to feeding on Betulaceae lacking salicin; these therefore rely exclusively on this mixture of butyric esters. Within the *interrupta* group, *Chrysomela lapponica* is unique in having some populations that feed on willow and others that feed on birch (Termonia *et al.*, 2001).

The shift to production of salicylaldehyde offered the advantage of exploiting a pre-synthesized precursor from the host plant and avoiding the energetic demands of *de novo* iridoid synthesis. Salicin is taken up from the plant and is transported to the lumen of the defensive gland, where a β-glucosidase cleaves off the glucose, yielding salicyl alcohol. The final oxidation to salicylaldehyde is performed in the lumen by an abundant salicyl alcohol oxidase (SAO) (Figure 3.5a). The gene for this enzyme has been cloned from *Chrysomela tremulae* and *C. populi* and heterologously expressed (Michalski *et al.*, 2008). It is a member of the large class of GMC (glucose-methanol-choline) oxidoreductases, which use FAD (flavin adenine dinucleotide) as a prosthetic group, along with molecular oxygen. This class has a very wide range of substrates, but the SAO from the two *Chrysomela* species is highly specific for salicyl alcohol (Michalski *et al.*, 2008).

To examine the selectivity of uptake and transport of different substrates from the midgut to the secretory glands, beetle larvae were fed leaves painted with thioglucosides. These are *S*-mimics of the *O*-glucosides, similar enough to be acted on by the same transport systems but resistant to cleavage by β-glucosidases. They can thus can reach their destination intact, where they can be detected and quantified.

For some species, the *S*-mimic selectively imported in the experiment corresponded to the naturally imported *O*-glucoside precursor of the predominant defensive compound in the gland. The salicylaldehyde-secreting *Chrysomela populi* and *Phratora vitellinae* preferentially imported the *S*-mimic of the precursor salicin, and *Phratora laticollis* and other iridoid-secreting species preferentially imported the *S*-mimic of glucosylated 8-hydroxygeraniol, an early intermediate in iridoid metabolism (Kuhn *et al.*, 2004).

Further experiments, in which the *S*-mimics were incubated with midgut tissue or injected into the hemocoel, indicated nonspecific uptake by the midgut, and specific uptake by the defensive glands (Discher *et al.*, 2009). The hemolymph is not a storage depot because the thioglucosides were rapidly cleared from it. When the aglycone 8-hydroxygeraniol was fed or injected, several species glucosylated it for transport in the hemolymph. Thioglucosides which were not taken up by the defensive glands were excreted in the faeces (Discher *et al.*, 2009). Thus, uptake, transport and excretion pathways have co-adapted to enable the different strategies of chemical defence (Figure 3.6).

Chrysomela lapponica was far less selective in uptake, however, and several different *S*-mimics fed to the larvae could be imported into the glands as efficiently as *S*-salicin. This applied both for populations feeding on willow that primarily secrete salicylaldehyde (Kuhn *et al.*, 2007) and for populations

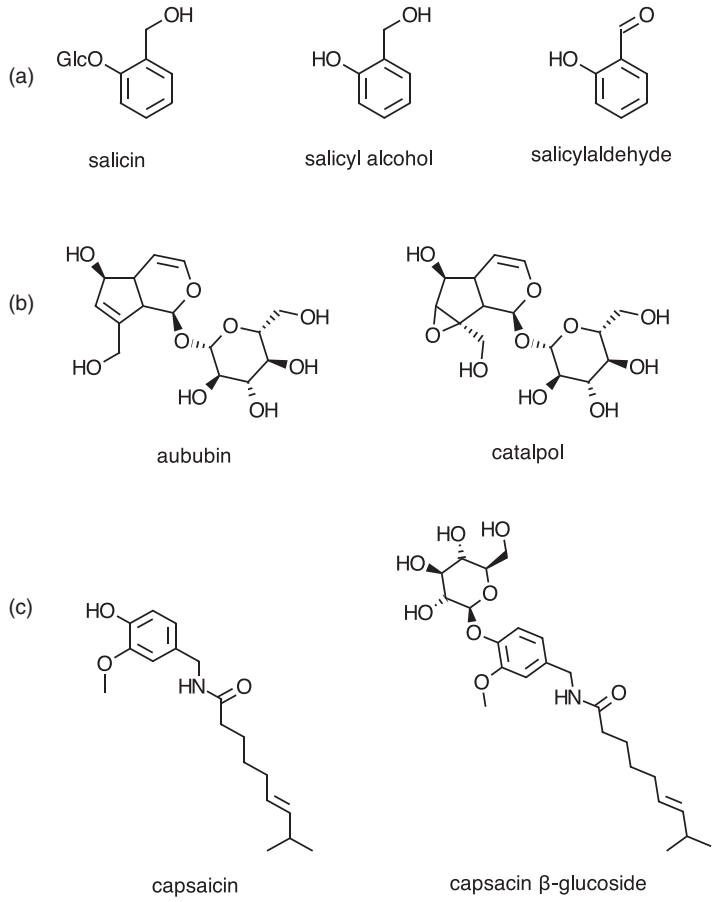
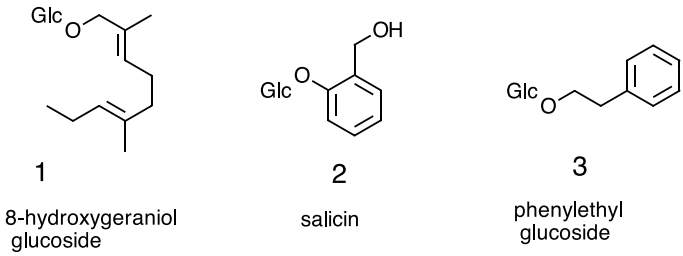


Figure 3.5 O-glucosides, products and precursors. (a) Salicin, salicyl alcohol, salicylaldehyde. (b) Iridoid glucosides: aububin, catalpol. (c) Capsaicin, capsaicin glucoside.

feeding on birch that primarily secrete butyrate esters (Tolzin-Banasch *et al.*, 2011).

This versatile species has further adapted to the use of multiple hosts, via a polymorphism in the SAO gene. Willow-feeding populations have a functional SAO enzyme similar in sequence and expression in the defensive gland to that of *C. tremulae* and *C. populae*. However, birch-feeding populations have a non-functional version of the same gene, due to a deletion resulting in alternate splicing (Kirsch *et al.*, 2011a). The non-functional transcript is still expressed in the defensive gland but at a much reduced level. Thus, *C. lapponica* represents a polymorphic, intermediate stage in the shift of the *interrupta* group from willow to birch, retaining the ability to import precursors of both salicylaldehyde and butyrate esters, which it deploys according to host



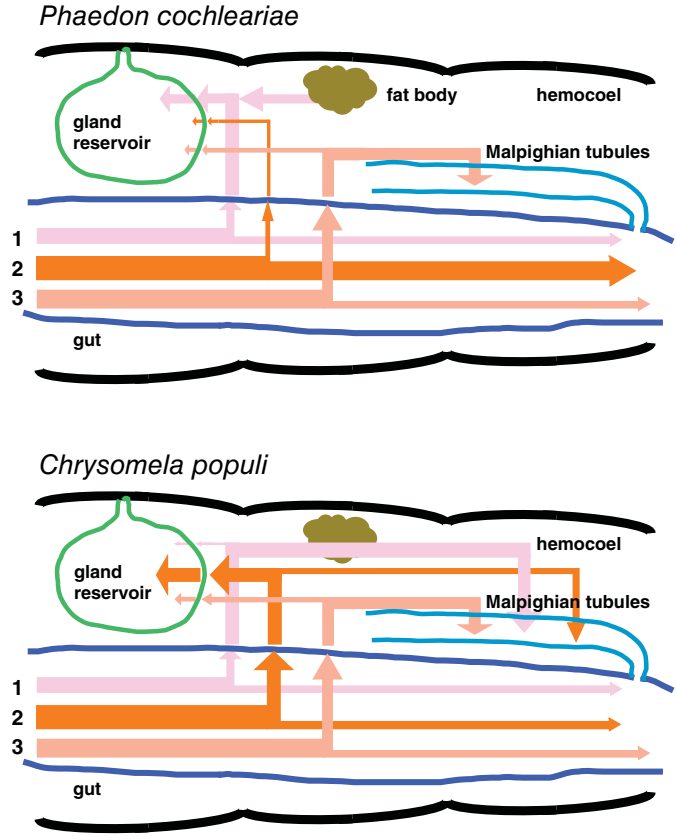


Figure 3.6 Transport and sequestration pathways in two species of leaf beetles. (*For colour details please see colour plate section.*)

plant, and reducing the cost of producing an unnecessary enzyme for salicylaldehyde production in populations feeding on birch, where no salicin is available.

Among the clade of beetles primarily producing iridoids, *Phratora vitellinae* stands out as the only one that has shifted to using predominantly salicylaldehyde in its defensive glands (Termonia *et al.*, 2001). *P. vitellinae* possesses

large amounts of a functional SAO in its defensive gland, encoded by a gene that is very similar in sequence and intron-exon structure to the previously-characterized SAOs, demonstrating evolutionary convergence on the gene level (Kirsch *et al.*, 2011b). Since an oxidase is also required for oxidation of 8-hydroxygeraniol in the production of iridoids, it was proposed that SAO evolved from this reductase by a change in substrate specificity (Pasteels *et al.*, 1990). Sequencing of GMC-oxidase genes from other species in the phylogeny showed, however, that SAO-like genes could be found in *Phratora laticollis* and *Phaedon cochleariae*, both iridoid-producing species (Kirsch *et al.*, 2011b). Thus, instead of parallel evolution by a switch in function of an oxidase in the iridoid pathway to a salicylaldehyde-producing one, the parallel evolution may be explained by recruitment of a gene from this pre-existing gene family, which has other, probably non-defensive functions in iridoid producers. The cloning of the oxidase involved in iridoid synthesis will be required to resolve this issue.

3.6 Pyrrolizidine alkaloids

Pyrrolizidine alkaloids (PA) are commonly found in Apocynaceae, Asteraceae, Boraginaceae, Fabaceae, Orchidaceae and, occasionally, in several other plant families. These disjunct occurrences are believed to be due to convergent evolution. Homospermidine synthase, which catalyzes the first step in PA synthesis, appears to have been recruited independently at least four times from deoxyhypusine synthase which occurs universally in eukaryotes (Reimann *et al.*, 2004).

Senecionine *N*-oxide (Figure 3.7a) from *Senecio* species in the Asteraceae is an example of the more than 350 different types of PA known (Hartmann & Witte, 1995). The hydrophilic *N*-oxide is itself non-toxic but, in the gut of herbivores, it is reduced to senecionine (Figure 3.7a), which is lipophilic. Senecionine penetrates cell membranes and is activated by the herbivore's cytochromes P450 to dehydrosenecionine, an unstable dehydropyrrolizidine ester which forms a carbenium ion. This, in turn, forms adducts with DNA and proteins. Thus, two separate transformations in the herbivore are required to activate the compound to its toxic form.

Insects have evolved several ways of dealing with PAs (reviewed in Langel & Ober, 2011). The generalist lepidopteran *Spodoptera littoralis* rapidly excretes the reduced PA from its cells before it can be bioactivated by P450s and damage cellular targets. The specialist chrysomelid beetle genus *Platyphora* efficiently clears the reduced PA from its hemolymph and accumulates it in defensive glands. Another chrysomelid genus, *Oreina*, somehow suppresses reduction of the *N*-oxides in the midgut and actively transports the *N*-oxides into the hemolymph and into defensive glands; any reduced PA that escapes this sequestering mechanism are glycosylated in the hemolymph. An African grasshopper, two danaid butterflies, and several arctiid moths have

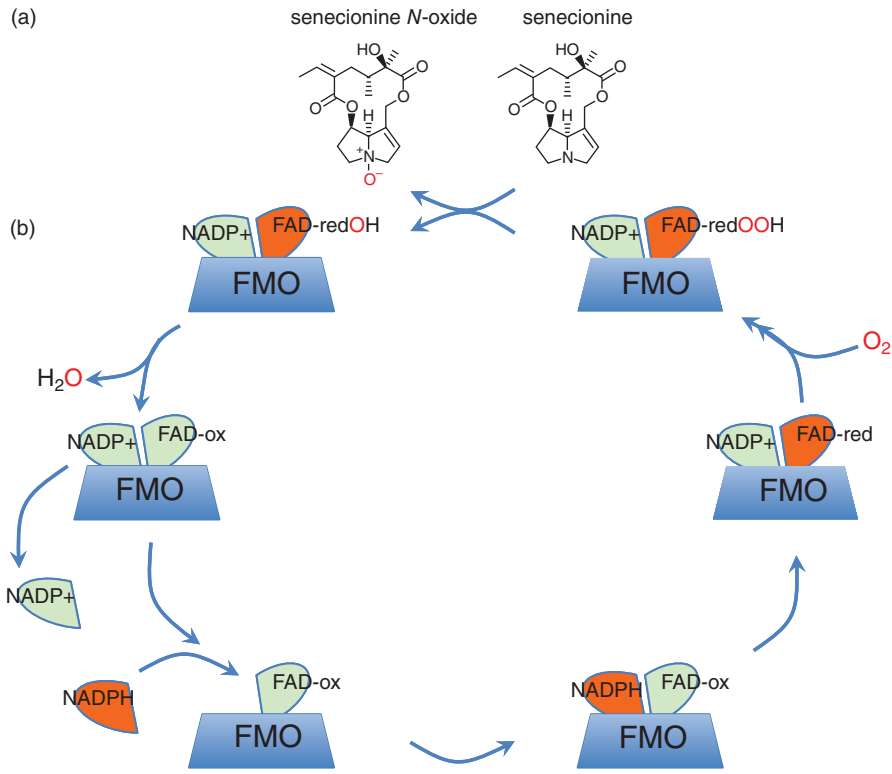


Figure 3.7 Pyrrolizidine alkaloids. (a) Senecionine, senecionine N-oxide. (b) FMO redox cycle.

been found to re-oxidize the reduced PA back into the nontoxic *N*-oxide. This latter strategy has been most thoroughly studied in the specialist arctiid moth *Tyria jacobaeae*, which acquires PAs from its host plant, the tansy ragwort, and sequesters the *N*-oxide as both larva and adult.

The enzyme used by *T. jacobaeae* to re-oxidize senecionine is called senecionine *N*-oxidase (SNO). SNO occurs in the hemolymph of the insect and is highly substrate-specific, *N*-oxidizing only PAs containing moieties that are bioactivated by P450s to the toxic form (Lindigkeit *et al.*, 1997). SNO was found to be a novel flavin-dependent monooxygenase (FMO) that has been recruited for this detoxicative function (Naumann *et al.*, 2002).

FMOs are active in oxidation of nucleophilic heteroatoms with the aid of a FAD prosthetic group and NADPH as cofactor (Figure 3.7b). Phylogenetic sequence analysis has revealed three major groups in Lepidoptera, FMO1, FMO2, and FMO3, the functions of which are unknown (Sehlmeyer *et al.*, 2010. Note: these are not orthologous to the mammalian enzymes of the same name.) The FMO1 clade is split in arctiids, each of which has one gene similar

to FMO1 of other lepidopterans and one gene in a cluster along with SNO. These derivative clusters have been termed PNOs – pyrrolizidine-alkaloid-*N*-oxygenizing enzymes (Sehlmeyer *et al*., 2010). Heterologous expression of the PNO from the generalist arctiid *Grammia geneura* shows that it also acts as an *N*-oxidase upon PAs, but with wider substrate specificity than SNO of the specialist *T. jacobaeae* (Sehlmeyer *et al*., 2010).

Unlike most FMOs, which are intracellular, the PNOs possess a signal peptide and are secreted into the hemolymph, enabling the maintenance of high concentrations of the non-toxic *N*-oxides there. Continued oxidation would require a source of NAPDH, which does not cross the cell membrane and is normally not found in the hemolymph. In the case of *Bombyx mori*, which possesses a hemolymph reductase catalyzing the formation of the hormone ecdysone, it has been suggested that an $NADP^+$-linked polyol phosphate dehydrogenase acting on sorbitol-6-phosphate may generate the necessary NADPH (Nomura *et al*., 1996).

Phylogenetic analysis (Sehlmeyer *et al*., 2010) suggests a single origin of PNOs in arctiids by gene duplication from FMO1. This 'key innovation' was probably the first evolutionary step enabling the detoxification and then sequestration of PAs for the insect's own defence in this group. This sequestration takes a variety of forms, including carrying over PAs to the adult stage after metamorphosis, with some species even incorporating PAs into sex pheromones and transferring PAs from the male to the female during mating for incorporation into, and protection of, the eggs (Hartmann & Witte, 1995; Eisner & Meinwald, 1995).

Recently, an independent recruitment of FMOs for the same purpose was discovered in the highly polyphagous grasshopper *Zonocerus variegatus*, which encounters pyrrolizidine alkaloids in its diet (Wang *et al*., 2012). Three distinct FMO genes were found, encoding intracellular enzymes expressed in the fat body. One of these showed high *N*-oxidizing activity towards senecionine and several other types of pyrrolizidines, and was named ZvPNO. The other two could also oxygenize pyrrolizidines, but with 400-fold lower specific activity. This provides another example of convergent evolution, in which one member of a gene family resulting from gene duplication has evolved optimized activity for detoxification of pyrrolizidine alkaloids (Wang *et al*., 2012).

3.7 Glycosylation of host plant compounds

As shown by the many examples of glycosylated chemical defences in plants, sugar residues attached to a molecule affect its properties by diminishing the reactivity of functional groups and by increasing solubility. Likewise, one way of combating a toxic aglycone is to attach a sugar to it. There is a large gene family encoding proteins specialized in this function, the UDP-glycosyl transferases (UGTs) (Ahn *et al*., 2012). After a monosaccharide is activated

by attachment to uridine diphosphate, UGTs catalyze the conjugation of the sugar donated by the UDP-glycoside to small lipophilic molecules. UGTs have been extensively studied in plants, where they participate in the synthesis of some of the glucosides already discussed, but their role in insects is less well known. UGT33A1 has already been mentioned as catalyzing the last step in the biosynthesis of cyanogenic glucosides in the burnet moth (Jensen *et al.*, 2011). Larval tissues of the tobacco hornworm *Manduca sexta* were shown to use UDP-glucose for glucosylation of several plant phenolics, including phenolic acids, phenolic aldehydes, coumarins and flavonoids, including quercetin (Ahmad & Hopkins, 1993). Two additional cases illustrate the role of UGTs in detoxification of plant secondary compounds in insects.

Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is an alkaloid found in the fruits of *Capsicum* spp. (Solanaceae). It and related compounds, the capsaicinoids, are responsible for the pungency of hot pepper fruits and are unique to the genus. Capsaicin binds to and activates the mammalian TRP (transient receptor potential) channel, causing the sensation of heat, but it has no such effect on avian TRP channels. It is believed to deter mammalian frugivores, whose digestive systems would damage the seeds, but not avian frugivores, through which the seeds pass intact and are thus dispersed. Variation in the capsaicin concentration of wild chilli peppers was also suggested to be an adaptive response to a fungal pathogen of the *Capsicum* fruit (Tewksbury *et al.*, 2008).

The oriental tobacco budworm, *Heliocoverpa assulta*, is a specialist on Solanaceae and a significant pest of hot peppers, aided by a high tolerance of dietary capsaicin. Its generalist relative *H. armigera* can also tolerate high concentrations (Ahn *et al.*, 2011a). Both species conjugate capsaicin to glucose (Figure 3.5c) using UGTs with highest activity in the Malpighian tubules and fat body, and they excrete the glucoside in the faeces (Ahn *et al.*, 2011b).

Quercetin and its many glycosylated derivatives are flavonoids, widely distributed in the plant kingdom and with mixed effects on insects, retarding growth of some and acting as feeding stimulants for others (Simmonds, 2001). Mulberry leaves contain quercetins *O*-glycosylated at the C-3 position and are consumed by larvae of the domesticated silkworm, *Bombyx mori*. The quercetin is eventually sequestered in the silk of the cocoon. A gene encoding a UGT responsible for the green cocoon colour of a mutant strain of silkworm has been identified (Daimon *et al.*, 2010). BmUGT-10286 catalyzes the *O*-glucosylation of quercetin at the C-5 position, producing a glucoside that is not present in its mulberry host. This is transported to the silk gland, where some is further glucosylated at the C-4 position, and both are incorporated into the silk of the cocoon. These compounds impart a green colour to the silk and protect the sensitive prepupa against ultraviolet radiation, as shown by higher pupation rates when prepupae were placed in cocoons from the mutant strain and irradiated with UV-B light (Daimon *et al.*, 2010).

3.8 Non-protein amino acids

Not all amino acids are incorporated into proteins; in addition to the 20 common amino acids utilized by the translational machinery, plants produce several non-protein amino acids. Some of these apparently have a defensive function, as they harm herbivores that consume them by being misincorporated into proteins. For example, L-canavanine, found in the seeds of many plants in the Fabaceae, is toxic to a wide range of organisms due to its misincorporation into proteins in place of L-arginine (Rosenthal, 1977).

The polyphagous tobacco budworm *Heliothis virescens* can tolerate high concentrations of L-canavanine, with a medial lethal concentration of 300 mM in the diet (Berge *et al.*, 1986). It possesses an enzyme in its midgut that converts L-canavanine to L-homoserine and hydroxyguanidine (Melangeli *et al.*, 1997). A second enzyme catalyzes the NADH-dependent reduction of hydroxyguanidine to guanidine (Rosenthal, 1992; see Figure 3.8). The first enzyme was purified from the cytosolic fraction of midgut cells and tested for substrate specificity. It also degraded L-canaline, but with a thousand-fold lower efficiency than for L-canavanine, and it exhibited no activity on compounds with a longer or shorter aliphatic chain length. Hydrolysis of the O-N bond is very unusual, but the reaction was verified by isolation and quantification of the products (Rosenthal, 1992). A second, minor detoxicative

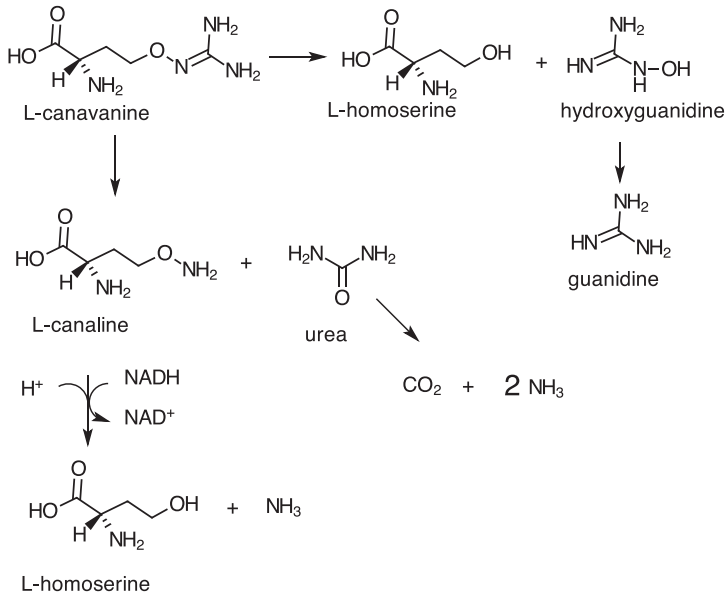


Figure 3.8 Detoxification of the non-protein amino acid L-canavanine. Conversion to L-homoserine and hydroxyguanidine by tobacco budworm, hydrolysis to L-canaline and urea in bruchid beetle.

mechanism possessed by this species is phosphorylation of L-canavanine by arginine kinase (Gindling *et al.*, 1995).

Larvae of the bruchid beetle *Caryedes brasiliensis* consume the seeds of the neotropical legume *Dioclea megacarpa*, which contain high concentrations of L-canavanine. One component of resistance is an arginyl-tRNA synthetase that discriminates effectively between canavanine and arginine (Rosenthal *et al.*, 1976). L-canavanine was observed to be hydrolyzed to L-canaline and urea, and the toxic L-canaline is further eliminated by reductive deamination to produce homoserine and ammonia (Rosenthal *et al.*, 1978; see Figure 3.8).

A highly active urease converted the urea to ammonia and carbon dioxide (Rosenthal *et al.*, 1977). When ^{15}N-labelled urea was provided, the labelled nitrogen was subsequently observed in several amino acids (Rosenthal *et al.*, 1982). The beetle would thus appear to derive some nitrogen from the metabolism of L-canavanine; however, it was not clear whether these reactions are catalyzed by beetle-derived enzymes or by prokaryotes residing in the larval midgut (Rosenthal *et al.*, 1982).

3.9 Iridoid glucosides

Iridoid glucosides provide another example of a plant defence that is activated by hydrolysis of an attached glucose molecule. They are cyclopentanoid structures of 8–10 carbon atoms derived from monoterpenes, with a glucose attached to C-1 (Figure 3.5b). Several hundred structures are known from plants in the Asteridae (Boros & Stermitz, 1990, 1991). The aglycone iridoid produced by hydrolysis of the sugar is a dialdehyde which binds to nucleic acids, proteins and amino acids via imine linkages. Activation is primarily by the herbivore's own digestive β-glucosidases; whether a plant-supplied enzyme also participates is unknown.

When activated, the aglycones have antibacterial and antifungal activity, as well as deterring most generalist herbivores. They are, however, oviposition or feeding stimulants to several specialists, some of which sequester the glucosides for their own defence. Sequestration of iridoid glucosides from the host plant is known in Lepidoptera, Hymenoptera, Homoptera, and Coleoptera.

There is no evidence that the sequestering Lepidoptera can synthesize their own iridoids, and concentrations in the body vary with amounts present in the host. However, ratios of the different compounds in the insect may differ from the host plant, e.g. *Plantago lanceolata* has twice as much aucubin as catalpol, and larvae of its herbivore *Junonia coenia* have equal amounts due to more efficient uptake or sequestration of catalpol (Bowers & Collinge, 1992). Many iridoid glucosides are not taken up by the herbivore at all, but the identities of the selective transporters responsible for differential uptake are unknown.

The mechanism most frequently invoked by which the herbivore could reduce its intoxication is suppression of β-glucosidase activity in the midgut. When larvae of the generalist arctiid *Spilosoma virginica* fed on three plant species with different amounts of iridoid glucosides, general midgut β-glucosidase activity measured using the standard substrate 4-nitrophenylglucose (4NPG) was inversely correlated with iridoid content of the host plant. However, aucubin-hydrolyzing activity was low and constant (Pankoke *et al.*, 2010).

A heat-inactivation experiment provided evidence of at least two forms of β-glucosidase in the midgut. In a similar experiment with larvae of the polyphagous arctiid species *Grammia incorrupta*, 4NPG hydrolysis was lower among larvae feeding on *P. lanceolata* containing iridoids than on the non-producer *Taraxacum officinale* (Pankoke *et al.*, 2012). However, aucubin-hydrolyzing activity was higher in the former group and negatively correlated with growth rate among insects feeding on *P. lanceolata*. Thus, down-regulation of some isozymes of β-glucosidase occurs, but is not particularly adaptive in these two generalists.

3.10 Cardenolides

Cardenolides are famous for two reasons: the medical use of digitoxin and digoxin for the treatment of heart failure in humans, and their role in the chemical defence of the iconic monarch butterfly. These two aspects are linked by the highly specific biological target of these compounds, the transmembrane sodium pump Na^+/K^+-ATPase (Horisberger, 2004). This protein is located in the plasma membrane of all animal cells, and it transports three sodium ions from the cytoplasm to the cell exterior and two potassium ions in the reverse direction for every molecule of ATP hydrolyzed. This energy-driven ion exchange is vital for the maintenance of the resting potential of the cell, the concentration gradient of ions across the membrane that powers other secondary transport mechanisms and enables the generation of action potentials in neurons.

Cardenolides bind to a conserved site on the extracellular side of the protein and block the ion transport. Unlike other defensive glycosides, they are not activated by cleavage of the sugar; in fact, the sugar participates in binding to the target (Yatime *et al.*, 2011). In therapeutic doses, this blockage has beneficial effects in ameliorating heart failure via an indirect effect on intracellular calcium concentrations; higher concentrations are universally toxic to animals. Plants can synthesize and store cardenolides with impunity, since they lack the vulnerable Na+/K+-ATPase. The digoxin isolated from the foxglove *Digitalis* is undoubtedly made for its chemical defence.

Structurally, cardenolides are built around a steroid framework, with a five-membered lactone ring at position C17 and a mono- or polysaccharide at position C3 (Figure 3.9). Related compounds are the bufodienolides, with a

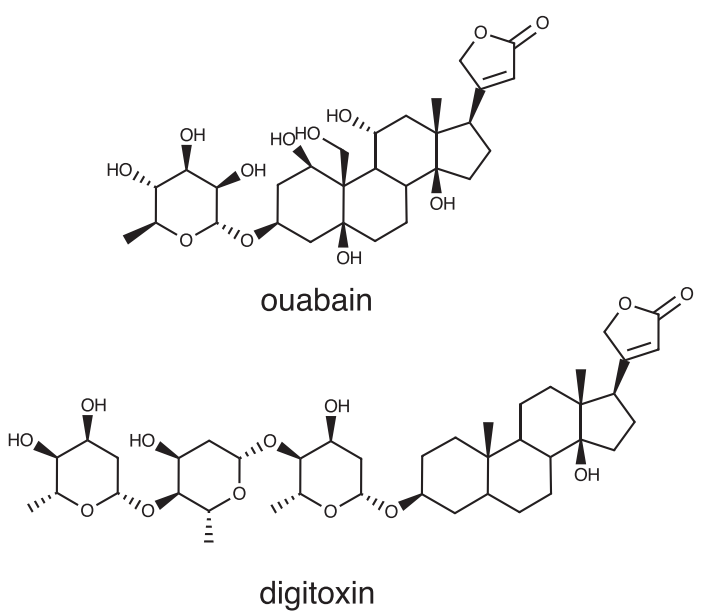


Figure 3.9 Cardenolides. Oubain, digitoxin.

six-membered lactone instead; these two types of compounds are collectively termed cardiac glycosides, because of their similar action on the vertebrate heart.

Sixty genera and 12 families of angiosperms collectively produce the more than 500 cardiac glycosides known. Chrysomelid beetles (*Chrysolina*) and fireflies (*Photinus*) synthesize them for defence, as do some toads (*Bufo*) (Dobler *et al.*, 2011). Mammals have been found to synthesize endogenous cardenolides to modulate cardiac function, including ouabain and digoxin, identical to those made by plants (Schoner & Scheiner-Bobis, 2007). Thus, the ability to produce cardiac glycosides has appeared repeatedly due to convergent evolution. Several remarkable cases of convergent evolution characterize the adaptation of cardenolide-sequestering insects as well.

The classic example of sequestration is the monarch butterfly *Danaus plexippus,* whose larvae take up cardenolides from their *Asclepias* host plants and retain them in the adult stage for protection against vertebrate predators. Several species of Coleoptera, Hemiptera, Orthoptera, and other Lepidoptera also sequester them for defence (Opitz & Mueller, 2009). Efficient and selective uptake mechanisms must be responsible, but the nature of these is unknown. Since cardenolides are toxic to insects as well, detoxification mechanisms must also be in place, but many sequestering species avoid intoxication by another mechanism – evolution of target site insensitivity.

Because of its clinical significance, the ATPase has been intensively studied in vertebrates to identify the amino acid residues responsible for cardenolide

activity, by targeted mutagenesis and cardenolide binding assays with the heterologously expressed ATPase. The highly conserved structure of ATPase and its universal occurrence in animals has enabled surveys for similar amino acid substitutions that could confer target-site insensitivity in insects.

One such survey sequenced most of the coding sequence of the α subunit in several species in four insect orders that encounter dietary cardenolides, along with related species that do not (Dobler *et al.*, 2012). The first extracellular loop is known to interact with cardenolides, and residue 122 is known from mutagenesis studies to affect binding. An asparagine occupies that position in mammals and most insects, but *D. plexippus* and another cardenolide-adapted species, *Chrysochus auratus*, were previously shown to have a histidine instead. The same N122H substitution was also found in the dipteran leafminer *Liriomyza asclepiadis* and two heteropterans, the milkweed bugs *Oncopeltus fasciatus* and *Lygaeus kalmii*.

Position 111 usually has an asparagine, but 11 of the 18 cardenolide-adapted species had a substitution there, usually to leucine or valine and twice to threonine. Additional substitutions at otherwise universally conserved residues in other transmembrane domains that contribute to the cardenolide-binding pocket are also found in some adapted species. Some of these substitutions of Q111 and N122 were tested in various combinations by mutating the *Drosophila* gene in those positions and expressing the mutants in cultured cells; the double mutation Q111T and N122H was significantly more effective in cell survivorship of ouabain treatment than the separate mutations. Not all cardenolide-adapted species showed amino acid substitutions in the region of the gene surveyed, and these are likely to have other mechanisms of circumventing toxicity. However, the species in four different insect orders that do so demonstrate widespread convergent evolution at a few critical sites in the target.

Additional aspects of convergent evolution were uncovered in another survey, which examined gene duplication events in herbivores feeding on Apocynaceae, which are rich in cardenolides (Zhen *et al.*, 2012). Most insects have two types of genes encoding the α subunit of the Na^+/K^+-ATPase, ATPα1 and ATPα2. ATPα1 has been duplicated to yield two copies in dogbane beetles (*Chrysochus auratus*) and milkweed stem weevils (*Rhyssomatus lineaticollis*), and three copies in the milkweed bugs *O. fasciatus* and *L. kalmii*. The two duplications in the latter species have been estimated to have occurred 60 and 125 million years ago and are likely shared by other representatives of the Lygaeidae.

The rate of amino acid replacement at sites in the ouabain binding pocket is significantly greater among lineages that feed on the Apocynaceae than in related lineages that do not, and several lineages showed substitutions at positions 111 and/or 122. Substitutions that were observed only once occurred significantly more often in lineages with a recent ATPα1 duplication, consistent with the idea that gene duplication may allow fixation of substitutions that would be deleterious in a single-copy gene, because the

ancestral unmutated copy is retained and remains functional. Docking simulations based on molecular modelling of the proteins encoded by the different genes were used to predict which duplicate copy would be less sensitive to ouabain. In the dogbane beetle and milkweed stem weevil, the less sensitive form is expressed more in the digestive tract, and the more sensitive form is expressed more in the head and thorax. A similar pattern was found among the three forms in the milkweed bugs. Thus, ATPα genes differentially sensitive to ouabain are differentially regulated across tissues, and this regulation pattern also exhibits convergent evolution.

3.11 Conclusions

A few common themes emerge from the diversity of mechanisms discussed here, which can only represent a small fraction of those that have evolved in insect herbivores. Diversification of gene families provides both the ability to synthesize novel toxins by plants and the ability to detoxify them by insects. Gene duplication can provide the opportunity for housekeeping enzymes to acquire novel detoxicative functions if needed. The evolution of transcriptional control allows detoxicative functions to be applied effectively and efficiently when needed. The ability to avoid activating a plant-derived toxin can be as useful as detoxifying the activated toxin. Some insects can mimic the biochemical pathways present in plants to synthesize their own versions of chemical protectants, and they use detoxicative mechanisms to regulate this synthesis for effective and safe sequestration. When a class of plant toxins affects one specific molecular target in the insect, gene duplication and mutation can provide resilient versions of the target that are less affected by the toxin. This 'passive defence' can be as effective, and energetically less demanding, than the 'active defence' of detoxification.

The cases surveyed here have illustrated how applications of molecular methods have greatly accelerated the study of insect detoxification and sequestration strategies, and how they will continue to do so. Following the leads provided by classical chemical and biochemical approaches has led to the isolation of the enzymes involved and the genes that encode them. Identifying the underlying genes has been facilitated by next-generation DNA sequencing techniques, which were used to identify candidate P450s and UGTS to be tested for involvement in the biosynthesis of cyanogenic glucosides in the burnet moth (Jensen *et al.*, 2011) and enabled rapid comparison of Na^+/K^+-ATPase sequences across many species (Zhen *et al.*, 2012).

Identifying these underlying genes has benefits on many levels. It enables heterologous expression of their protein products to rigorously examine substrate specificity and sensitivity to inhibitors. Knowledge of the predicted protein sequence, in connection with molecular modelling, can be used to probe properties of enzymes that cannot be isolated or expressed, or which are unavailable today because they existed in hypothetical ancestors in the

past. It also allows examination of the acquisition of new functions in the context of gene families created by gene duplication and functional divergence. The increasing availability of whole genome sequences will reveal members of gene families that have escaped detection by functional or transcriptomic analysis and are available for mutation and adaptive change. And sequence comparison of homologous genes in different organisms, combined with phylogenetic analysis, can provide estimates of the time of appearance of new key adaptations in evolution and can establish the degree of convergent evolution.

Finally, integrating knowledge of insect detoxification and sequestration strategies with growing information from plants about the evolution of the deployment of their chemical defence strategies enables a holistic approach to studying the interactions that have been ongoing for the past 300 million years or so (Ehrlich & Raven, 1964), putting the 'co' in co-evolutionary studies on a firm footing that can only continue to amaze us with its innovation and diversity.

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