Chapter 1
Phytoecdysteroids: Diversity, Biosynthesis and Distribution

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Abstract We review the current status of knowledge on phytoecdysteroids, with particular emphasis on their occurrence, diversity, functions, biosynthesis and biotechnological production. We also consider evolutionary aspects with regard to their probable role in the deterrence of phytophagous invertebrates and in respect to their relationship to other classes of phytosteroids.

Keywords Allelochemical • biosynthesis • cell culture • deterrence • distribution • diversity • ecdysteroid • insect-plant relationships • phytosteroid

Abbreviations 2dE: 2-deoxyecdysone; 22dE: 22-deoxyecdysone; 2,22dE: 2,22-dideoxyecdysone; 2,22,25dE: 2,22,25-trideoxyecdysone; 20E: 20-hydroxyecdysone; BR: Brassinosteroid; DAH: Diacylhydrazine; E: Ecdysone; LBD: Ligand-binding domain; makA: Makisterone A; NMR: Nuclear magnetic resonance; poA: Ponasterone A; polB: Polypodine B.

1.1 Introduction

The discovery of ecdysteroids in plants (phytoecdysteroids) followed soon after the elucidation by X-ray analysis of the structure of ecdysone (Huber and Hoppe, 1965). Almost simultaneously, four independent laboratories reported

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the isolation of closely related molecules from two gymnosperms (Galbraith and Horn, 1966; Nakanishi et al., 1966), one fern (Jizba et al., 1967) and one angiosperm (Takemoto et al., 1967a). Such an unexpected finding was the impetus for extensive investigations of many plant species to examine the distribution of these molecules in the plant kingdom (see Ecdybase [Lafont et al., 2002] for an extensive bibliographic survey of >2,350 species; Dinan and Lafont, 2007). At that time, there was great hope that ecdysteroids would form the basis for a new class of safe and specific endocrine disruptors for controlling insect pests. Indeed, these molecules are able to interfere with insect development/reproduction (see Chapter 6), but for various reasons (the complexity of the molecules, the difficulty of their chemical synthesis, environmental and metabolic lability, penetration problems) this has not led to any practical use of ecdysteroids *per se* in the area of pest control (which is not the case for synthetic ecdysteroid agonists [e.g. diacylhydrazines], which have much simpler, and unrelated, structures).

These extensive investigations allowed the isolation of a wide array of molecules (see Ecdybase), i.e. more than 300 different phytoecdysteroids, and showed the surprisingly wide distribution of these molecules in the plant kingdom (Dinan, 2001). The presence of ecdysteroids is not restricted to terrestrial species, and several aquatic plants also contain ecdysteroids (Chadin et al., 2003), which may be ecologically relevant (see Chapter 23 of this book).

Ecdysteroids are also present in fungi (reviewed in Kovganko, 1999), and closely related molecules (pinnasterols) have been isolated from a red alga (Fukuzawa et al., 1981, 1986).

Ecdysteroids are somewhat related to brassinosteroids (BRs), i.e. plant growth steroid hormones, in their chemical structures, although it is apparent that this similarity is not so strong when one considers 3D-representations of the molecules. While BRs may act as weak ecdysteroid antagonists in insects (Dinan and Hormann, 2005), the reverse is not true, since ecdysteroids do not appear to be active on BR receptors. This makes biological sense when considering (i) the uneven distribution of phytoecdysteroids in the plant world and (ii) the huge concentrations of ecdysteroids reached in certain plant species, when compared with the minute amounts of BRs (Adam and Marquardt, 1986). This does not however preclude other biological activities of ecdysteroids in the plant itself (Lehmann et al., 1988), although convincing experimental evidence for this is lacking (Dinan, 2001).

In this chapter, we shall first consider the diversity and the distribution of ecdysteroids. We will then discuss in detail the biosynthesis of these molecules and identify the remaining open questions. We will then consider the *in vitro* systems developed for the production of ecdysteroids. The putative allelochemical functions of phytoecdysteroids will then be examined. Finally, we shall consider phytoecdysteroids in relation to the diversity of non-ecdysteroidal plant sterol derivatives.

1.2 Distribution and Diversity of Phytoecdysteroids

1.2.1 General

The literature concerning this aspect has been comprehensively reviewed recently (Lafont, 1997; Dinan, 2001). Thus, the main features of the distribution of phytoecdysteroids within the world flora and their distribution within plants will be just briefly summarised, with emphasis on publications appearing since 2001, and attention will be drawn to the larger surveys of plant species to aid those wishing to ascertain if particular species have already been investigated for the presence of ecdysteroids. A good resource in this respect is Ecdybase (http://www.ecdybase.org), which provides chemical and biological data for ecdysteroid analogues and listings of the literature concerning the occurrence of ecdysteroid in plants, their effects on various organisms and their applications.

Ecdysteroids or ecdysteroid-like compounds have been found in gymnosperms, angiosperms, fungi, algae and certain marine organisms, in addition to arthropods and other invertebrate groups (see Chapter 2). The most extensive studies of ecdysteroid distribution within these categories of organisms have focussed on terrestrial plants, but even here only a very small percentage of the world's 250,000 species have been analysed, and, of course, the published literature tends to report only the ecdysteroid-positive species, rather than those not found to contain them. Additionally, one needs to take the method and protocol used to detect ecdysteroids into account, since they can possess very different thresholds for detection, as plants can contain vastly different levels of ecdysteroids, and the method may detect only particular types of ecdysteroids. Thus, a new class of ecdysteroid conjugate (glucosyl-ferulate) has recently been detected in the fern *Microsorum membranifolium* (Ho et al., 2008), which could easily have been missed in earlier studies on other species. Also, one has to ask if the ecdysteroids are naturally produced by the organism. For example, do fungi produce mycoecdysteroids *de novo*, or do they represent modifications of phytoecdysteroids taken up by the fungus? Only in a few terrestrial plants has the biosynthetic capacity properly been demonstrated (see later).

1.2.2 Phytoecdysteroid Distribution Within the Natural World

Within the higher plants, ecdysteroids are found in gymnosperms and angiosperms (mono- and di-cotyledonous plants). It appears that 5–6% of terrestrial plant species contain significant levels of ecdysteroids (Imai et al., 1969; Dinan, 1995). However, use of sensitive immunoassays has suggested their presence in the leaves of 40% of randomly selected species

(Dinan et al., 2001a). Additionally, individual ecdysteroid-positive plants in species generally regarded as ecdysteroid-negative (e.g. *Arabidopsis thalliana*; Dinan et al., 2001a) can be found. Put together, this suggests that most, if not all, plants retain the genetic capacity to produce ecdysteroids, but that the accumulation is suppressed in most species. It was originally thought that there was a preponderance of ecdysteroid-containing species amongst the ferns, but it has come to be realised that this just reflected the large number of ferns in early surveys (Hikino et al., 1973; Yen et al., 1974). Few plant families do not contain at least some ecdysteroid-positive species; where none has been found, it is probably because the family is very small, or that too few species have been examined. Levels of phytoecdysteroids in ecdysteroid-positive plant species vary enormously, from the barely detectable to the staggeringly high, where ecdysteroids can make up 2–3% of the dry weight (e.g. seeds of *Rhaponticum* [*Leuzea*] *carthamoides* [Koudela et al., 1995] or stems of *Diploclisia glaucescens* [Bandara et al., 1989]). Even low concentrations may be ecologically significant in the deterrence of invertebrate predators, since they may act in synergy with other classes of secondary compounds to bring about effective deterrence. Equally, there is not a simple relationship between the frequency of occurrence of ecdysteroid-positive species in a genus/family and the levels found in individual species. Thus, there are a few individual species in the Compositae (*Leuzea carthamoides*, *Serratula coronata*), which contain very high levels of ecdysteroids, but in general the vast majority of composites are ecdysteroid-negative (Dinan, unpublished). On the other hand, in the genus *Silene*, where there is a high preponderance of ecdysteroid-positive species, there are several ecdysteroid-rich species (Zibareva et al., 2003). As more species are examined within certain large families/genera, patterns are beginning to be discerned between their presence/absence and the taxonomic position of the species e.g. in the Chenopodiaceae (Dinan et al., 1998) or the genus *Silene* (Zibareva et al., 2003).

1.2.3 Phytoecdysteroid Distribution Within Plants

Phytoecdysteroid levels are not uniform in ecdysteroid-positive species. Rather, they vary from organ to organ, and can undergo changes according to season or geographical location. Further, the ecdysteroid profiles of different organs may vary. It is not known where in the plant cell or tissue that ecdysteroids accumulate, although it is often speculated that it is in the vacuole. Data on the relative biosynthetic capacity of different plant organs are also scarce, as is information about the kinetics and mechanisms of synthesis and distribution around the plant.

It has been suggested that the qualitative and quantitative aspects of ecdysteroid presence or profiles could be used for chemotaxonomic purposes (Zibareva, 2000). However, the fluctuations associated with organ type, season and geographical

location may confound this. In spite of this, levels and profiles in seeds may truly have some potential in this regard (Dinan et al., 1998; Zibareva, 2000). The taxonomic value of ecdysteroids in the chemosystematics of mushrooms, particularly in genera *Paxillus* and *Tapinella*, has been discussed and used as an additional chemosystematic character (Vokáč et al., 1998a, b).

Evidence is gradually accumulating that ecdysteroid concentrations are highest in tissues which are most important for the survival of the plant, or, in the case of annuals, of the species into the next generation. Thus, in the wind-pollinated annual *Chenopodium album*, high levels of ecdysteroids are found in the anthers protecting the developing pollen, in the seeds and in young leaves (Dinan, 1992).

1.2.4 Large-Scale Surveys of Phytoecdysteroid Distribution

It is estimated that there are more than 250,000 species of terrestrial plants, but only a small percentage of these has been surveyed for the presence of phytoecdysteroids. Further, since the possibility for varietal, developmental, ecological or geographical variation of ecdysteroid content exists within a species, very few species indeed have been examined under different circumstances, and certainly not enough to be able to state categorically that a particular species does not accumulate ecdysteroids under any circumstances.

The first large-scale survey was conducted by Imai et al. (1969) who assessed methanolic extracts of 1,056 taxonomically diverse species and 351 crude drug preparations by means of the lepidopteran *Chilo suppressalis* 'dipping' test. They found that 24 (from 47) pteridophytes, 7 (from 42) gymnosperms and 30 (from 967) angiosperms were positive. The publication names and presents data only for the positive species. Hikino et al. (1973) examined methanolic extracts of 283 species (871 specimens) of Japanese ferns by means of the dipteran *Sarcophaga peregrina* test, whereby the plant extract is injected into the abdomen of ligated last instar larvae to see if pupariation is induced (positive response). 170 species proved positive, of which 51 gave high activity. The paper reports the data for all positive and negative species. The *Sarcophaga* test was also employed by Yen et al. (1974), who assessed methanolic extracts of 115 species (164 specimens) of Taiwanese ferns. Sixty-four species were positive, of which 18 were highly so. Again, all the tested species are named.

During the period 1995–2002, the Insect Biochemistry Group at Exeter University conducted a survey for the presence of ecdysteroid agonist and antagonist activities in a large number of plant extracts. The plant material (25–30 mg dw) was extracted with methanol, partially purified by addition of water (30%) and partition of the aq. methanol phase against hexane. The aq. methanol phase was then assessed by means of the dipteran *Drosophila melanogaster* B_{II} cell-based microplate assay for ecdysteroid agonist and antagonist activities (Clément et al., 1993) and in two or three ecdysteroid-specific immunoassays (Dinan, 1995).

Phytoecdysteroid-containing extracts were positive in the agonist bioassay and in the immunoassays. The plant material surveyed consisted of:

- 2,454 randomly selected seed samples
- 2,111 targeted seed samples
- Parts of plants grown from 180 randomly selected species (Dinan et al., 2001a)
- Seeds of 290 species assessed for the presence of hydrolysable ecdysteroid conjugates (Dinan et al., 2001a)
- Circa 200 species of the Chenopodiaceae (Dinan et al., 1998)
- Seeds and plant parts of 128 species of the Solanaceae (Savchenko et al., 2000)
- Circa 110 species of the Caryophyllaceae (Zibareva et al., 2003)
- 470 species of the Compositae

Some of this data has been published over the years in the scientific literature (e.g. Savchenko et al., 1997, 1998, 2001; Whiting et al., 1998; Dinan et al., 2001b, c, 2002), and detailed summary tables of the findings have now being incorporated into the Ecdybase website (http://www.ecdybase.org), which permits one, with significant certainty, to identify the species which contain (i) phytoecdysteroids (agonist bioassay positive, RIA-positive; 5–6% of species based on seed extracts), (ii) ecdysteroid antagonists (antagonist bioassay positive, RIA-negative; 2% of species), or (iii) non-steroidal agonists (agonist bioassay positive, RIA-negative; very rare), and to use the data to consider the taxonomic significance of the distribution of ecdysteroid agonists/antagonists.

The approach of the Exeter Survey has been extended by the Biochemistry and Biotechnology Laboratory of the Komi Research Centre to examine the ecological and geographical distribution of ecdysteroid-containing plant species in a specific geographical region (European North-east Russia). The findings of this study have been published (Volodin et al., 2002; Volodin, 2003). Seven hundred samples collected from eight geographical locations and representing 411 species were investigated. Four percent of the species tested positive for phytoecdysteroids. All the data are presented and analysed for the impact of ecological/geographical factors on phytoecdysteroid distribution.

1.2.5 Diversity of Phytoecdysteroids

To date, somewhere in the region of 300 different phytoecdysteroids have been identified (Lafont et al., 2002). Ecdybase provides an up-to-date listing of all their structures, together with spectral and biological data, where available. The diversity of ecdysteroid analogues has also been reviewed previously (Lafont and Horn, 1989; Lafont, 1997, 1998; Dinan, 2001). They differ in the number of C-atoms present (24C–29C), the number, position and location of hydroxyl and keto groups on the steroid skeleton, whether the ecdysteroid is free or conjugated and, if conjugated, whether the conjugating moiety is polar or non-polar. Multiple

conjugates also exist. This diversity is summarised in Fig. 1.1. By far the most commonly occurring phytoecdysteroid is 20-hydroxyecdysone, followed by polypodine B. Typically, a phytoecdysteroid-containing species will contain 1–3 major ecdysteroids, which together make up ca. 95% of the total ecdysteroid, together with a plethora of minor ecdysteroids, forming a diverse 'cocktail' of ecdysteroid structural analogues. The number of minor ecdysteroids may be very large, since in few cases have only the most major of them been isolated and identified. In *Leuzea carthamoides*, which is rich in ecdysteroids, has been extensively studied and where very large amounts of plant material have been extracted to provide a commercial source of the major ecdysteroids present, the total number of ecdysteroid analogues so far identified is >20 (Buděšinský et al., 2008). Figure 1.2 demonstrates the ecdysteroid profile present in fronds of the fern *Microsorum membranifolium* (Ho et al., 2008), including the presence of novel ecdysteroid glucosylferulate conjugates. This species is characterised by high ecdysteroid concentrations (0.65% of the dry weight of the fronds) and, unusually, by significant proportions of a number of ecdysteroid analogues, where 20E is not the major component. Although far fewer ecdysteroids have been isolated from fungi (mycoecdysteroids) than from plants, it would appear that, although there is some overlap in the structural analogues found (20E, ajugasterone C etc.), some structural variations (e.g. side-chain epoxide groups) appear to be unique to fungi. It is currently not clear if fungi possess the ability to generate ecdysteroids from sterols, or whether they take in phytoecdysteroids from their substratum and modify them.

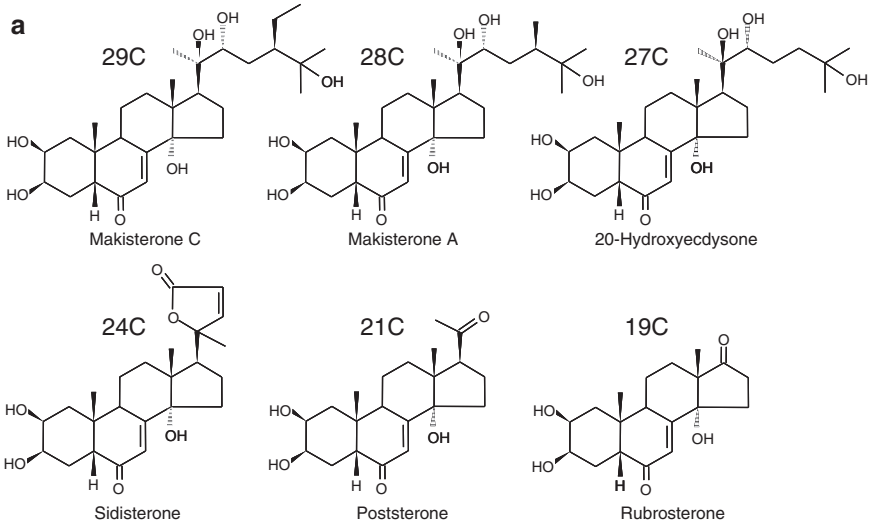

Fig. 1.1 Summary of the diversity of phytoecdysteroid structures, show examples of variety in steroids (**panel a**)

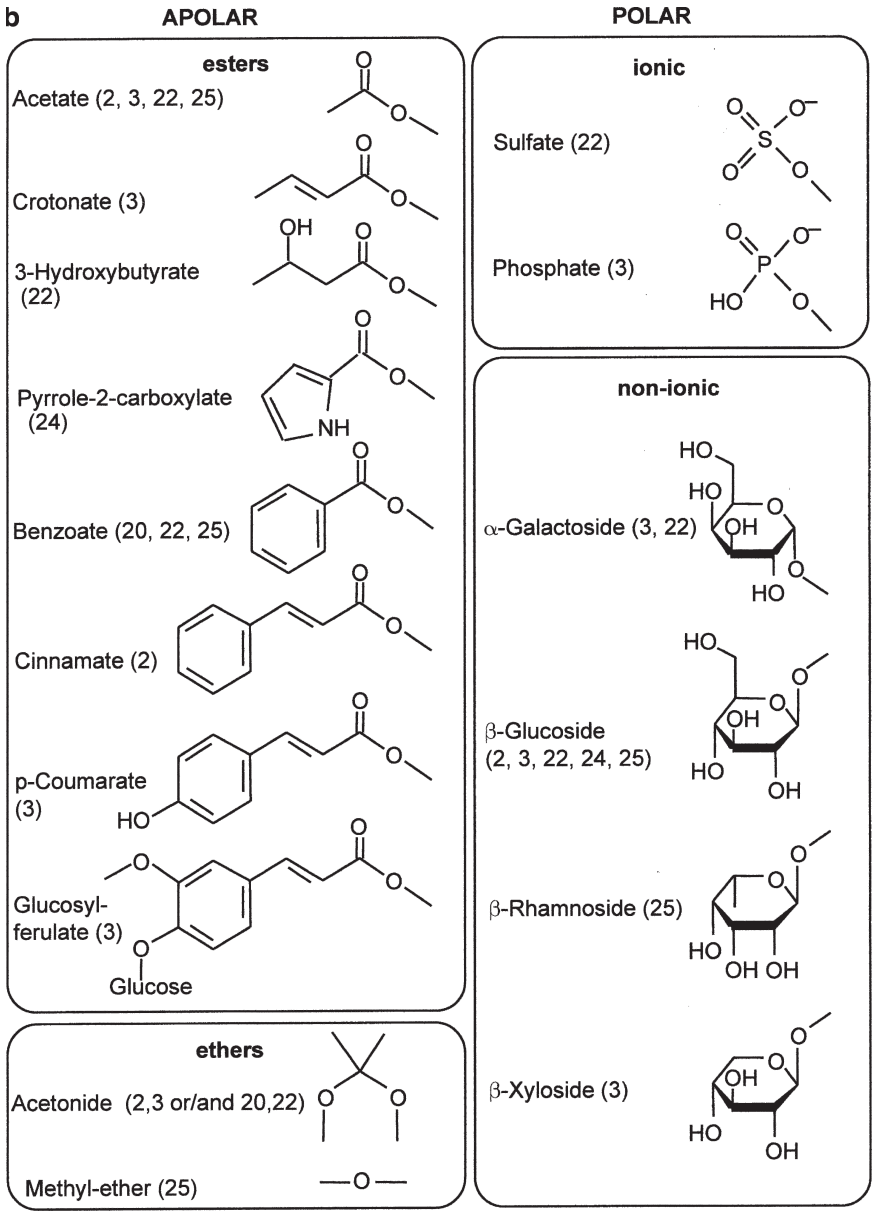

Fig. 1.1 (continued) and conjugating moieties (**panel b**).The numbers in brackets in panel B refer to the C-atoms of the steroid which can be modified

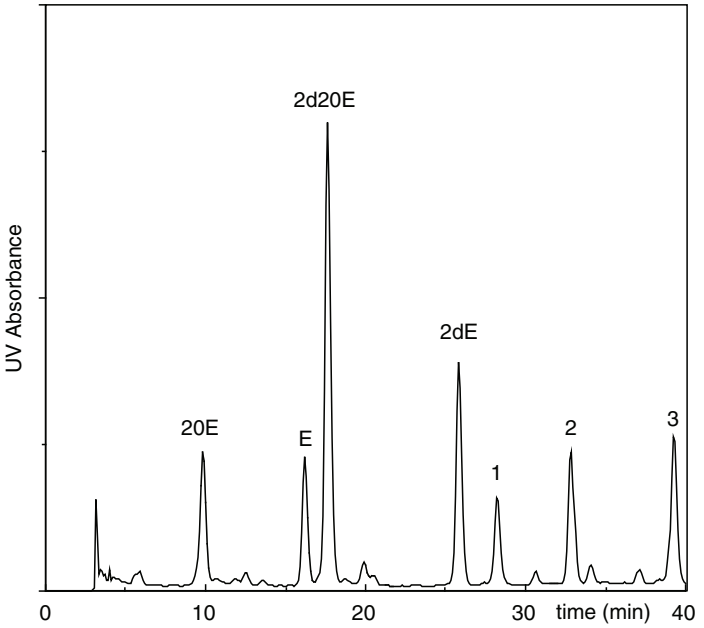

Fig. 1.2 Ecdysteroid profile in fronds of *Microsorum membranifolium* as determined by C_{18} RP-HPLC (15 cm × 4.6 mm; 5 µm particle size), eluted at 1 ml/min with a gradient from 15–30% acetonitrile in water over 40 min; peak 1 = 2dE 25-rhamnoside, peak 2 = 2d20E 3-glucosylferulate and peak 3 = 2dE 3-glucosylferulate

1.3 Pathways and Regulation of Phytoecdysteroid Biosynthesis

1.3.1 Introduction

Studies on phytoecdysteroid biosynthesis started ca. 30 years ago, i.e. soon after their discovery, and, in fact, at about the same time as in insects. Given the large amounts of edysteroids found in some plant species, it could be thought that elucidation of the pathway could be easily addressed. In reality, the high amounts are the result of ecdysteroid accumulation over a long period, which does not require such high rates of synthesis when compared to that in insect moulting glands; moreover, the sites of ecdysteroid production have not been defined, and we do not know whether biosynthesis takes place in all, or only is some, specialized cells (yet to be identified). It will become possible to address such a question only with a molecular biological approach, when genes encoding some of the biosynthetic enzymes have been identified, which is not yet the case for the following reasons: (1) none of the plant species for which genomic sequence information is available accumulate ecdysteroids and (2) unlike brassinosteroids, we cannot expect that mutants will

display a specific phenotype. Thus, very few attempts have been made so far to purify biosynthetic enzymes (e.g. ecdysone 20-monooxygenase) using classical biochemical approaches (Grebenok et al., 1996; Canals et al., 2005).

1.3.2 Methods

Ecdysteroids are derived from sterols, and different strategies can be used to elucidate their biosynthetic pathway, which are aimed at providing different types of information:

1. Use of radioactive (^{3}H or ^{14}C) molecules and testing whether they are converted into ecdysteroids (mainly 20-hydroxyecdysone); this can be done with very early sterol precursors (acetate, mevalonate), sterols (cholesterol) or any available putative intermediate; radioactive intermediates/substrates can also be used to characterize enzymes (organ distribution, subcellular localization, cofactors etc.) involved in some biosynthetic steps (e.g. hydroxylases).
2. Use of doubly-labelled (^{3}H + ^{14}C) molecules, where tritium is introduced at specific positions of the A/B-rings with the aim of understanding the mechanism of the formation of the (5β–H)14αOH-7-en-6-one structure, or the stereochemistry of a given reaction (e.g. 7-dehydrogenation).
3. Use of molecules labelled with stable isotopes (^{2}H, ^{13}C) followed by NMR analysis of 20-hydroxyecdysone; this gives information about effective incorporation and, in the case of ^{2}H, it allows the establishment of a possible migration of deuterium atoms to another position.

1.3.3 Biological Systems

In addition, labelling experiments are faced with the problem of compound delivery to/uptake by the plant. Topical applications have a limited efficiency, owing to the low permeability of plant cuticle. Whole plant experiments can allow uptake by the roots. Excised leaves can be efficiently loaded through their petiole thanks to evaporation. Hairy roots (from *Serratula tinctoria* [Delbecque et al., 1995; Corio-Costet et al., 1996] or *Ajuga nipponensis* [Fujimoto et al., 2000]) and ecdysteroid-producing cell cultures from *Ajuga reptans* and *Serratula coronata* (Filippova et al., 2003) should provide more efficient biological systems. *Polypodium vulgare* prothalli also represent a good model for metabolic studies (Reixach et al., 1996, 1997, 1999).

1.3.4 Biosynthetic Pathway(s)

Early on, it was shown that cholesterol was a precursor of C_{27} ecdysteroids, but this may not be true for all species; for instance, *Spinacia oleracea* contains mainly Δ^{7}-sterols and lathosterol (a Δ^{7}-sterol) is a more likely ecdysteroid precursor (Grebenok

and Adler, 1993). Acetate and mevalonate are also converted into ecdysteroids, and acetate may give C_{27}-, C_{28}- and C_{29}-ecdysteroids (Tomás et al., 1993) (Table 1.1). It is conceivable that C_{28}- or C_{29}-sterols are the precursors for the corresponding C_{28}- and C_{29}- ecdysteroids (e.g. clerosterol for cyasterone; Okuzumi et al., 2003). The use of hydroxylated cholesterol derivatives (25-hydroxycholesterol, 22*R*-hydroxycholesterol) has been essentially restricted to *Polypodium vulgare* prothalli and these molecules were very efficiently converted into ecdysone and 20-hydroxyecdysone in this fern (Reixach et al., 1999), whereas 25-hydroxycholesterol labelling experiments with *Podocarpus elata* (Joly et al., 1969), *Serratula tinctoria* hairy roots (J.-P. Delbecque, personal communication), or *Silene otites* seedlings (R. Lafont, unpublished data) were negative. In *P. vulgare*, this means that early hydroxylation at the 22*R*- or 25-positions does not prevent further reactions taking place.

The terminal steps have been investigated by using available tritium-labelled intermediates previously used with insects and crustaceans (5β-diketol, 5β-ketodiol, 2,22dE, 2dE, E etc.), but the limited number of species investigated does not allow general conclusions to be drawn. In *P. vulgare*, 5β-ketodiol and 2,22dE were converted only up to 22dE, but no further, which means that 22-hydroxylation must take place at an early stage (in this species at least, but not in *Achyranthes fauriei*, *Ajuga reptans* or *Spinacia oleracea*). On the other hand, 25-hydroxylation may take place at any time in *P. vulgare* (where both 25-hydroxycholesterol and ponasterone A [25-deoxy-20-hydroxyecdysone] can be converted to 20E), whereas 25-hydroxycholesterol is not converted in other species. These two examples illustrate possible differences in the biosynthetic pathway among plant species (pteridophytes *vs*. spermatophytes), possibly connected with the narrow substrate-specificity of some hydroxylases. In fact, similar differences have also been recorded in the case of arthropods. 20-Hydroxylation may also be a point of divergence between plants and animals; this step is the last one in insect larvae, because it takes place outside the moulting glands (e.g. in the fat-body), but there is no reason to think that the whole plant pathway to 20E should not take place in the same cell, thus allowing 20-hydroxylation to take place at an earlier stage (hence the presence of 22-deoxy-20-hydroxyecdysone [taxisterone] in several plant species. However, it is possible that in some animal systems the same situation exists, e.g. in the case of *Bombyx* ovaries where, for instance, 2,22d20E has been isolated (Ikekawa et al., 1980), or the presence of taxisterone in a marine arachnid, *Pycnogonum litorale* (Bückmann et al., 1986). The few enzymatic studies performed with subcellular fractions (mitochondria, microsomes, cytosol) have shown differences with insects concerning the localization of some hydroxylases or oxidoreductases (Reixach et al., 1999; Bakrim, 2007).

During metabolic studies, special attention was given to the, as yet unsolved (but in insects too), early-step problem, i.e. the formation of the (5β–H)14αOH-7-en-6-one chromophore characteristic of ecdysteroids. This question was initially addressed in parallel experiments performed on plants and insects, at first by using putative intermediates (cholest-4-en-3-one, cholesterol 5α,6α-epoxide, cholesterol 5β,6β-epoxide), then doubly-labelled cholesterols: [4-^{14}C,3α-^{3}H] to test whether a 3-oxo-Δ^4 intermediate was formed (Lloyd-Jones et al., 1973), [4-^{14}C,7α-^{3}H], [4-^{14}C,7β-^{3}H] to understand the stereochemistry of Δ^7-bond formation

Table 1.1 Biosynthetic studies performed with radioactive or non-radioactive labelled precursors

LABELLED PRECURSOR	PLANT SPECIES	ECDYSTEROIDS PRODUCED	REFERENCE
Acetate	*Ajuga reptans*	20E + Cyasterone + 29-Norcyasterone	Tomás et al., 1993
	Sesuvium portulacastrum	E + 20E	Sipahimalani et al., 1972
	Spinacia oleracea	20E + Polypodine B	Grebenok and Adler, 1993
Mevalonate	*Cyathula capitata*	only sterols, no Cyasterone	Boid et al., 1975
	Polypodium vulgare	E + 20E + Polypodine B	De Souza et al., 1970
	Serratula tinctoria	20E	Reixach et al., 1996
	Sesuvium portulacastrum	20E	Delbecque et al., 1995
	Spinacia oleracea	E + 20E	Sipahimalani et al., 1972
	Taxus baccata	20E + Polypodine B, E + E conjugate	Grebenok et al., 1994
		20E + Ponasterone A	De Souza et al., 1969
Cholesterol	*Ajuga reptans*	20E	Nagakari et al., 1994a, b; Yagi et al., 1996; Fujimoto et al., 1997; Nakagawa et al., 1997; Nomura and Fujimoto, 2000
	Podocarpus elata	20E	Sauer et al., 1968; Joly et al., 1969
	Podocarpus macrophyllus	20E + Ponasterone A	Hikino et al., 1970
	Polypodium vulgare	20E	Cook et al., 1973; Lockley et al., 1975; Davies et al., 1980; Reixach et al., 1996
	Serratula tinctoria	E + 20E	Delbecque et al., 1995
	Sesuvium portulacastrum	E + 20E	Sipahimalani et al., 1972
	Spinacia oleracea	20E + Polypodine B	Grebenok et al., 1994
	Taxus baccata	20E	Lloyd-Jones et al., 1973; Cook et al., 1973
	Zea mays	20E conjugate	Devarenne et al., 1995
Cholest-4-en-3-one	*Podocarpus elatus*	no conversion	Sauer et al., 1968
	Sesuvium portulacastrum	no conversion	Sipahimalani et al., 1972
7-Dehydrocholesterol	*Ajuga reptans*	20E (low)	Ohyama et al., 1999
25-OH-Cholesterol	*Podocarpus elata*	None	Joly et al., 1969
	Polypodium vulgare	E + 20E	Reixach et al., 1999

(continued)

Table 1.1 (continued)

LABELLED PRECURSOR	PLANT SPECIES	ECDYSTEROIDS PRODUCED	REFERENCE
22*R*-OH-Cholesterol	*Polypodium vulgare*	E + 20E	Reixach et al., 1999
22*S*-OH-Cholesterol	*Polypodium vulgare*	none	Reixach et al., 1999
Clerosterol	*Ajuga reptans*	Cyasterone + Isocyasterone + 29-Norcyasterone	Okuzumi et al., 2003
Lathosterol	*Ajuga reptans* *Spinacia oleracea*	20E 20E	Ohyama et al., 1999 Grebenok and Adler, 1993
3β-Hydroxy-5β-cholestan-6-one	*Ajuga reptans*	20E	Hyodo and Fujimoto, 2000
2β,3β-Dihydroxy-5β-cholestan-6-one	*Ajuga reptans*	20E	Hyodo and Fujimoto, 2000
3β-Hydroxy-5β-cholest-7-en-6-one	*Ajuga reptans*	20E	Hyodo and Fujimoto, 2000
3β,14α-Dihydroxy-5β-cholest-7-en-6-one	*Ajuga reptans*	no 20E 20E	Hyodo and Fujimoto, 2000 Fujimoto et al., 2000
5β-Cholest-7-ene-3,6-dione	*Polypodium vulgare* *Spinacia oleracea*	22,25dE + 22dE 20E	Reixach et al., 1999 Bakrim, 2007
22,25-Dideoxyecdysone	*Achyranthes fauriei*	20E + Inokosterone	Tomita and Sakurai, 1974
2,22-Dideoxyedysone	*Polypodium vulgare*	22dE	Reixach et al., 1999
2-Deoxyecdysone	*Polypodium vulgare* *Spinacia oleracea*	E + 20E 2d20E + E + 20E	Reixach et al., 1999 Bakrim et al., 2008
Ponasterone A	*Polypodium vulgare*	20E	Reixach et al., 1999
Ecdysone	*Polypodium vulgare* *Sesuvium portulacastrum* *Spinacia oleracea*	20E (+ polypodine B + abutasterone) 20E 20E + polypodine B + E conjugate	Reixach et al., 1996 Sipahimalani et al., 1972 Grebenok and Adler, 1993; Grebenok et al., 1994

(Cook et al., 1973), then [4-^{14}C,3α-^{3}H] or [4-^{14}C, 4α-^{3}H] or [4-^{14}C,4β-^{3}H] for understanding the formation of the chromophore (*Polypodium vulgare*; Davies et al., 1980) (Fig. 1.3).

More recently, molecules labelled with stable isotopes have allowed a more convenient and exact approach with the *A. reptans* hairy root system. After labelling with [3α-^{2}H]-, [4α-^{2}H]- or [4β-^{2}H]cholesterol, it was shown that, in

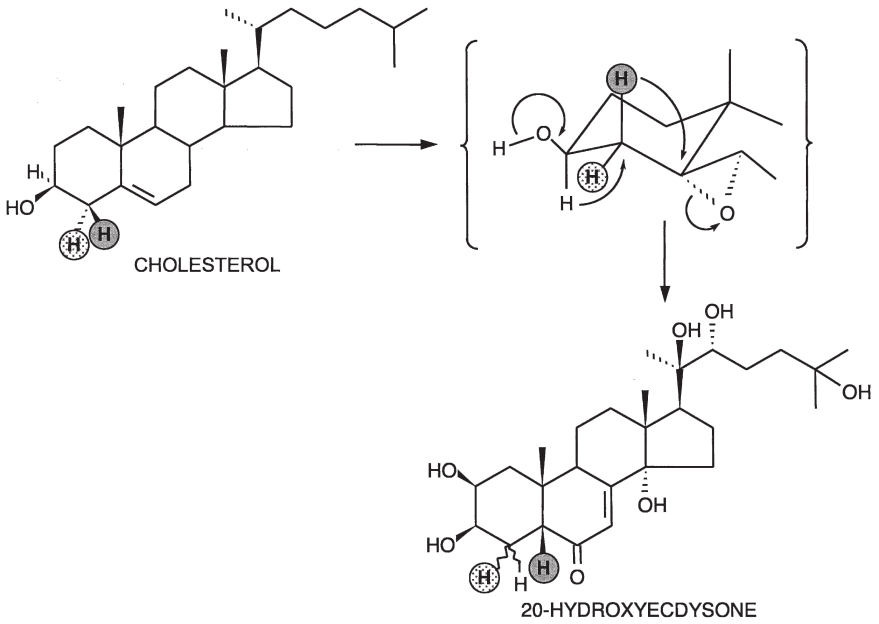

Fig. 1.3 Proposed early steps of ecdysteroid biosynthesis in *Polypodium vulgare* (Davies et al., 1980)

these three cases, deuterium atoms remained in the same positions, suggesting a direct mechanism involving carbons 5 and 6, and no oxidation at C-3 (Nagakari et al., 1994a). Further labelling experiments with [6-^{2}H]- or [3α,6-$^{2}H_{2}$]cholesterol showed that the 6-H proton of cholesterol was found in position-5β in 20E (Fujimoto et al., 1997). These data were consistent with the intermediate formation of a 5α,6α–epoxide. However, the mechanism is probably not so simple, because only 70% of the deuterium is conserved, and this non-stoichiometric behaviour has received no explanation.

Labelling experiments with a Δ^{7}-sterol, [3α,6α-$^{2}H_{2}$]- or [3α,6β-$^{2}H_{2}$]lathosterol, have shown that the 6β-proton migrates to the 5β–position, whereas the 6α-H is eliminated (Ohyama et al., 1999) (Fig. 1.4). These data, taken together with the above, suggest that cholesterol and lathosterol are converted into 20E through the formation of 7-dehydrocholesterol. Here again, 30% of the deuterium is lost during the migration of the 6β-^{2}H to C-5. However, this means that direct oxidation of C-6 of lathosterol is not involved. According to this scheme, 14α-hydroxylation would represent an independent step, which is confirmed by the conversion of 3β-hydroxy-5β-cholest-7-en-6-one (5β-ketol) into 20-hydroxyecdysone (Hyodo and Fujimoto, 2000). In this respect, we are reminded that 14β-hydroxylation is a separate step in cardenolide biosynthesis in plants (Kreis et al., 1998). In the same way, the origin

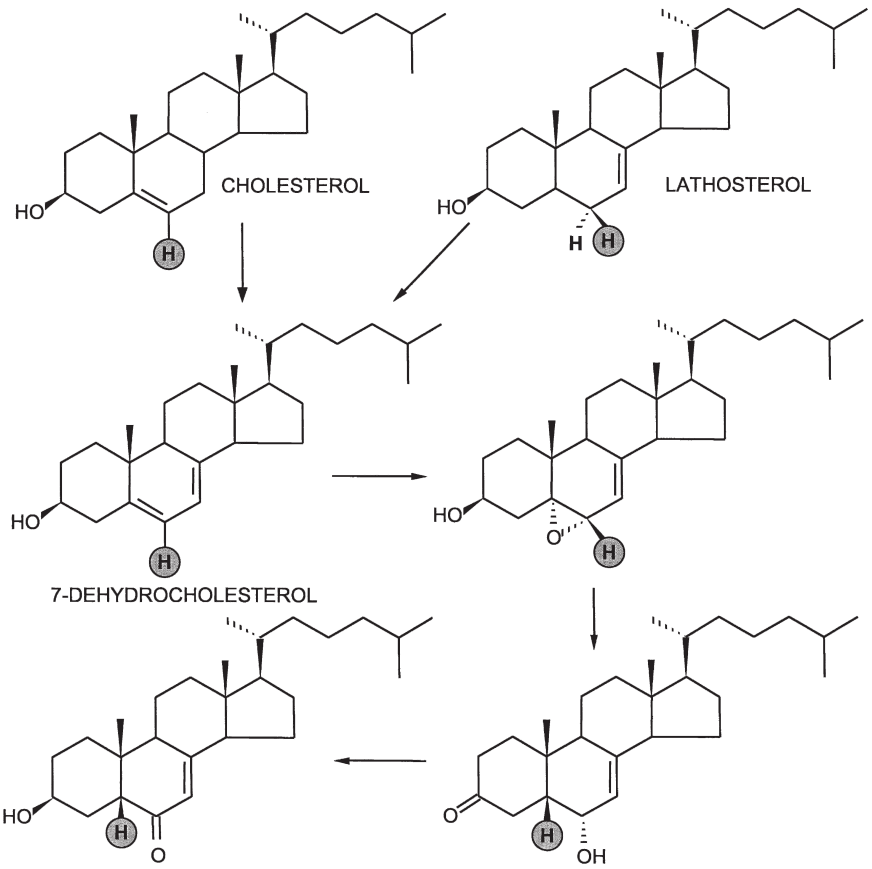

Fig. 1.4 Proposed early steps of ecdysteroid biosynthesis in *Ajuga reptans* (Fujimoto et al., 1997; Ohyama et al., 1999)

of polypodine B, and more generally of 5βOH-ecdysteroids, might result from a late 5β-hydroxylase, as evidenced by the (low) conversion of labelled ecdysone into polypodine B in *Spinacia oleracea* (Grebenok and Adler, 1993) and *Polypodium vulgare* (Reixach et al., 1996).

Finally, and most unexpectedly, [3α-^{2}H]3β-hydroxy-5β-cholestan-6-one and [5β,7α,7β-^{2}H]2β,3β-dihydroxy-5β-cholestan-6-one are efficiently incorporated into 20E, which means that the introduction of the Δ^{7}-bond may take place at a late stage in the biosynthetic sequence (Hyodo and Fujimoto, 2000).

These data permit the conclusion that formation of the (5β–H)14α–OH-7-en-6-one chromophore may proceed by at least two different routes in plants (both differing from that in animals?). Whether this means that the capacity to synthesize

ecdysteroids has appeared several times independently remains to be established. To address this question, it will be absolutely necessary to develop molecular tools. Further steps (mainly hydroxylations) may also occur in different sequences; so, like the biosynthesis of brassinosteroids (Bishop, 2007), we should perhaps consider the biosynthetic pathway for phytoecdysteroids as a grid rather than a linear sequence, with possibly privileged ways in any given species.

Finally, we wish to underline the absolute need for molecular biological approaches in this area in order to address the following basic questions: (1) In which plant cells (all or specialized ones?) does ecdysteroid biosynthesis take place? (2) Does the whole pathway take place in the same cells or does it involve some 'cooperation', as is often the case for animal steroids? (3) How conserved are the concerned genes in the plant kingdom and in fungi? (4) Are these genes present in all plants species, but silent (or very poorly expressed) in most of them?

1.3.5 Biogenetic Diversity of Phytoecdysteroids

The current biosynthetic studies have so far not explained the formation of the main feature of ecdysteroid structural specificity, i.e. the 14α-hydroxy-7-en-6-one moiety, but have provided some information on the sequence of hydroxylations. Some evidence exists for the occurrence of multiple or branching pathways (see Figs. 1.3 and 1.4). In some studies the main effort has concentrated on the elucidation of the formation of the 5β–H configuration, which is characteristic for ecdysteroids, or the 5β-OH substituent present in certain analogues. However, the structural variability of ecdysteroids, even if maintaining the above indicated characteristic features, is much higher. Some variability is associated with the basic ecdysteroid carbocyclic part of the skeleton, e.g. the presence of hydroxyls at positions C-1, C-11, and less frequently also at C-9 or C-15. Further variability is elicited by 3-*epi*-OH, or a 2,3-*diepi*-OH configuration, C-2 deoxy or C-2 dehydro formation, occasionally also in combination with a C-9(11) double bond. However, the 9(11)-double bond may be an artefact arising from the ready dehydration of 11α-hydroxyecdysteroids (Szendrei et al., 1988).

Much more variability, however, is found in the side-chain. The $C_{27}/C_{28}/C_{29}$ homology, which has already been mentioned, has been studied at various levels and types of sterols and steroids, including ecdysteroids, as reviewed by Goad and Akihisa (1997) and Brown (1998). This homology together with various hydroxy, oxo, oxy, isopropyloxy, acyloyl, aryloyl, glycosyl and other substituents, significantly enhances the structural variability. Moreover, the oxygenated side-chain derivatives are further appropriate for generating five- or six- membered cyclic ethers or lactones, depending on the side-chain homology (C_{28} or C_{29}) and on the oxygenation level and positions of participating substituents. Different configurations of the participating alkyloxy- and oxygen-containing groups induce additional increase

of variability. Formation of such a structural variability has so far only been very marginally studied by biosynthetic methods. Often, the biogenetic relations of similar structural types can only be inferred from tentative chemical relationships between the compounds. The assumed structural relationship of selected minor ecdysteroids from *Leuzea carthamoides* (Vokáč et al., 2002; Buděšínský et al., 2008) can serve as an illustration. This tentative biosynthetic scheme (Fig. 1.5) is based on the structural relations of the constituents so far identified from *L. carthamoides*, and on feasible biochemical reaction sequences. Such a sequence of reactions could be proposed only because an unusually large amount of plant material was processed (Vokáč et al., 1999), enabling identification of a large number and variety of minor constituents, which would be unattainable from amounts customarily used for routine laboratory extractions. This is why it has not been possible to derive such relationships for other rich ecdysteroid-containing genera, e.g. *Ajuga*, *Silene* or *Serratula* (see Ecdybase; Lafont et al., 2002). On the other hand, the biosynthesis of some side-chain lactones, e.g. cyasterones in the genus *Ajuga*, has already been experimentally explored (Okuzumi et al., 2003).

Even when a large range of constituents has been identified from certain species, a tentative biogenetic relationship for the so far identified ecdysteroid analogues could not be proposed, since some of the identified ecdysteroids might only be artefacts obtained by simple oxidation, dehydration or other reactions performed during plant processing or compound isolation; especially the cyclic ethers might be formed in such a way. Indirect evidence is provided by the formation of shidasterone and congeners by catalytic thermal reactions (Harmatha et al., 2002a,b), or by specific anhydride-induced dehydration (Odinokov et al., 2002). Many more biosynthetic studies are required to explain the large, and still growing, structural diversity of natural ecdysteroids, especially those of plant origin, which are collated and thoroughly characterised in Ecdybase (Lafont et al., 2002).

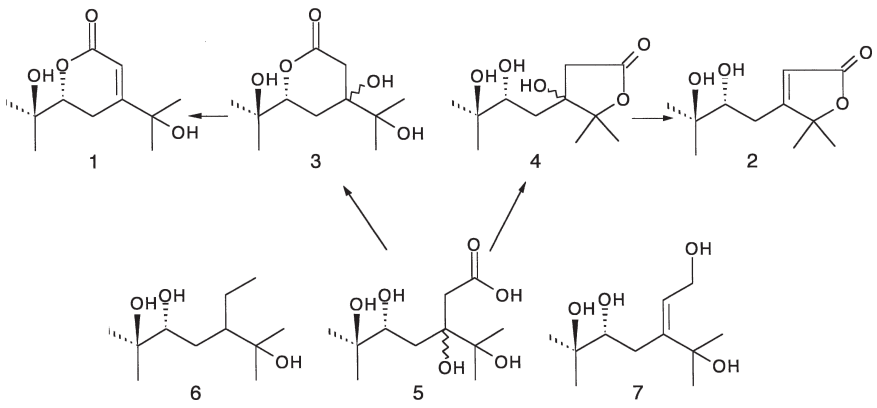

Fig. 1.5 Assumed biogenetic relations of *Leuzea carthamoides* ecdysteroids
1. leuzeasterone, 2. carthamosterone, 3. 24-hydroxy-dihydroleuzeasterone, 4. 24-hydroxy-dihydrocarthamosterone, 5. hypothetical precursor, 6. makisterone C, 7. 24(28)-dehydro- 29-hydroxy-makisterone C

The biological activities of natural ecdysteroids, or those artificially formed from the main natural metabolites, or those structurally modified by chemical transformations, are all interesting and important for structure-activity relationship studies. Almost all types have been tested for their activity at the ecdysteroid receptor (Harmatha and Dinan, 1997; Dinan et al., 1999) and the summed data evaluated by appropriate computational methods (Ravi et al., 2001), which permits mapping of the ecdysteroid receptor ligand-binding domain. Both natural ecdysteroid metabolites and the artefactual analogues derived from them serve as source compounds or leads for the preparation of new structural types by targeted chemical transformations (Bourne et al., 2002) or by unspecific, and rather unconfined, phototransformations (Harmatha et al. 2002a,b, 2006). In this way, new and unusual structural classes have been obtained, e.g. epimeric 14-*epi* analogues or dimeric derivatives (Harmatha et al., 2002a,b).

The synthetic or transformed derivatives of native ecdysteroids with unusual configurations, conformations or constitutions (not currently included in Ecdybase) significantly increase the structural variability of ecdysteroids, as well as their bioactivity value and potential. They represent new possibilities and unconventional outlooks for ecdysteroid research and for practical utilization.

1.3.6 Regulation of Biosynthesis

Any biosynthetic pathway requires regulatory mechanisms in order to control the accumulation of terminal metabolites. In the present case, we know that ecdysteroids are stable molecules which undergo, at best, a very slow turnover (Schmelz et al., 2000). As a consequence, ecdysteroids accumulate during plant life, but, owing to their possible migration within the plant, their distribution may vary greatly during ontogeny; thus, in developing spinach, ecdysteroids are produced in older leaves, but transported and accumulated in the young apical leaves (which, on the other hand, are unable to synthesize them) (Grebenok et al., 1991; Grebenok and Adler, 1993). Removal of the apical leaves (= the sink) will result in a cessation of ecdysteroid production in the older leaves (Bakrim et al., 2008). Further, loading excised leaves with unlabelled ecdysteroids will also induce a cessation of ecdysteroid production (Grebenok et al., 1991; Grebenok and Adler, 1993; Adler and Grebenok, 1995), which might result from the formation/accumulation of ecdysteroid (poly)phosphates.

Similar evidence for feed-back mechanisms which do not involve phosphate conjugates has been obtained with *Polypodium vulgare* prothalli (Reixach et al., 1997, 1999). The prothalli actively synthesize ecdysteroids (mainly 20-hydroxyecdysone) up to a certain concentration. By immersing the prothalli in hot water (45 °C for 60 min; i.e. heat shock), it is possible to induce an almost complete release of ecdysteroids, which will result in the stimulation of *de novo* ecdysteroid production up to their initial level (Reixach et al., 1997). This treatment was used

to enhance the conversion of 25-hydroxycholesterol into ecdysteroids (Reixach et al., 1999) (Fig. 1.6).

It is possible to observe both quantitative and qualitative changes during development/ageing, as described for shoots of *Taxus cuspidata* (Ripa et al., 1990) (Fig. 1.7).

Phytoecdysteroids are believed to represent a chemical protection of plants against phytophagous insects (and soil nematodes). It is therefore logical to expect that their production will be enhanced by mechanical wounding or insect attack. Indeed, this was demonstrated in *Spinacia oleracea*, but, unexpectedly, only in roots (Schmelz et al., 1998, 1999). Similarly, it was shown that jasmonate, a signalling lipid produced by plants in response to insect attack, was able to increase ecdysteroid production (Schmelz et al., 1998).

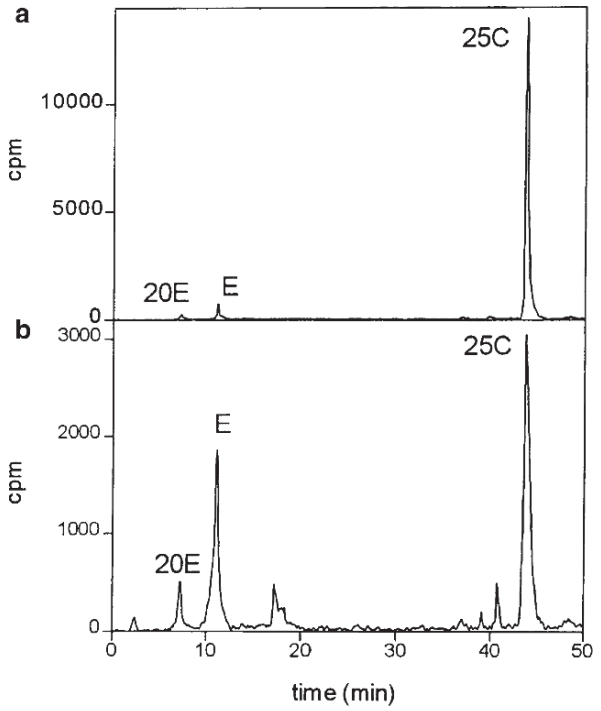

Fig. 1.6 Comparative ability of control (**panel a**) and heat-shocked (**panel b**) prothalli of *Polypodium vulgare* to convert 25-hydroxycholesterol (25C) into ecdysteroids (E and 20E). Separation occurred on a C_{18} RP-HPLC column (25 cm × 4.6 mm; 5 µm particle size) eluted at 1 ml/min with a gradient of acetonitrile/isopropanol (5:2 v/v; Solvent B) in 0.1% TFA in water (Solvent A), pre-equilbrated at 15% B and eluted as follows from the time of injection: linear gradient from 15–25% B over 2 min, isocratic at 25% B for 6 min, linear gradient from 25–75% B over 20 min, isocratic at 75% B for 22 min, linear gradient from 75–90% B over 5 min, linear gradient from 90–100% B over 5 min and isocratic at 100% B for 15 min (Reixach et al., 1999)

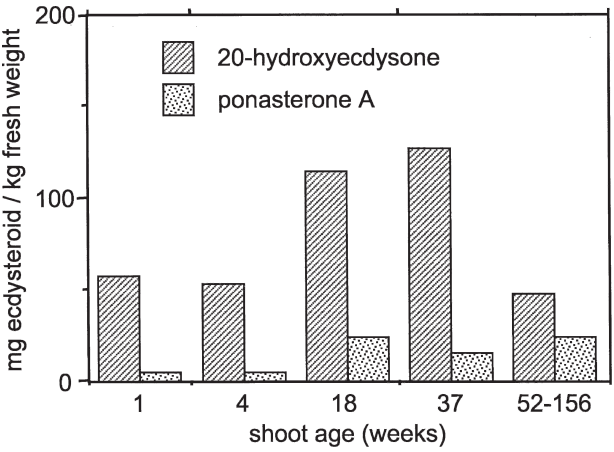

Fig. 1.7 Variation in ecdysteroid content with ageing in *Taxus cuspidata* shoots (Redrawn from Ripa et al., 1990)

Could feed-back mechanisms be responsible for the inhibition of ecdysteroid production in non-accumulating plant species? This question was raised by the data obtained by Devarenne et al. (1995) working with maize plants (*Zea mays*). This plant does not accumulate detectable amounts of ecdysteroids; however, after long-term labelling with [^{14}C]mevalonate, the authors isolated labelled ecdysone and 20-hydroxyecdysone conjugates which released the free ecdysteroids upon glycosidase treatment. If confirmed, these data would mean that possibly every plant species is able to produce ecdysteroids and explain the surprising (uneven?) distribution of ecdysteroid-accumulating species in the plant kingdom. Another possible inhibition may be explained by assumedly preferential biosynthesis of steroid saponins, which can bind simple sterols into water insoluble complexes, and thus eliminate them from the possibility of being ecdysteroid precursors. Such a mechanism was already suggested at a plant-insect interaction level (Harmatha, 2000) for *Allium porrum* (Arnault et al., 1986), belonging to *Liliaceae* family, known as a generally ecdysteroid-negative plant group.

1.4 Plant Cell Cultures as Model Systems for Ecdysteroid Production

1.4.1 Introduction

The transition from intact plant to cultivated plant cells *in vitro*, a process involving repression of cell differentiation as well as specialization, is accompanied, as a rule, by a sharp decline in the biosynthesis of specialized secondary metabolites

(Zaprometov, 1981). This is accounted for by de-differentiated plant cells partly losing the ability to express genetic information related to secondary metabolism. The loss is not irreversible, but certain conditions are required for its continued expression. Understanding these conditions is an important challenge.

Ecdysteroid production by plant cell cultures is of both scientific and practical interest. On the one hand, plant cell cultures are an amenable experimental model for the elucidation of ecdysteroid biosynthetic pathways and their regulation mechanisms; this is also important for understanding ecdysteroid function in plants. On the other hand, cell cultures could be used for the production of specific ecdysteroids by bioengineering techniques. Moreover, the possibility of obtaining significant amounts of 20-hydroxyecdysone (20E), which has potential in human medicine (Lafont and Dinan, 2003), as well as other rarer ecdysteroid analogues, which may also possess interesting biological properties (Báthori and Pongrácz, 2005), is promising.

1.4.2 Callus and Suspension Cultures

Ecdysteroid formation *in vitro* was originally demonstrated in callus cultures of *Achyranthes fauriei* (Amaranthaceae; Hikino et al., 1971) and *Trianthema portulacastrum* (Aizoaceae; Ravishankar and Mehta, 1979), as well as in gametophytes of the fern *Pteridium aquilinum* cultivated in liquid medium (McMorris and Voeller, 1971). More intensive research into secondary metabolism in tissue and cell cultures of ecdysteroid-containing plants started in 1990s. Species with high phytoecdysteroid content were introduced into culture *in vitro*. Prothalli of the fern *Polypodium vulgare* cultivated under aseptic conditions produced the phytoecdysteroids characteristic of intact plant roots (20E, polypodine B [polB] and ecdysone [E]); the 20E content in prothalli (as high as 0.8% of the dry mass) was twice that found in the roots and the 20E:polB ratio varied from 7:1 to 10:1 (in wild plants, the ratio is between 1:1 and 2:1) (Camps et al., 1990). Five further phytoecdysteroids (inokosterone, pterosterone, abutasterone, 24-hydroxyecdysone and 5β-hydroxyabutasterone) previously unknown in the fern *P. vulgare* were released from prothalli in culture (Coll et al., 1994). The high biosynthetic quotient of prothallus cultures permitted the development of a method for obtaining 20E (J.-J. Bonet, IRTA Institute, Barcelona, Spain, personal communication).

P. vulgare prothallus and callus cultures are good experimental systems for ecdysteroid biosynthetic studies. Calluses, however low their ecdysteroid content may be, efficiently transformed E added to the medium into 20E (Irrure-Santilari et al., 1996a). Tracer precursors (mevalonate, cholesterol and E) added to the calluses were incorporated into all ecdysteroids synthesized by this culture (Irrure-Santilari et al., 1996b). Compounds less polar than E were also investigated as possible precursors; poA and 2dE were transformed in both prothallus and callus cultures, whereas 2,22dE and 2,22,25tE were transformed into ecdysteroids atypical for *P. vulgare* (Reixach et al., 1999).

Callus and suspension cultures were obtained from *Pteridium aquilinum* fern spores (Vanek et al., 1990; Maček and Vanek, 1994). The concentration of ecdysteroids in suspension culture exceeded that in the intact plant by 20-fold in a medium optimized for nutrient components and phytohormones. The synthetic ability of callus cultures was much lower. PoA, 20E, polB, E and five unidentified compounds were found in the cell cultures. Allowing for the ecdysteroid accumulation rate by suspension culture during the cultivation cycle, a procedure was developed which increased the yield of the target products by elimination of the lag-phase from the cultivation cycle and by maintaining the cells at the optimal growth rate (Svatos and Maček, 1994); the cells accumulated up to 0.89 mg ecdysteroid per gram dry weight, poA being the major ecdysteroid.

Cell cultures of *Serratula coronata* (Karnachuk et al., 1991) and *S. tinctoria* (Corio-Costet et al., 1996) are of great interest as a source of ecdysteroids. Wild plants of these species have high contents of various bioactive ecdysteroids. In *S. coronata* suspension culture, the total cell ecdysteroid content amounted to 0.074% of the dry matter, while the ecdysteroid concentration in the callus tissue was an order of magnitude lower (Saad et al., 1992a, b). The highest concentration of ecdysteroids was present during the exponential phase of cell growth. A comparative study of the composition and content of ecdysteroids and their precursors (i.e. sterols) in intact *S. tinctoria* plants and callus and suspension cultures showed that plant roots contain 20E, 20E 3-acetate and polB at a total concentration of 1.2–1.5% of the dry weight, whereas cell cultures contained only 20E, the content being 0.003–0.01% (Corio-Costet et al., 1993a). Based on the data obtained, a biosynthetic scheme from expected ecdysteroid precursors could be proposed.

A steroidogenetic study (including the detection of sterols which are most likely to be ecdysteroid precursors) was carried out on a suspension culture of *Chenopodium album* (Corio-Costet et al., 1993b, 1998). The cells produced 20E in much lower quantities than the intact plants. However, the possibility of increasing the level of ecdysteroid biosynthesis by using some cultivation procedures was noted.

Cell cultures of different species of *Ajuga* have been obtained. Synthesis of 20E and turkesterone was established in both callus and suspension cultures of *A. turkestanica*, the 20E concentration in the cells being several times higher than that in the roots and leaves of the plants, whereas the turkesterone concentration was somewhat lower than in the intact plant (Lev et al., 1990). A number of studies has been carried out on sterile culture and *A. reptans* shoots and roots cultivated separately (Tomás et al., 1992, 1993; Camps and Coll, 1993). The plants cloned *in vitro* retained the ecdysteroid composition inherent to both intact wild plants and glasshouse plants. Ajugalactone and cyasterone (C_{29}-ecdysteroids), 29-norsengosterone and 29-norcyasterone (C_{28}-ecdysteroids) and 20E (C_{27}-ecdysteroid) were found. PolB was not found in plants *in vitro*, but was detected in intact plants. The ecdysteroid content in the roots of the plants *in vitro* amounted to 0.4% of the dry weight and was 1.5–2.5 times higher than that in intact plant roots. The ratio of C_{28}/C_{29}-phytoecdysteroids in intact and in *in vitro* cloned plants was substantially different. *A. reptans* roots were established to be the site of ecdysteroid biosynthesis. Based on the overall data obtained both under *in vivo* and *in vitro* conditions, the existence of two major metabolic pathways in *A. reptans* was pro-

posed (Camps and Coll, 1993), involving side-chain dealkylation and differing in hydroxylation at C-5, and resulting in two groups of biogenetically related C_{29}/C_{28}/C_{27} compounds (cyasterone/29-norcyasterone/20E and sengosterone/29-norsengosterone/polB), which differ in the absence or presence of an OH-group at C-5. Dealkylation occurs mainly in the roots of the plant. The presence of ecdysteroids was not detected in *A. reptans* callus cultures by Tomás et al. (1992). However, trace amounts of 20E were detected in *A. reptans* callus and suspension cultures by Mboma et al. (1986).

Recently, cell suspension cultures of *Vitex glabrata* have been used to obtain evidence that 7-dehydrocholesterol and ergosterol are ecdysteroid precursors since supplementation with these two sterols enhanced 20-hydroxecdysone production by the cells, whereas addition of cholesterol did not (Sinlaparaya et al., 2007).

1.4.3 'Hairy-Root' Cultures

Hairy root culture models, i.e. a genetically modified "bearded" root culture resulting from the treatment of plants by *Agrobacterium rhizogenes*, have several advantages over normal isolated root cultures (Kuzovkina, 1992).

A hairy-root culture with stable and sufficiently high ecdysteroid synthesis level (0.1–0.2% of dry weight) was obtained from a sterile sprout of *S. tinctoria* by means of modification with *A. rhizogenes*. The roots of intact plants, as well as the hairy roots, produced 20E and its 3-acetate. In both cases, a concentration gradient increasing toward meristematic zones was detected. After inclusion of labelled precursors, such as cholesterol and mevalonate, into *S. tinctoria* hairy roots, they synthesized labelled ecdysteroids, making this system an appropriate model for research into ecdysteroid metabolism (Delbecque et al., 1995; Corio-Costet et al., 1996).

Hairy root cultures have also been obtained for a number of other ecdysteroid-producing plant species: *Ajuga reptans var. artropurpurea*, *Achyranthes fauriei*, *Pfaffia iresinoides* and *Vitex strickeri* (Matsumoto and Tanaka, 1991). All of the *A. reptans* hairy root clones were shown to synthesize 20E, norcyasterone, cyasterone and isocyasterone, which are characteristic ecdysteroids of intact plant roots. The component ratio was also similar to that in intact plant roots, with 20E prevailing. There was a positive correlation between the ecdysteroid content and the clone growth-rate. Selection resulted in the isolation of the rapidly growing Ar-4 clone, where the 20E concentration amounted to 0.14% of the dry weight. When growing the Ar-4 clone in an Airlift-type fermenter for 45 days, the weight of the culture increased 230-fold, and the 20E content was as much as 0.12% of dry weight.

Regenerated plants cultivated from high-producing hairy root clones of *A. reptans var. atropurpurea* were shown to have higher ecdysteroid accumulation than untransformed regenerants (Tanaka and Matsumoto, 1993). The transformed plants had root/total plant weight ratios of 68–75%, compared to 50% for normal regenerant plants, the ecdysteroid content being as high as in the parent clones. Thus, the possibility of obtaining microclonal reproduction of modified plants with

an increased ability to produce ecdysteroids was demonstrated, with the associated possibility of creating phytophagous insect-resistant plants.

Genetically modified rhizogenic culture of *A. reptans var. atropurpurea* also proved to be a boon for ecdysteroid biosynthesis research (see Section 1.3.4).

Transformed *Rhaponticum carthamoides* (Willd.) Iljin root cultures were obtained by modifying sterile shoots with *A. rhizogenes*, the cultures differing qualitatively and quantitatively in ecdysteroid content from that in the intact plant roots (Orlova et al., 1998). These authors also developed an effective system for regeneration from leaf explants and carried out genetic modification of this plant species with a *A. rhizogenes* GV 3101 recombinant strain containing *rolC*-gene plasmid under a 35S CaMV promoter and with the inherent dwarf phenotype (Orlova et al., 2000). In the authors' opinion, the known multiple effects of *rolC*-gene, e.g. changing the hormonal status of modified plants (Estruch et al., 1991), are the probable cause of the changes in the qualitative and quantitative content of secondary metabolites, i.e. ecdysteroids.

1.4.4 General Conclusions and Prospects for Future Research

The potential and convenience for ecdysteroid biosynthesis research has been demonstrated with various experimental systems *in vitro*, including non-differentiated cells, morphogenic structures, isolated organs and sterile plant culture. The data obtained show the possibility of regulating phytoecdysteroid synthesis both at cell and organ levels. *In vitro* cultures with high biosynthetic activity are of interest from the point of view of developing bioengineering techniques for obtaining valuable bioactive ecdysteroids, few of which are commercially available.

A comparative study of ecdysteroid formation in cell cultures of three species of plants has been conducted with *Rh. carthamoides*, *S. coronata* (both belonging to the Asteraceae) and *Ajuga reptans* (Lamiaceae). They differ in both the composition and levels of ecdysteroids in the intact plants (Volodin, 2003). *Rh. carthamoides* callus cultures obtained from different organs of the plant were found to be incapable of ecdysteroid synthesis or to synthesize them only in trace quantities. The level of ecdysteroid synthesis in *Rh. carthamoides* cell cultures increased relative to that of the intact plant only by utilizing cell cultivation in suspension culture. Of major importance was a correlation between the highest ecdysteroid synthesis level and the expotential growth phase of suspension cultures. In contrast to *Rh. carthamoides*, young *S. coronata* callus cultures retain the ability to synthesize ecdysteroids. However, there is a significant decrease in their concentration in the callus in comparison with the intact plant (five-fold, on average). *A. reptans* plants are characterized by a low ecdysteroid content. However, in contrast to *Rh. carthamoides* callus cultures which are incapable of synthesizing significant amounts of ecdysteroids, strains of *A. reptans* cultures were obtained which surpassed both plants cultivated *in vitro* and wild plants in their ecdysteroid content. The content of 20E was found to increase significantly during long-term

(for 9 years) cultivation of *S. coronata* and *A. reptans* callus cultures. This is likely to be a result of cell differentiation over time. Thus, ecdysteroid-producing cell cultures were shown to retain their biosynthetic abilities to different extents. In young callus cultures with high or moderate ecdysteroid content (*S. coronata* and *Rh. carthamoides*), a decrease in ecdysteroid synthesis occurs. After being introduced into culture, species with low ecdysteroid content, such as *A. reptans* match, or even surpass, intact plants as far as ecdysteroid accumulation is concerned. Moreover, ecdysteroid accumulation dynamics in cell cultures of phylogenetically distant species is similar in spite of the difference in morphological features. The data obtained are of considerable importance for a better understanding of ecdysteroid functions in plants. If ecdysteroids act as toxins/deterrents towards phytophagous insects in plants with high or moderate content of the former, the repression of ecdysteroid synthesis occurring in de-differentiated-cell cultures could be accounted for by the fact that, in the absence of organismal control, there is no longer a need for these compounds as eco-regulators (Volodin, 2003).

In future studies, attention should be given to the regulation of ecdysteroid synthesis in plant cell cultures. An opportunity was opened up by the isolation of ecdysone-20-monooxygenase from the leaves of spinach, *Spinacia oleracea L.*, and demonstration of the induction of ecdysteroid accumulation in plants attacked by insects. The effect demonstrated in spinach subjected to attack by the fungus gnat *Bradysia impatiens* (Johannsen) (Schmelz et al., 2002) is triggered by methyl jasmonate, which is a plant pathogen signal transducer. Encouraging results have been achieved from the use of cytochrome-P_{450} inducers (manganese salts, exogenic sterols, phytohormones, methyl jasmonate) for promoting ecdysteroid synthesis in cell cultures of ecdysteroid-containing plants (Alexeeva, 2004, 2005, 2006).

1.5 Do Phytoecdysteroids Have a Physiological Role in Plants?

Based on the hormonal role of ecdysteroids in arthropods, it was suggested that phytoecdysteroids might regulate physiological processes in plants. The early evidence has been summarised previously (Lafont, 1998; Dinan, 2001) and it is not convincing for such a role. Basically, ecdysteroids have been employed in a series of standard plant bioassays used for determination of the activities of phytohormones (auxins, brassinosteroids, cytokinins, ethylene formation, giberellins: Hendrix and Jones, 1972; Dreier and Towers, 1988; Macháčková et al., 1995; Golovatskaya, 2004). Activities were either absent or slight. *A priori*, it seems unlikely that ecdysteroids could possess a hormonal role in plants, since their occurrence is not universal and yet, when they do occur, the levels can be very high, far surpassing those expected of hormonal molecules. The varying distributions within ecdysteroid-containing plants and seasonal variations also accord better with an allelochemical function (see below), and it is, of course, now known that the hormonal steroids in plants are the brassinosteroids. However, ecdysteroids do, by an

as yet unexplained mechanism, appear to significantly affect growth, cell size and biochemical properties both in the cyanobacterium *Nostoc* 6720 (Maršálek et al., 1992) and in *Chlorella vulgaris* (Bajguz and Dinan, 2004). Also, it is possible that phytoecdysteroids could be released by plants to have allelochemical effects on other plants or microbes in their environment, since 20E has been shown to affect seed germination and seedling growth (DellaGreca et al., 2005; Bakrim et al., 2007), and ecdysteroids have been shown to possess antimicrobial activity (Ahmad et al., 1996).

1.6 Allelochemical Functions of Phytoecdysteroids

1.6.1 Probable Function of Phytoecdysteroids

The most generally accepted hypothesis for the function of phytoecdysteroids in plants is that they act, either alone or in conjunction with other classes of secondary metabolites or even together with physical defence mechanisms, to protect the plant against non-adapted invertebrate predators, bringing about reduced consumption of the plant or even endocrine disruption and death of the phytophagous invertebrate. In general, it has been the effects on phytophagous insects which have been considered, but plant nematodes and even crustaceans have also been examined to a lesser extent.

1.6.2 Ecdysteroid Effects on Insects

1.6.2.1 Effects of 20-Hydroxyecdysone on Insects

Ingested ecdysteroids (predominantly 20E) have been shown to have a range of detrimental effects on the development and survival of a number of insect species (*Bombyx mori* [Kubo et al., 1983], *Pectinophora gossypiella* [Kubo et al., 1981, 1983], *Spodoptera frugiperda* [Kubo et al., 1981], *Acrolepiopsis assectella* [Arnault and Sláma, 1986; Harmatha, 2000], *Agrius convolvulus* [Tanaka and Naya, 1995]), including inhibition of growth, supernumerary larval instars, death without moulting, death associated with promoted moulting and prothetely. However, certain insect species (e.g. *Heliothis virescens*, *H. armigera*, *Locusta migratoria*, *Manduca sexta*, *Spodoptera littoralis*, *Lacanobia olereaceae*, *Acherontia atropos*; summarised in Dinan, 1998) are remarkably tolerant to ecdysteroids in their diet, showing no apparent ill-effects when fed 400 ppm or more 20E in their diet (although *S. littoralis* larvae are affected by 1,000 ppm 20E; Ufimtsev et al., 2006a). However, several of these species (*H. virescens*, *H. armigera*, *S. littoralis*, *L. oleracea*) and *Ostrinia nubilalis* (Rharrabe et al., 2007) detoxify the ecdysteroid intake by conjugating them to fatty acids, thereby blocking the C-22 hydroxyl group, which

is important for the biological activity of ecdysteroids (Dinan and Hormann, 2005). Such a detoxification mechanism has a considerable energy cost, such that although the insect may develop normally when food it plentiful, the energy demand will be detrimental to insect development when food is more scarce. In addition to conjugation with fatty acids, insects use several other detoxification mechanisms for ingested ecdysteroids (22-glucosides, 2/22-phosphates, 3-acetates, 3-oxo/3-*epi* derivatives and side-chain cleavage; Rharrabe et al., 2007; see Fig. 1.8), or even excreting ingested 20E unmetabolised (e.g. *Acherontia atropos* [Blackford and Dinan, 1997c]). Other insect species are partially tolerant to ecdysteroids in their diet, being able to cope with low levels (e.g. *Cynthia cardui*, *Tyria jacobaeae*; Blackford and Dinan, 1997b), while higher levels are toxic. Yet other species are extremely sensitive to ecdysteroids in the diet, such that they eat and succumb at very low concentrations (e.g. *Aglais urticae*), or are so deterred from eating an ecdysteroid-containing diet that they die of hunger rather than consume ecdysteroid-containing food (e.g. *Inachis io*; Blackford and Dinan, 1997b). There seems, as might be expected, to be a relationship between the tolerance of the phytophagous insect species and the probability of it encountering ecdysteroids in its diet, such that highly polyphagous species are ecdysteroid-tolerant, while oligophagous and polyphagous species are semi-tolerant and monophagous species are ecdysteroid-sensitive.

Recently, Malausa et al. (2006) have examined the genetic variability of the response of six clones of the peach-potato aphid (*Myzus persicae*) to moderate (10^{-6}M) and high (10^{-3}M) concentrations of 20E by measuring fecundity (number of offspring produced) and found that they differ very considerably. The fecundity was decreased, unaffected or increased, depending on clone, at the moderate 20E concentration relative to controls (no 20E), whereas it was the same or reduced at the high 20E concentration.

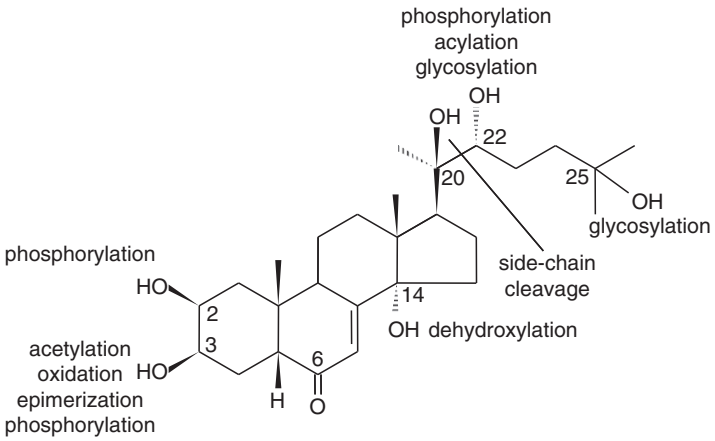

Fig. 1.8 Summary of the detoxification pathways for ingested phytoecdysteroids in insects

1.6.2.2 Effects of Other Purified Ecdysteroids on Insects

Most studies have used ecdysteroid preparations where 20E is the sole or major component. 20E is without doubt the most commonly occurring phytoecdysteroid, but an important question concerns the raison d'être of the many other phytoecdysteroid analogues found in plants; Do they possess differential activities? Are they active against different insect species? Are they detected differentially? Are they subject to different metabolic rates or fates? Are the minor components there to provide evolutionary flexibility for when potential predators become tolerant of the major ecdysteroids? There are few studies which compare the activities of a significant number of ecdysteroid analogues, and where such studies have been performed *in vivo* (Sláma et al., 1993), the ecdysteroids have been injected into the test species, which does not mimic the natural route of exposure to phytoecdysteroids.

1.6.2.3 Effects of *Serratula coronata* Extracts and Its Ecdysteroids on Insects

Long-term investigations on the impact of *Serratula coronata* L. (Asteraceae) leaves, extracts and total ecdysteroids, or of isolated major and minor ecdysteroids of the plant, incorporated into artificial nutrient media on the viability and behaviour of different age caterpillars of three species of polyphagous insects, namely *Ostrinia nubilalis* Hb., *Mamestra brassicae* L. and *Spodoptera littoralis* Boisd., have been performed. Significant antifeedant effects of ecdysteroids in different artificial nutrient media were observed for first instar larvae of *O. nubilalis* and *M. brassicae*. This was associated with mass migration of the caterpillars from the feedstuff and their death. A difference was found in the effect of ecdysteroid-containing nutrient media effect on third and fourth instar larvae of *M. brassicae*, when compared to that on the first instar larvae. Diets of different ecdysteroid contents first stimulated nutrition of the older larvae, and then rejection of the feedstuff occurred, resulting in an outburst of cannibalism and mass caterpillar lethality. Caterpillars kept on the ecdysteroid-containing diet revealed developmental defects, significantly impaired pupation and formation of nonviable pupae with various abnormalities. With *O. nubilalis* caterpillars of all age groups, the ecdysteroid-containing diet was more toxic than for *M. brassicae*, causing total lethality (Volodin, 2003; Ufimtsev et al., 2001).

A detrimental effect (reduced caterpillar weight, delayed pupation and reduced adult fecundity) of a diet containing 1,000 ppm 20E on sixth instar larvae of *S. littoralis*, previously considered to be resistant to high 20E concentrations (400 ppm; Blackford et al., 1996), was observed. Ecdysone at 1,000 ppm had no effect. When fourth instar larvae were fed on nutrient media containing 10% powdered parts of *S. coronata* (containing 100–2,200 ppm ecdysteroids), the detrimental effects were shown to occur in the absence of a pronounced antifeedant effect. However, distinct correlations between the expressivity of the effects and the ecdysteroid content in different parts of the plants (roots, stems, leaves and buds) were not found

(Ufimtsev et al., 2003, 2006a, b), which imply that other components of the plant are responsible for, or contribute to, the effects.

In comparative experiments on wild-type and the mutant line 147 *Drosophila virilis* (with enhanced levels of E and 20E and diminished JH levels) an effect of exogenous ecdysteroids on the fecundity and a delay in egg-laying by wild-type imagos was shown to occur. Female flies with artificially enhanced 20E level were found to undergo a decrease in JH degradation (and consequently, an increase in its titre), mediated through the dopamine metabolism system (Rauschenbach et al., 2005).

The development of last instar larvae of *Ephestia kühniella*, after having been submerged in ecdysteroid-containing solutions, revealed diverse effects, depending both on the structure and the concentration of ecdysteroids, as well as on the nature of the solvent and the duration of submersion. Methanolic 20E solutions were less toxic than pure methanol (which caused total caterpillar lethality) or aqueous 20E solutions. This was attributed to the adaptogenic properties of 20E when in the presence of the damaging agent (methanol in this case). 20E 22-acetate and 25*S*-inokosterone do not possess this property. After submersion of caterpillars in methanolic 20E 22-acetate or methanolic or aqueous 25*S*-inokosterone solutions, pupation occurred much more rapidly than after submersion into 20E solutions. However, the number of abnormal pupae was far higher (Volodin, 2003; Ufimtsev et al., 2002a, b).

1.6.2.4 Ecdysteroid Taste Receptors in Insects

Where phytoecdysteroids act as deterrents, the insect must possess taste receptors to be able to recognise the presence of these compounds in the food. The location and properties of such taste receptors are now starting to be investigated (Ma, 1969; Tanaka et al., 1994; Descoins and Marion-Poll, 1999; Marion-Poll and Descoins, 2002).

Initially, it was shown for *Pieris brassicae* (Ma, 1969) and *Bombyx mori* (Tanaka et al., 1994) that a reduction in food intake is mediated by specialised sensory perception of ecdysteroids. With the earlier studies on these two species with specialised diets (containing no or low levels of ecdysteroids), Descoins and Marion-Poll examined taste detection of three ecdysteroids (E, 20E and poA) in three polyphagous (*Mamestra brassicae*, *Spodoptera littoralis* and *Ostrinia nubilalis*) and one monophagous (*B. mori*) species (Descoins and Marion-Poll, 1999; Marion-Poll and Descoins, 2002). Electrophysiological studies demonstrated that all four species possess contact chemoreceptor cells on the maxilla, which respond to ecdysteroids (but not necessarily to all three), indicating that perception of ecdysteroids by phytophagous lepidopterans is a common feature, even if the toxicity or antifeedant activity of phytoecdysteroids differs between the species, such that, when given a choice, larvae of these species will avoid ecdysteroid-containing food and show preference for an ecdysteroid-free diet.

Since the survival of first instar lepidopteran larvae is highly dependent on the quality of food available to them on hatching, the ability of the adult female to detect good host plants for oviposition is crucial. For both *Lobesia botrana* (Calas

et al., 2006) and *Ostrinia nubilalis* (Calas et al., 2007), it has been shown that 20E deters oviposition by adult females and that this is mediated by tarsal sensilla, which demonstrate a sensitivity similar to that of the first instar larvae to the same compound. In the European Grapevine moth (*Lobesia botrana*), 20E deters not only larval feeding, but also oviposition by adult females (Calas et al., 2006), the taste sensilla being located on the last tarsus of the prothoracic leg of the adult female. This is clearly an important strategy since neonate larvae of this species have limited dispersal capacities, such that the eggs should be deposited by the adult female on to an adequate food source for larval development.

1.6.2.5 Do High Levels of Phytoecdysteroids Deter Phytophagous Insects?

Although all parts of *L. carthamoides* contain very high levels of ecdysteroids (300–1,000 ppm 20E equivalents in the leaves), there is an extensive diversity of insect species which associate with this plant. Zeleny et al. (1997) found 126 species of arthropods on plants of this species in the Czech Republic (to where it had been introduced from Asia) over 2 years, of which 74 fed on the leaves and 34 could complete their development on the plant without any apparent problems. The most abundant arthropods on the plant were all oligo- or phytophagous and belonged to groups of arthropods (collembolans, psocopterans etc.) which have so far been poorly studied with regard to their response to phytoecdysteroids; extensively studied groups which are known to be, at least partially, susceptible to ecdysteroids e.g. lepidopterans were essentially absent from the plants. This study underlines how inadequate our current knowledge is in terms of plants being protected by even exceedingly high levels of phytoecdysteroids and our ability to extrapolate knowledge concerning the susceptibility of certain groups of holometabolous insects to arthropods in general.

1.6.2.6 A Commercial Application of Phytoecdysteroids

In the specific case of silkworms, particularly *Bombyx mori*, the response of last instar larvae to exogenous ecdysteroids has been exploited to hasten maturation and synchronise cocoon spinning. It is important to stress that the doses used to bring about the beneficial effects on silkworms are low relative to those having detrimental effects on insects (see Section 1.6.2.1). The ecdysteroids are extracted from ecdysteroid-containing plants (see Chandrakala et al., 1998) and sprayed on to mulberry leaves, which are then given to the larvae as the first ones in the batch are about to spin their cocoons (Ninagi and Maruyama, 1996; Maribashetty et al., 1997, 2002; Trivedy et al., 2003a, b, c). It is not certain whether mulberry (*Morus nigra* or *M. alba*) contains endogenous ecdysteroids (Takemoto et al., 1967b; Blackford and Dinan, 1997c), but if it does, the levels are low. Recently, the consequences of controlled ecdysteroid application (2 µg/larva) at other times during the fifth

instar have been determined (Nair et al., 2005), showing that the quantity of silk can be significantly increased (by about 10%) by treating the insects 48 h into the fifth instar, while the time taken for cocoon formation in the batch can be reduced from 60 to 36 h by treating when the first insects in the batch begin to spin. Even more recently (Trivedy et al., 2006), an extract of ecdysteroid containing *Silene gallica* has been used for the induction of spinning and uniform maturation in *B. mori*. Spraying of last instar larvae with ecdysteroid to induce uniform maturation does not affect silk yield or quality, but spraying twice during the instar to control both spinning and maturation does reduce cocoon traits, although it does shorten development time, thereby saving on mulberry leaves and reducing the possibility of crop loss owing to disease in the last phase of rearing.

Ecdysteroids have also been proposed as a treatment for the enhancement of fecundity of honey bees in apiculture (cited in Kholodova, 2001).

1.6.3 Ecdysteroid Effects on Crustaceans

As with insects, most studies of the effects of exogenous ecdysteroids on crustaceans have been performed by injection of the compounds. Where the animals have been externally exposed to the compounds, it has generally been with smaller crustaceans, such as shrimps, where injection would be more difficult. With the snapping shrimp *Alpheus heterochelis*, for example, exposure to 20E at 5 µg/ml in the seawater was able to reduce the length of the moult cycle by up to 65% (Mellon and Greer, 1987).

The possibility of improving prawn/shrimp aquaculture by exposure of the animals to ecdysteroids has been examined. In an early study (Kanazawa et al., 1972), *Penaeus japonicus* was exposed to the phytoecdysteroids 20E (0.5–2.5 mg%), inokosterone (0.1–12.5 mg%) or cyasterone (0.5–2.5 mg%), incorporated into an artificial diet. The ecdysteroids were found to enhance moulting rate, but were also found to reduce survival and growth rates in a concentration-dependent manner. In a more recent study (Cho and Itami, 2004), an ecdysteroid-containing extract of *Achyranthes* spp. (composition and ecdysteroid content not revealed) was incorporated into diet and fed to *Marsupenaeus japonicus*. The treated shrimps showed improved weight gain (29%) over the control animals.

The effects of exposure of crustaceans to ecdysteroid agonists (steroidal or non-steroidal) and antagonists is of increasing interest and concern because of the potential of such compounds as endocrine disruptors in the environment and the suitability of various crustacean species as signal species to monitor the quality of the environment (Hutchinson, 2002).

Certain decapod crustaceans (e.g. *Carcinus maenas*, *Cancer pagurus*, *Homarus americanus*, *Astacus astacus*) have been shown to reject ecdysteroid-containing food, indicating the presence of taste receptors for ecdysteroids associated with the mouthparts (Tomaschko, 1995). The origin of this discovery goes back to

the finding that the pantopod *Pycnogonum litorale*, in addition to using 20E to regulate its moulting, produces and accumulates in the gut and cuticle very large amounts of seven other ecdysteroids, which are released to the exterior when the animal is attacked (Bückmann et al., 1986; Bückmann and Tomaschko, 1992; Tomaschko and Bückmann, 1993; Tomaschko, 1994, 1995). This raised the question as to whether these ecdysteroids were acting as defensive chemicals and the demonstration that the crab *Carcinus maenas* was indeed deterred from feeding on the pantopod by the ecdysteroids excreted from the pantopod. Further studies developed a bioassay based on the crab's rejection mechanism (Tomaschko et al., 1995) and this was used to examine the ecdysteroid specificity of the taste receptors (Tomaschko, 1995). The available evidence indicates that the ligand specificity is very different to that of nuclear ecdysteroid receptors (Dinan and Hormann, 2005), both in terms of structural specificity for ecdysteroids and recognition of diacylhydrazines. Since certain aquatic plants contain significant amounts of phytoecdysteroids (e.g. *Potamogeton* spp.; Chadin et al., 2003), these may also be protected against crustacean predators.

1.6.4 Ecdysteroid Effects on Plant Nematodes

Whether ecdysteroids possess a hormonal role in nematodes is uncertain (Chitwood, 1999), but studies on several species of nematodes have shown effects of exogenous ecdysteroids upon them, including plant nematodes which could be exposed to phytoecdysteroids. Soriano et al. (2004) demonstrated that cereal cyst nematodes (*Heterodera avenae*) exposed to exogenous 20E (>ca. 10^{-6}M) were far less able to invade roots of *Triticum aestivum*, and that exposure of *H. avenae*, *H. schachtii* (sugarbeet cyst nematode), *Meloidogyne javanica* (root-knot nematode) and *Pratylenchus neglectus* (root lesion nematode) to 20E at 5.2×10^{-5}M brought about abnormal moulting and/or mortalilty. The authors also demonstrated that *Spinacia oleracea* (spinach) plants in which ecdysteroid levels had been enhanced (Schmelz et al., 1999) by treatment with methyl jasmonate suffered reduced damage after inoculation with *H. schachtii*, *M. javanica* and *P. neglectus*. The spraying of tomato plants with a solution of ecdysone reduced infestation by the root-knot nematode *Meloidogyne incognita* (Udalova et al., 2004).

1.7 Possible Relationships Between Ecdysteroids with Other Phytosteroids

The available evidence, although far from complete or conclusive, indicates that ecdysteroids have arisen independently in arthropods and plants, and possibly several times in plants and fungi. The generally accepted explanation for this is the need for sessile plants to protect themselves against predation after the evolution of

the insects during the Devonian Period and the subsequent ensuing chemical race between plants and arthropods. This can, on the one hand, account for the large amounts of ecdysteroids found in certain plant species, the wide occurrence of phytoecdysteroids in the plant world and the large diversity of analogues present, and, on the other hand, for the diversity of biochemical and behavioural strategies found in insects to avoid or overcome phytoecdysteroids when present in their host plants (see Fig. 1.8 for a summary of the metabolic strategies). Neither side in the battle will be fully successful, since this would be too costly either in limiting necessary interactions or in energetic terms. Plants' interactions with insects are ambivalent, since many are dependent on insects for pollination. It suffices for plants to reduce predation and phytophagous insects to consume enough plant material to a level where the species are able to maintain themselves from generation to generation. The complex cocktail of phytoecdysteroids typically found in ecdysteroid-containing species can be viewed as a resource for the generation of new potent analogues to be selected for when, and if, the current major ecdysteroids become ineffective, owing to a change of predator or its ability to overcome the major ecdysteroids. Further, ecdysteroids may not act alone in the defence of the plant, but in conjunction with other classes of secondary compound or even physical defence mechanisms to give synergistic interactions, such that the level of phytoecdysteroids required to provide effective protection may be low. This appears to be the case for *Kochia scoparia* (burning bush; Dinan, 1994) and *Pteridium aquilinum* (bracken; Jones and Firn, 1978), which contain only low levels of ecdysteroids together with many other defensive components to provide good general resistance to insect attack. In fact, it appears that plants can use phytoecdysteroids in the full spectrum of defence possiblities ranging from full emphasis on one class of defensive chemicals ('all eggs in one basket') to a complex mixture of chemically highly diverse components ('hedging one's bets'), depending on species and presumably a consequence of the range, aggressivity and susceptibilities of predators they have been/are exposed to.

There are chemical and structural similarities between ecdysteroids and other classes of phytosteroids (brassinosteroids, withanolides, etc.) beyond the fact that they are all steroidal. These similarities include the types and locations of functional groups. The most marked similarities are between the ecdysteroids and the brassinosteroids, where (i) a full sterol side-chain is retained, (ii) diols are found on the A-ring and the side-chain and (iii) an oxygen-containing functional group is generally associated with ring-B. However, these similarities are more superficial than real, deriving from the 2D-representations of the molecules, rather than through thorough consideration of their 3D-structures. It is the 3D-structure which reveals particular and individual biochemical features specific to the steroid class, and these then impart specific biological properties. Thus, the stereospecific orientation of the hydroxyl groups is different, as is the nature of the A/B-ring junction (*cis* in ecdysteroids and *trans* in brassinosteroids). Admittedly, a combination of 2α,3α-diol and *trans*-ring junction in brassinosteroids or a 2β,3β-diol and *cis*-ring junction in ecdysteroids put O-2 and O-3 in similar (but not identical) spatial locations if the C- and D-rings are superimposed, but given that even small changes can significantly alter

chemical reactivity and biological potency, even within a class of steroids (e.g. just changing the stereochemical orientation of the 3β-hydroxyl group of 20E to give 3-*epi*-20-hydroxyecdysone is associated with a 20-fold reduction in biological activity; Dinan, 2003), the summed structural differences between classes of phytosteroid would be expected to reduce activity in heterologous assays to low or non-existent levels. Thus, ecdysteroids do not appear to show activity in brassinosteroid bioassays and, where activity (agonist or antagonist) of brassinosteroids in ecdysteroid assays has been described, it only occurs at very high concentrations ($>10^{-5}$M) i.e. it is not truly specific and could even be a consequence of impurity of the test compound or metabolism in the assay system. Also, in this specific case, any activity has no biological relevance, since the levels of brassinosteroids found in plants are so low that no phytophagous arthropods would be naturally exposed to adequate levels to bring about any possible effects. Several groups have synthesised steroids which are chemically hybrid between ecdysteroids and brassinosteroids and determined their activities in ecdysteroid- and brassinosteroid-specific bioassays to determine which features are responsible for specific activities and to determine which are essential to obtain a cross-over in activity (Voigt et al., 2001; Watanabe et al., 2004).

In contrast to the brassinosteroids, most classes of phytosteroid do not possess phytohormonal roles and, although not ubiquitous, where they do occur, they occur at much higher concentrations than the brassinosteroids. Predominantly defensive roles have been ascribed to these other classes and their associated biological activities are manifold, acting against a wide range of organisms and at a wide range of biochemical sites. It is therefore perhaps not surprising then that some of these can interfere with the hormonal actions of ecdysteroids, even if they do not generally mimic the ecdysteroids as agonists (Dinan et al., 2001a). The ecdysteroid receptor antagonist activities of cucurbitacins (triterpenoids, but not strictly steroids) and withanolides are cases in point (Dinan et al., 1996, 1997), where the features of size and general shape and polarity with certain chemical similarities are adequate to permit interaction with the LBD to prevent ecdysteroid binding, but not to bring about the conformational changes associated with agonism i.e. these compounds act as weak antagonists.

It should be borne in mind that agonism is not compulsorily associated with an ecdysteroidal structure, since DAHs and other classes of non-steroidal agonists and antagonists exist, which bear no obvious chemical or biochemical similarity to ecdysteroids (Dinan and Hormann, 2005).

One is struck by the wide array of natural analogues in each phytosteroid class and how the profile can vary between plant species. The continuing ability to find new analogues suggests that many analogues still remain to be discovered. For example, over 300 phytoecdysteroid analogues are currently known and 10–20 new ones are described each year in the literature. Given this vast array of analogues in each phytosteroid class, it is perhaps not surprising that the same chemical functional groups occur across the classes of phytosteroids in at least some of the analogues. One is left wondering if this represents a biosynthetic relatedness between the different classes of phytosteroids, such that at least some of the biosynthetic enzymes might

be common to the pathways for the different classes. Circumstantial evidence exists that most, if not all, plant species have the genetic capacity to produce phytoecdysteroids (see above). Perhaps this is also the case for the other classes of defensive steroids/triterpenoids and certainly seems highly probable for the phytohormonal brassinosteroids. Whether a particular plant species produces a particular class of defensive steroid would then depend on whether the particular pathway is activated or repressed. It is conceivable that these pathways are not mutually exclusive, since some may use enzymes with lesser specificity to modify intermediates in similar locations. In the extreme case, one could envisage that each class might have a distinct key early intermediate, which is acted on by a number of common enzymes (hydroxylases etc.) to generate the cocktail of molecules of that class. We currently know very little about the genetic organisation of the defensive steroid pathways in plants, so it is not possible to answer this question at the moment. However, progress in the elucidation of the brassinosteroid pathway(s) (Bishop, 2007) is providing some of the biological tools to begin to investigate possible commonalities in the pathways.

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