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Authors: Lafont, R., and Dinan, L.

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Practical uses for ecdysteroids in mammals including humans: and update

Lafont R.¹ and Dinan L.²

¹*Université Pierre et Marie Curie, Institut de Biologie Intégrative, Laboratoire d'Endocrinologie Moléculaire et Évolution, 7 Quai Saint Bernard, Case Courrier N° 29, 75252 Paris Cedex 05, France.*

²*University of Exeter, Department of Biological Sciences, Hatherly Laboratories, Prince of Wales Road, Exeter, Devon, EX4 4PS, U.K.*

Rene.Lafont@snv.jussieu.fr

L.N.Dinan@exeter.ac.uk

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Abstract

Ecdysteroids are widely used as inducers for gene-switch systems based on insect ecdysteroid receptors and genes of interest placed under the control of ecdysteroid-response elements. We review here these systems, which are currently mainly used *in vitro* with cultured cells in order to analyse the role of a wide array of genes, but which are expected to represent the basis for future gene therapy strategies. Such developments raise several questions, which are addressed in detail.

First, the metabolic fate of ecdysteroids in mammals, including humans, is only poorly known, and the rapid catabolism of ecdysteroids may impede their use as *in vivo* inducers.

A second set of questions arose in fact much earlier with the pioneering “heterophylic” studies of Burdette in the early sixties on the pharmacological effects of ecdysteroids on mammals. These and subsequent studies showed a wide range of effects, most of them being beneficial for the organism (e.g. hypoglycaemic, hypocholesterolaemic, anabolic). These effects are reviewed and critically analysed, and some hypotheses are proposed to explain the putative mechanisms involved.

All of these pharmacological effects have led to the development of a wide array of ecdysteroid-containing preparations, which are primarily used for their anabolic and/or “adaptogenic” properties on humans (or horses or dogs). In the same way, increasing numbers of patents have been deposited concerning various beneficial effects of ecdysteroids in many medical or cosmetic domains, which make ecdysteroids very attractive candidates for several practical uses.

It may be questioned whether all these pharmacological actions are compatible with the development of ecdysteroid-inducible gene switches for gene therapy, and also if ecdysteroids should be classified among doping substances.

Abbreviation:

20E	20-hydroxyecdysone
2d20E	2-deoxy-20-hydroxyecdysone
2dE	2-deoxyecdysone
BAH	bisacylhydrazine
BmEcR	<i>Bombyx mori</i> EcR
CfEcR	<i>Choristoneura fumiferana</i> EcR
CfUSP	<i>Choristoneura fumiferana</i> USP
CHO	Chinese hamster ovary
CMV	cytomegalovirus
DBD	DNA-binding domain
DmEcR	<i>Drosophila melanogaster</i> EcR

Abbreviations continued on next page

Abbreviations continued from previous page

AbbE	ecdysone
EcR	ecdysteroid receptor
EcRE	ecdysteroid response element
EHT	effective half-time
ERE	oestrogen response element
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HEK	human embryonic kidney
HvEcR	<i>Heliothis virescens</i> EcR
LBD	ligand binding domain
murA	muristerone A
PKA	protein kinase A
polB	polypodine B
ponA	ponasterone A
PPAR	peroxisome proliferator-activated receptor
RAR	retinoic acid receptor
RXR	retinoid X receptor
TR	thyroid receptor
USP	ultraspiracle
VDR	vitamin D receptor
VEGF	vascular endothelial growth factor

Introduction

Ecdysteroids (zooecdysteroids) are steroid hormones that control moulting and reproduction of arthropods. Whether they fulfil hormonal functions in other invertebrate groups is still a matter of debate. In 1966, the discovery of the same molecules (phytoecdysteroids) in several plant species made them easily available in large amounts, and this allowed pharmacological studies to be initiated on mammals. Such studies were at first undertaken in the hope of developing safer and more specific insecticides, and it was quickly shown that these molecules were not toxic to mammals. On the other hand, they displayed a wide array of rather beneficial pharmacological effects (e.g. against diabetes or asthenia), thus providing a plausible explanation for the properties of several plant species widely used in traditional medicine. Although they have been detected in ca. 6% of plant species analysed so far (Dinan, 2001), phytoecdysteroids are not so frequent in plant species used as human food (with the noticeable exception of spinach; Bathory *et al.*, 1982; Grebenok *et al.*, 1991). More than 300 different ecdysteroids have been isolated from animal and plant sources (all their structures can be found in the Ecdybase, <http://ecdybase.org>).

Ecdysteroids are structurally quite different from mammalian steroids, and they are not expected to bind to vertebrate steroid receptors. Soon after the isolation and cloning of *Drosophila melanogaster* ecdysteroid receptor proteins, it appeared very attractive to use them for designing inducible gene systems in mammalian cells. Such a system has been commercially developed by Invitrogen® and the potential use of ecdysteroid receptors for gene therapy is being investigated. The different ecdysteroid-based gene-switch systems will be reviewed in the first part of this article.

The *in vivo* use of ecdysteroids as inducers taken orally raises questions about their uptake, metabolism and half-life in mammals including humans, a topic which has not been extensively investigated up to now (Sláma and Lafont, 1995), and this question

will be addressed in the second part of this review.

The development of ecdysteroid-regulated gene switches seems, however, to have neglected much of the previous pharmacological studies which showed the interference of ecdysteroids with many physiological processes in mammals and humans. All these effects will be summarised in the third part, paying special attention to the protocols used and the significance/limitations of the results obtained. In the light of recent data, we will present in the fourth section some working hypotheses, which could explain how ecdysteroids might act on mammalian cells.

The reported effects (mainly the anabolic effects) led initially to a (doping ?) use for high-performance sportsmen in the Eastern Bloc Countries, but nowadays a large number of ecdysteroid-based preparations are freely available on the market. Most of them are proposed as legal and non-toxic muscle-promoting substances for bodybuilders, but an extensive search on the web has led to more surprising findings (e.g. recommended use for golfers or for domestic animals). So, whether ecdysteroids should be considered as doping substances and whether their use should be controlled will be finally discussed.

Ecdysone-inducible gene expression systems

Basic requirements

Spatial and temporal control of heterologous gene expression is an area of considerable and growing interest with relevance to basic and applied biological and medical research, including gene therapy and functional genomics. However, these heterologous regulatory systems should interfere minimally with the complex endogenous regulatory networks. Ideally, heterologous modification of gene expression in host cells should give rapid, robust, precise and reversible induction (or suppression) of the target gene(s). The necessary criteria are thus (Saez *et al.*, 1997; Bohl and Heard, 1998; DeMayo and Tsai, 2001; Fussenegger, 2001; Graham,

2002):

1. Specificity: the system should not interfere with endogenous regulatory networks and should be activated exclusively by exogenous nontoxic compounds.

2. Inducibility: the system should possess a low baseline expression and a high induction ratio.

3. Bioavailability of the inducer: control should be effected by a drug that readily penetrates tissue.

4. Reversibility: the elicitor should possess high pharmacokinetic turnover to enable reversal and permit repeated cycles of induction.

5. Low immunogenicity: the components of the system should not elicit immune responses in the host.

6. Flexibility: it should be possible to modify the system to take account of different tissue applications and to optimise the system for each of these.

7. Dose-dependence: the extent of the response should be dependent on the dose of elicitor applied.

Ecdysteroid receptors in arthropods

Ecdysteroid receptors are members of the nuclear receptor superfamily (Laudet, 1997), which are characterised by a domain structure. The N-terminal A/B-domain is highly variable and is associated with transcriptional activation. The C-domain is highly conserved and is involved in binding the receptor complex to specific response elements in the DNA. The D-domain is variable and represents a hinge region between the DNA-binding domain and the ligand-binding domain (E-domain). The E-domain is not only responsible for ligand binding, but also has been implicated in receptor dimerisation and interactions with other transcriptional activators. There may also be a C-terminal F-domain, which, if present, is highly variable between even closely related nuclear receptors (Kumar and Thompson, 1999). Nuclear receptors regulate gene expression as dimers, either as homodimers or as heterodimers with another member of the nuclear receptor superfamily. One of the most promiscuous heterodimeric partners for vertebrate nuclear receptors is RXR, of which the equivalent in insects is Ultraspiracle (USP; Oro *et al.*, 1990). In the case of ecdysteroid receptors, only the EcR:USP (or EcR:RXR) (Yao *et al.*, 1993) complex is able to bind the ecdysteroid ligand with high affinity and the presence of ecdysteroid promotes complex formation. The ecdysteroid binds to the EcR protein. No definitive ligand for USP has been identified, but it has been suggested that juvenile hormones (or methyl farnesoate in Crustacea) may bind to this receptor component and modify the transactivation capacity of the complex (Jones and Jones, 2000).

The most extensively studied ecdysteroid receptor system in arthropods is that of *Drosophila melanogaster*, where three isoforms (A, B1 and B2) of EcR occur (Koelle *et al.*, 1991; Talbot *et al.*, 1993). These isoforms arise through alternative promoter usage and differential splicing, resulting in different A/B-domains, but they all possess common DNA- and ligand-binding domains. The EcR isoforms show tissue- and stage-specificity. Although there is only one form of USP in *D. melanogaster*, two or more isoforms have been found in other arthropods. USP isoforms also show tissue- and stage-specificity (Kapitskaya *et al.*, 1996).

EcR and USP gene homologues have now been

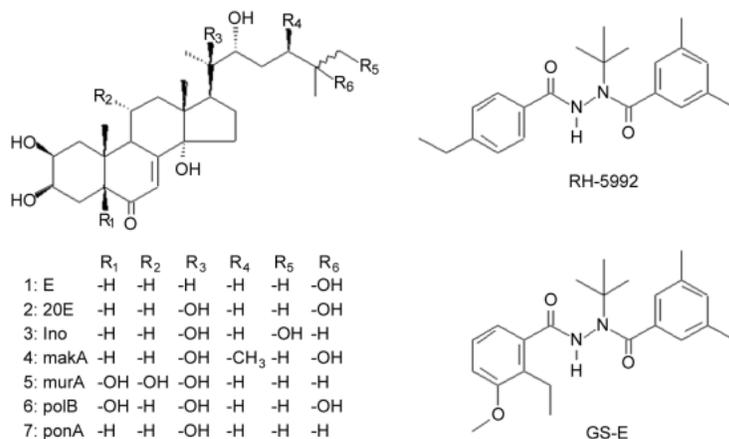


Figure 1. Structures of ligands used for ecdysteroid-inducible gene expression systems in mammalian and plant cells.

characterised from a variety of arthropod species: *Aedes aegypti* (Cho *et al.*, 1995; Kapitskaya *et al.*, 1996), *Amblyomma americana* (Palmer *et al.*, 1999), *Bombyx mori* (Swevers *et al.*, 1996), *Ceratitis capitata* (Verras *et al.*, 1999), *Chironomus tentans* (Imhof *et al.*, 1993; Vöggtli *et al.*, 1999), *Choristoneura fumiferana* (Kothapalli *et al.*, 1995; Perera *et al.*, 1999), *Heliothis virescens* (Martinez *et al.*, 1999c), *Locusta migratoria* (Saleh *et al.*, 1998; Hayward *et al.*, 1999), *Lucilia cuprina* (Hannan and Hill, 1997; 2001), *Manduca sexta* (Fujiwara *et al.*, 1995; Jindra *et al.*, 1997), *Ostrinia nubilalis* (Albertsen *et al.*, 2000), *Sarcophaga crassipalpis* (Rinehart *et al.*, 2001), *Tenebrio molitor* (Mouillet *et al.*, 1997; Nicolai *et al.*, 2000), *Uca pugilator* (Durica *et al.*, 2002).

The biochemical characterisation of ecdysteroid receptor complexes lags well behind that of vertebrate steroid hormone receptors and has been in a period of quiescence for the past decade, as emphasis has been placed on the characterisation and expression of the genes. The generally accepted ligand for ecdysteroid receptors in arthropods is 20E, but this does not preclude the other ecdysteroids being significant at particular stages of development or in certain tissues (Wang *et al.*, 2000). In fact, ecdysteroid receptor complexes recognise a wide range of ecdysteroid structural analogues and sophisticated structure-activity and molecular modelling studies are now beginning to be performed (Dinan *et al.*, 1999a; Wurtz *et al.*, 2000; Ravi *et al.*, 2001; Kumar *et al.*, 2002). Owing to the importance of ecdysteroid receptors in the regulation of arthropod development, they are seen as an appropriate target for the development of new pest control agents. In the context of this review, the identification of bisacylhydrazines as non-steroidal ecdysteroid agonists (Wing, 1988; Dhadialla *et al.*, 1998) is particularly worth mentioning as, in addition to several of these molecules being commercialised as insecticides, other analogues appear appropriate as gene switching elicitors. Antagonists for ecdysteroid receptors are also being identified (Dinan *et al.*, 1999b).

Ecdysteroid-responsive expression systems

Mammalian systems: Ecdysteroids are apparently not endogenously generated components of mammalian systems. However, they are normal components of the diets of many animals.

The low mammalian toxicity of these compounds (Sláma and Lafont, 1995), together with the specificity of the ecdysteroid receptor complex (EcR and USP proteins), indicate that a successful gene-switching system might be developed from this system (Fig. 2). With regard to plant systems (see below), there are a significant number (ca. 6% of higher terrestrial species) of plants which accumulate phytoecdysteroids (Dinan, 2001). This may restrict the use of steroidal and non-steroidal ecdysteroid analogues as elicitors in plant systems.

Initial reports appeared in the early 1990s (Christopherson *et al.*, 1992; Thomas *et al.*, 1993; Yao *et al.*, 1992; 1993). Christopherson *et al.* (1992) transfected a human embryonic kidney cell line (HEK293) with DmEcR and a reporter gene and assessed the ability of various ecdysteroids and vertebrate steroids (all at 1 μM) to induce reporter activity; E, 20E and ponB and the vertebrate steroids were inactive, while ponA and murA were active. The domain structure of nuclear receptors allows the domains to operate autonomously (however, this should not be taken to mean that the domains operate exactly the same under all circumstances). This permitted the ligand binding domain of EcR to be fused with the DNA-binding and A/B-regions of the GR (GGEc) and the demonstration of the induction of a GRE-containing reporter gene by murA and with the same ecdysteroid specificity as for EcR in the same mammalian cells. MurA could also induce a reporter gene via a chimeric receptor recognising a consensus oestrogen response element (ERE). They also demonstrated that replacement of a portion of the GR N-terminal activation domain in GGec with the activation domain of the *Herpes simplex* viral protein (VP16) resulted in 5-fold greater activity (Christopherson *et al.*, 1992).

Yao *et al.* (1992) showed that USP could substitute for RXR as a heterodimeric partner for RAR, TR, VDR and PPAR and showed that, for many mammalian cells types, cotransfection of USP with EcR was necessary to make the cells ecdysteroid-responsive, demonstrating that USP is an essential part of the ecdysteroid receptor complex.

Thomas *et al.* (1993) found that certain mammalian cell lines (e.g. HeLa) could support ecdysteroid-responsive transactivation while others (e.g. CV-1) could not. They demonstrated that the factor responsible for this was RXR. RXR could not be replaced by RAR- α , TR- α or COUP-TF, but USP was

an effective partner for EcR. MurA was effective at enhancing the DNA-binding activity (as assessed by gel-shift assays) of EcR:RXR, but not EcR:USP. Interestingly, the ligand of RXR 9-*cis*-retinoic acid, also enhanced the DNA-binding activity of EcR:RXR complexes.

The system has been further developed (No *et al.*, 1996). The final form of this development (VgEcR) was more specific and gave a lower basal activity than tetracycline-responsive systems. The starting point for the developments of No *et al.* (1996) was the observation that mammalian cells cotransfected with EcR and USP only produce a 3-fold induction on treatment with murA (1 μM). To improve the induction ratio they carried out a number of modifications. Replacement of USP by RXR gave 34-fold induction. Creation of a fusion protein consisting of an N-terminal truncation of EcR attached to the VP16 activation domain (generating VpEcR) gave 212-fold induction. Inclusion of binding sites for the transcription factor Sp1 into the reporter vector between minimal promoter and the EcREs enhanced the induction by a further 5-fold.

Since the ecdysteroid response element might be weakly activated by endogenous farnesoid X receptors FXR, the EcRE (to give 2 different half-sites with a 1-nucleotide spacer, AGGTCA-AGAACA, generating E/GRE) and DBD of EcR (by mutating 3 amino acids in the P-box of the DNA-binding domain, generating VgEcR) have been modified to ensure that the response element will only bind the modified EcR. The transcription-regulatory potential of EcR has been enhanced by replacing the endogenous activation domain by the *Herpes simplex* virus VP16 activation domain (DeMayo and Tsai, 2001). The final system gives a 1200-fold induction with 1 μM murA, without interference from glucocorticoid or farnesoid.

No *et al.* (1996) also generated transgenic mice harbouring an ecdysteroid-inducible promoter or a T-cell-specific expression construct of VpEcR and RXR. Crossing of these two strains of mice gave double transgenic offspring, which were induced to generate the reporter gene transcript specifically in the thymus by injection of murA (10 mg/mouse). Mice expressing VpEcR and RXR were healthy, fertile and apparently normal.

Yang *et al.* (1995) produced a Chinese hamster ovary (CHO) cell line stably transfected with EcR isoform B1 and showed that the cells produce functional receptor of the correct M_r (105 kDa), which is recognised by specific antibodies, binds to EcRE in gel-shift assays and mediates reporter gene expression in a ligand-dependent manner (ponA; 4 - 100 μM). The authors suggest that CHO cells produce high levels of RXR, which can heterodimerise with EcR to generate functional receptor complexes.

A parallel system using the *Bombyx mori* receptor (Swevers *et al.*, 1995; Swevers *et al.*, 1996) has been developed (Suhr *et al.*, 1998), who found that BmEcR, in conjunction with murA (1 μM) or RH5992 (tebufenozide; 1 μM) could effect high level transactivation of a reporter gene in the absence of exogenous heterodimeric partner in mammalian cells (HEK293 cells and African green monkey CV-1 cells). BmEcR is much shorter (616 amino acids; Swevers *et al.*, 1995) and has less than 42% overall amino acid identity with the B1 isoform of DmEcR (878 amino acids; Suhr *et al.*, 1998). It has been recognised that RXR (which is present to at least some extent in most, if not all, mammalian cells)

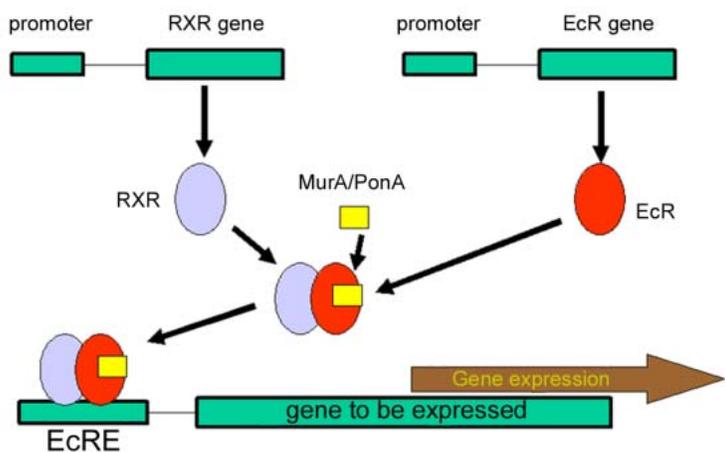


Figure 2. General scheme for ecdysteroid-based gene switches.

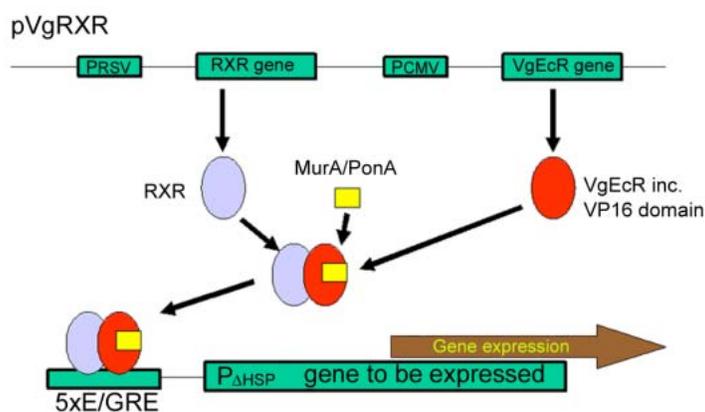


Figure 3. The Invitrogen system for mammalian cells; the elicitor is muristerone A (murA) or ponasterone A (ponA). See text for further details (www.invitrogen.com).

is a reluctant heterodimerisation partner for DmEcR (Thomas *et al.*, 1993; Yao *et al.*, 1993), but it appears to be less reluctant for BmEcR. By creating chimaeric EcRs consisting of Dm and Bm domains in various combinations, Suhr *et al.* (1998) could demonstrate that the regions responsible for high affinity, ligand-dependent heterodimerisation in BmEcR were present in the hinge region (D) and the ligand-binding (E) domain. Only the D-region appears to be involved in heterodimerisation of BmEcR with USP.

Hoppe *et al.* (2000) created a hybrid *Drosophila/Bombyx* ecdysteroid receptor (DB-EcR), which is independent of recombinant RXR, and demonstrated its efficacy *in vitro* and *in vivo*.

The commercially available Invitrogen system (<http://www.invitrogen.com>: Fig. 3) has been used to regulate the expression of a wide range of transfected genes in mammalian cells (Sawicki *et al.*, 1998; Chen *et al.*, 2000; Lüers *et al.*, 2000; Niikura *et al.*, 2000; Rampazzo *et al.*, 2000; Abeysinghe *et al.*, 2001; Baba *et al.*, 2001; Cole *et al.*, 2001; Gill *et al.*, 2001; Hennigan and Stambrook, 2001; Iwata *et al.*, 2001; Jana *et al.*, 2001; Kondo *et al.*, 2001; Patrick *et al.*, 2001; Schmidt and Fan, 2001; Shi *et al.*, 2001; Sparacio *et al.*, 2001; Stauffer *et al.*, 2001; Stolarov *et al.*, 2001; Wang *et al.*, 2001; Xu *et al.*, 2001; Yam *et al.*, 2001; Yarovoi and Pederson, 2001; Zhu *et al.*, 2001; Chen *et al.*, 2002; Coulthard *et al.*, 2002; Davis *et al.*, 2002; Hashimoto *et al.*, 2002; Kuate *et al.*, 2002; Kudo *et al.*, 2002; Meents *et al.*, 2002; Mellon *et al.*, 2002; Odero-Marrah *et al.*, 2002; Plows *et al.*, 2002; Vickers and Sharrocks, 2002; Wang *et al.*, 2002; Wolter *et al.*, 2002; Xiao *et al.*, 2003; Xu and Mellgren, 2002; Zhang *et al.*, 2002) and in a tissue-specific manner in mice (No *et al.*, 1996; Albanese *et al.*, 2000;). The literature on the application of ecdysteroid-regulated transgenic systems is currently growing exponentially.

Wyborski *et al.* (2001) developed a bicistronic expression vector from which VgEcR and RXR can be co-expressed. They used the general cytomegalovirus (CMV) promoter, but this can be replaced with a cell-type specific promoter.

Albanese *et al.* (2000) developed a system for mammary gland-specific expression of an ecdysteroid-regulatable gene in mice and have examined the pharmacokinetics of injected ponA in the animals. Serum clearance was rapid (activity half-life = 48 min).

Karns *et al.* (2001) have developed an alternative to the

Invitrogen system. The basic system (Figure 4) consists of the (i) plasmid pGAL4-EcR, encoding a fusion protein of the yeast GAL4 DNA-binding domain and the ligand-binding domain of the ecdysteroid receptor from *Choristoneura fumiferana*, (ii) plasmid pVP16-mRXR, encoding a fusion protein of the *Herpes simplex* transcriptional transactivator VP16 and mouse RXR protein, and which, in the presence of ecdysteroid-type ligands heterodimerises with GAL4-EcR, (iii) an indicator and selection plasmid, either pGAL4-EGFP-SV40-neo (consisting of 5 copies of the GAL4 response element, followed by the minimal promoter region of the major late promoter from adenovirus, the coding region of enhanced green fluorescent protein [EGFP], the SV40 promoter and the neomycin resistance locus) or pGAL4-SEAP (for stable transformation, containing 5 x the GAL4 response element and the coding region of the SEAP protein as reporter gene) iv) the BAH GS-E (1 - 15 μ M). This system forms the basis of RHeoGene's RHeoswitch Technology. As RHeoGene have access to a large number of BAH analogues (RHeoChem Ligands) and EcR genes from a wide variety of insect species (RHeocept Receptors), they are able to identify BAH analogues which are specific for particular EcR LBDs and, thus, have the possibility to regulate multiple genes in a coordinated manner, and this is being developed under the title of RHeoPlex Systems (www.rheogene.com). Karns *et al.* (2001) also considered the suitability of GS-E as an *in vivo* inducer in mice and obtained maximal induction of reporter protein in 6-12 hrs and return to basal expression levels by 12-24 hrs.

Plant systems: Most of the published research in this area has been conducted by the industrial research labs at Zeneca Agrochemicals (now Syngenta) and has been based on the ecdysteroid receptor protein from *Heliothis virescens* (HvEcR), which was cloned and characterised (Martinez *et al.*, 1999c). This protein has most similarity to EcRs from other lepidopteran species and is closely related to the B1 isoform from *D. melanogaster* (DmEcRB1). Transfection of mammalian HEK293 cells (RXR-containing) with HvEcR and a reporter gene resulted in induction of the reporter gene by murA (50% response at ca. 5 μ M), but not by 20E (Martinez *et al.*, 1999a). For the development of the plant

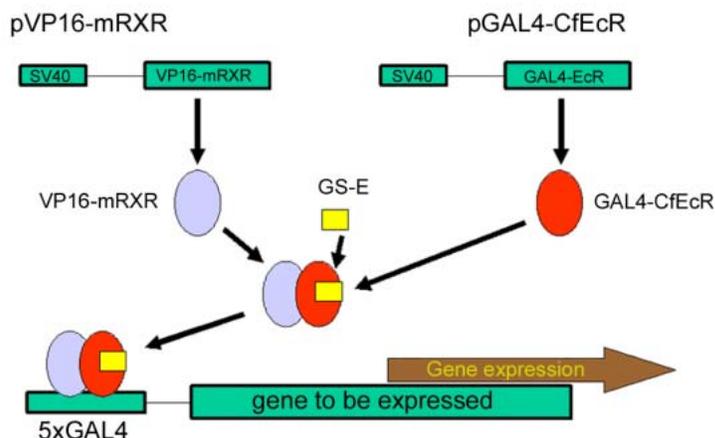


Figure 4. The RHeoGene system for mammalian cells; the elicitor depicted is the bisacylhydrazine GS-E (1-[3-methoxy-2-ethylbenzoyl]-2-[3,5-dimethylbenzoyl]-2-*tert*-butylhydrazine). See text for further details (Karns *et al.*, 2001).

system (Fig. 5), a chimeric receptor consisting of the hinge and ligand-binding domains of HvEcR was fused to the transactivation domain of the *Herpes simplex* VP16 protein and the DNA-binding domain of the glucocorticoid receptor and transfected into tobacco protoplasts. The use of the GR DNA binding domain circumvents the need to incorporate USP/RXR into the system, since glucocorticoid receptors bind to their response elements as homodimers. The second component of the gene regulatory system consisted of 6 copies of the glucocorticoid response element fused to the minimal 35S cauliflower mosaic virus (35SCaMV) promoter (conferring expression in all tissues and throughout development) and a β -glucuronidase gene. Although induction was observed with murA (100 μ M), its steroidal nature precludes its use under field conditions. Consequently, the non-steroidal BAH RH5992 (1 - 10 μ M) was used as an elicitor. In addition to being ecdysteroid agonists, these compounds are not phytotoxic. Incorporating the regulatory and reporter components via *Agrobacterium tumefaciens* transformation generated transgenic lines of tobacco plants. Germination of the transformed seeds in the presence of murA or RH5992 resulted in induction of the reporter gene activity (up to 420-fold). RH5992 is 100-fold more potent than murA in this system, giving maximal activation at 12.5 μ M and 50% activation at ca. 1 μ M (Martinez *et al.*, 1999a). Parallel studies using maize protoplasts compared chimeric receptors involving ligand and hinge regions of either the *D. melanogaster* or *H. virescens* EcR fused to the A/B/C-domains of the glucocorticoid receptor, showed that RH5992 activates in the presence of GRH, but not GRD (Martinez *et al.*, 1999b). On the other hand, murA (100 μ M) activates in the presence of GRD, but not GRH. The preferential activation of GRH by RH5992 is in accord with the higher affinity of this BAH for lepidopteran EcR/USP complexes than for dipteran complexes (Dhadialla *et al.*, 1998), but the lack of activation of GRH by murA is not readily explained and seems to indicate that the conformation of the LBD of the chimeric receptor is significantly altered.

Unger *et al.* (2002) have developed a BAH-regulated system for the control of male fertility in maize. Ms45 is a nuclear male fertility gene, which is expressed in anthers. Homozygous recessive mutants are male sterile. The aim was to create a Ms45 construct which would allow male fertility to be restored after application of

an elicitor. The hinge and ligand-binding domains (domains D-F) of the *Ostrinia nubilalis* EcR gene were linked to the VP16-GAL4 or C1-GAL4 transcription activators, under the regulation of the Ubiquitin 1 promoter, which gave constitutive expression of the receptor construct. The Ms45 promoter region was replaced by 5 copies of the yeast 17 bp UAS_G. Regulatory proteins containing the GAL4 DNA-binding domain bind to UAS_G. The authors demonstrate that treatment of transformed maize callus with methoxyfenozide (10 μ M) induces Ms45 expression. Further, when incorporated into plants, the plants were male sterile in the absence of methoxyfenozide, but fertility was restored by treating plants with methoxyfenozide.

Padidam *et al.* (2002) have recently developed an ecdysteroid receptor-based gene expression system based on a modified *Choristoneura fumiferana* EcR and the BAH methoxyfenozide and demonstrated its effectiveness in transgenic *Arabidopsis thaliana* and *Nicotiana tabacum*.

In addition to ecdysteroid/BAH controllable systems, other chemically inducible gene expression systems for plants are also being investigated (reviewed in Gatz and Lenk, 1998; Jepson *et al.*, 1998). These systems have been recently compared and reviewed (Padidam, 2003).

Fungi: When transfected into *Saccharomyces cerevisiae*, DmEcR is able to transactivate a reporter gene in the absence of USP/RXR or ecdysteroid (ponA or murA) (Dela Cruz and Mak, 1997). Activation is EcRE-dependent, but, unexpectedly, ecdysteroid- and heterodimerisation partner-independent. Interestingly, high affinity specific binding of [³H]ponA (K_d = 1.8 nM) by yeast extracts was dependent on coexpression of EcR and USP (or RXR). Radiolabelled hormone displacement assays for yeast-expressed EcR/USP with ponA, murA, 20E and RH5849 (Dela Cruz and Mak, 1997) indicate similar specificity and affinity to *D. melanogaster* ecdysteroid receptor complexes in insect systems (Bidmon and Sliter, 1990). Thus, the situation prevailing when ecdysteroid receptors are expressed in yeast cells is apparently very different to that for mammalian or plant cells.

Using the ecdysteroid receptor genes from *Choristoneura fumiferana* (CfEcR and CfUSP) coexpressed in yeast with a reporter gene containing EcREs, Tran *et al.* (2001) showed that EcR and USP together (but not individually) induced reporter gene expression in the absence of ligand, with RH5992 (10 μ M) only providing a small enhancement in reporter gene expression. Deletion of the A/B-regions of CfEcR, in conjunction with CfUSP, still gave ligand-independent transactivation with some enhancement on addition of RH5992. However, deletion of the A/B-regions of CfUSP (generating Cf Δ USP) abolished reporter gene expression, regardless of whether co-expression was with CfEcR or Cf Δ EcR and in the presence or absence of RH5992. Together, these data showed that EcR:USP is not suitable for a ligand-dependent transactivation assay in yeast. Replacement of USP with RXR α , RXR β or RXR γ , when co-expressed with EcR, resulted in no induction of the reporter gene in the presence or absence of RH5992. However, co-expression of GRIP1 (a member of the p160 family of coactivators) and Cf Δ EcR:RXR or Cf Δ EcR:Cf Δ USP resulted in significant ligand-dependent transactivation of the reporter gene activity. The system with RXR β appeared to have a low sensitivity to RH5992 and other

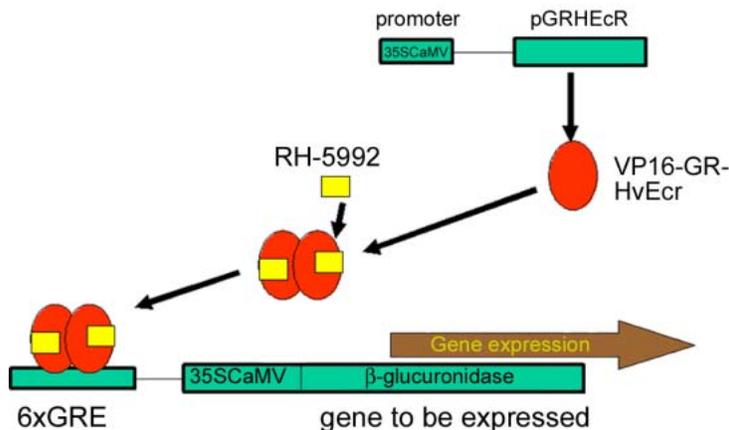


Figure 5. The Syngenta system for plant cells. See text for further details (Martinez *et al.*, 1999a&b).

BAHs and was not pursued further. Comparison of three yeast systems ($\Delta\text{EcR}:\Delta\text{USP}:\text{GRIP1}$, $\Delta\text{EcR}:\text{RXR}\alpha:\text{GRIP1}$ and $\Delta\text{EcR}:\text{RXR}\gamma:\text{GRIP1}$) with an insect cell CfEcR:USP-containing system (L57; DmEcR-negative Kc cells transfected with CfEcR and β -galactosidase reporter controlled by 6 x EcRE and a minimal promoter) using a range of BAHs and murA and ponA showed induction in all systems by active compounds, but i) the degree of induction was far lower in the yeast systems and ii) the ecdysteroids were very much poorer inducers in the yeast $\Delta\text{EcR}:\Delta\text{USP}$ system than in the insect cells and did not induce the $\Delta\text{EcR}:\text{RXR}$ (α or γ) systems. Further, 9-*cis*-retinoic acid (a natural ligand of RXR receptors) induced the $\Delta\text{EcR}:\text{RXR}\alpha:\text{GRIP1}$ and $\Delta\text{EcR}:\text{RXR}\gamma:\text{GRIP1}$ systems, complicating interpretation of results from these systems if they were used in screening processes. In part these problems may derive from poor access of test compounds through the thick yeast cell wall or rapid export from the cells. Tran et al. (2001) provide evidence that use of yeast strains with mutations in certain ABC transporter pathway loci results in improved sensitivity (100-fold for RH5992). Tran et al. (2001) propose their transactivation assay as a screen to identify potential insecticides with ecdysteroid agonist activity.

Commercially available systems: The system devised by No et al. (1996) has been developed and commercialised by Invitrogen (<http://order.invitrogen.com/>). The company provides kits consisting of mammalian cells (CV-1, HEK293 and CHO) stably expressing a functional ecdysteroid receptor from pVgRXR, the inducing agent (now ponA) and a vector by which the gene of choice can be introduced into the cells, after introduction of the gene into the vector by simple recombination using Cre recombinase. The components are also available individually. The pVgRXR expression vector includes VgEcR, RXR and a gene for Zeocin resistance, which allows for selection of stable cells expressing the heterodimeric receptor (VgEcR:RXR).

Stable ecdysteroid-inducible mammalian cell lines can be difficult to establish because of either high basal expression of the target gene or poor induction of gene expression, because of low expression of the receptors (VgEcR and RXR) or the transgene. Ideally, stable cell lines expressing the receptors should be established first and the cells should be screened by transient expression of an ecdysteroid-regulatable transgene to identify those expressing the receptor proteins effectively. An improvement on Invitrogen's pIND/lacZ reporter system (which generates β -galactosidase activity has been reported (Wakita *et al.*, 2001), which uses a firefly luciferase reporter system. This considerably reduces analysis time (15 s, rather than 2 h) and obviates background interference from endogenous β -galactosidase activity. A similar advance has been suggested by Lüers et al. (2000) who prepared an expression plasmid for green fluorescent protein (EGFP) and the reporter protein of interest. The co-inducible production of EGFP permits the visual verification of target gene expression and the selection of expressing cells by flow-cytometry.

As described above (Section 2.3.1), RHeoGene LLC are commercialising their ecdysteroid receptor-based gene switching system (www.rheogene.com) and are identifying specific receptors/ligand pairs which allow the simultaneous, but independent, regulation of transfected genes (Kumar et al., 2002). Further, hybrid

receptors are being optimised to give very low basal activity and high induction on addition of ligand (Palli et al., 2003). A two-hybrid format switch where the GAL4 DNA-binding domain was fused to CfEcR domains D, E and F and the VP16 activation domain was fused to mouse RXR domains E and F, transactivating a reporter gene under the control of GAL4 response elements and a synthetic TATAA promoter was found to give the best combination with rapid turn-on and turn-off responses on the addition and removal of RG-102240 (GS-E), respectively.

Ecdysteroid systems vs. other systems

Fussenegger (2001) provides a comprehensive description of the heterologous molecular switching systems currently under consideration. Each has its own advantages, but none fulfils all the desirable criteria perfectly. From the time of early studies, transgenic ecdysteroid-inducible gene expression systems in mammalian cells have appeared to possess lower basal activity and higher inducibility than tetracycline-based systems (No *et al.*, 1996). Senner et al. (2001) compared 3 inducer systems (tetracycline, dimerizer and ecdysteroid) in one system (rat C6 glioma cells) under identical conditions. Each system required transient transfection with two plasmids (a regulator plasmid and a reporter plasmid) and treatment with an elicitor (inducers for the ecdysteroid and dimerizer systems and a repressor for the tetracycline system). The ecdysteroid system provided the highest induced activity, but the authors conclude that each of the systems may be beneficial, depending on what the experimental goals are. Van Craenenbroeck et al. (2001) compared the tetracycline and ecdysteroid systems to regulate the expression of neurotransmitter receptors in mouse fibrosarcoma L929sA and HEK293 cells. The tetracycline system resulted in higher levels of the neurotransmitter receptors being expressed, but the ecdysteroid system gave more tightly regulated expression. Moreover, this study underlined the importance of the genetic background of the cells being used.

Morgan et al. (1999) compare several exogenously regulatable promoter systems for their suitability for the study of the functions of genes implicated in aging.

Ecdysteroid specificity

Gene expression systems in mammalian and plants cells possess markedly different ecdysteroid specificities to ecdysteroid receptors in insect systems. Both the affinity and specificity seem to be affected. Thus, the generally accepted endogenous hormone in insects, 20E, is inactive in transgenic systems. Two phytoecdysteroids, murA and ponA, are normally used to activate the transgenic systems, but even these are required at least 100-fold higher concentrations than in insect systems; e.g. EC_{50} values for murA and ponA in the *Drosophila melanogaster* B_{11} bioassay are $2.2 \times 10^{-8}\text{M}$ and $3.1 \times 10^{-10}\text{M}$, respectively (Dinan *et al.*, 1999a), while concentrations of 1 - 10 μM are required to induce transgenic expression. The basis of this altered affinity/specificity is not clear and it could derive from: (i) altered metabolism, (ii) use of RXR, rather than USP, (iii) altered transportation into cells, (iv) fusion of the EcR ligand-binding domain to the GR DNA-binding domain and/or VP16 to form VgEcR, (v) the different properties of mammalian transcription factors, enhancers, repressors etc.

A limited investigation of the ecdysteroid specificity of

VgEcR/RXR in CV-1 cells has been performed (Saez *et al.*, 2000). MurA, ponA and 14-deoxymuristerone A were almost equivalently active ($EC_{50} = ca. 5 \times 10^{-7}M$), with ponasterone C being moderately active ($EC_{50} = 2 \times 10^{-5}M$), polB being weakly active and 20E, inokosterone, makisterone A, E, 2-deoxyecdysone, 20E 22-acetate and 2-deoxy-20-hydroxyecdysone being inactive or only very weakly active at $10^{-4}M$. This study also showed that the presence of a natural (9-*cis*-retinoic acid) or synthetic (LG268 or LG1069) RXR ligands, while inactive in itself, potentiated the activity of ponA by 3- to 5-fold.

Availability of ligands

Ecdysteroids: MurA has only been isolated once in large amounts (Canonica *et al.*, 1972), and then from a Himalayan plant (*Ipomoea calonyction*). Consequently, world supplies of this phytoecdysteroid became very restricted and did not suffice for full-scale trials of ecdysteroid-induced transgenic systems. However, Sequoia Sciences (<http://www.sequoiasciences.com>) report on their website that they have recently re-isolated murA. PonA, which has been isolated from several named plant species and which can be chemically generated from 20E (Heinrich, 1970), is also active. Examination of the ecdysteroid specificity in more detail could result in the identification of more active inducers. While *in vitro* work may not suffer unduly from the poor activity of currently used ecdysteroid inducers, other than having to use much larger amounts of expensive chemicals, the *in vivo* prospects for transgenic systems using ecdysteroids would be enormously enhanced if they were as active as in insect systems.

Bisacylhydrazines (BAHs): Bisacylhydrazines were identified as non-steroidal agonists of ecdysteroid receptors in 1988 (Wing, 1988). Their chemical simplicity, low mammalian toxicity and selectivity for certain Orders of insects has led to several being developed as insecticides (Dhadialla *et al.*, 1998). They could also be used for the induction of transgenic systems (Carlson *et al.*, 2001) and, as is apparent above, RH-5992 has found application in this context. Further analogues (e.g. GS-E; Fig. 1) have been identified which appear to be more potent for use with mammalian systems (Carlson, 2000). However, the very limited water solubility of these compounds may limit their application *in vivo*.

Modified receptors: A further approach to overcoming the current lack of really potent ligands for transgenic induction would be to modify the ligand-binding domain of the transgenic EcR to either enhance the affinity for a particular analogue, or to alter the specificity, so that a readily available analogue (e.g. 20E) or a non-dietary ecdysteroid is recognised. Cloning and sequence data for ecdysteroid receptor proteins (EcR and USP) from a range of arthropod species provide the basis for site-directed mutagenesis to modify specific amino-acid residues. Both this and the previous approach require a more thorough understanding of ecdysteroid receptor recognition, not only in *D. melanogaster* (Dinan *et al.*, 1999a; Ravi *et al.*, 2001), but also in other arthropod species and in transgenic systems. The ultimate goal of such studies is to engineer a range of EcR proteins, some of which respond to non-steroidal inducers, but not to ecdysteroids, while others respond to selected

ecdysteroids, but not to other classes of agonists (Graham, 2002). Strategies are being developed for the synthesis of further non-steroidal ligands for selective activation of ecdysteroid receptors (Tice *et al.*, 2003) and for the targeted modification of ligand specificity of ecdysteroid receptors (Kumar *et al.*, 2002).

Registration problems

Development of ecdysteroid systems for human therapeutic use may be hampered by the steroidal nature of ecdysteroids and the insecticidal origin of BAHs, which may prejudice their use as elicitors, this being in spite of the fact that both ecdysteroids and BAHs have low mammalian toxicities and ecdysteroids are a normal (but small) component of the human diet. For plant systems, ecdysteroids *per se* cannot be considered because of penetration problems and BAHs may not be acceptable because of the enhanced risk of development of resistance to insecticidal analogues. However, use might be restricted to specified crops under conditions where exposure to sensitive insect species is minimal.

Biochemical problems

Although the systems developed to date are effective for use in *in vitro* expression systems, the requirements for an effective *in vivo* system are much more stringent. In this context, one can identify the following aspects of ecdysteroid-regulatable systems which would need to be improved in order to generate a medically viable system:

- Integration of heterologous DNA into host cells is not site-specific and is unpredictable with regard to copy number.
- The current systems are genetically complex, requiring both VgEcR and RXR.
- The artificial transactivator is potentially immunogenic.
- RXR is a reluctant dimerization partner for EcR and, therefore, very high cellular RXR concentrations are required. Overexpression of RXR may result in pleiotropic effects in mammals.
- The maximal expression levels achieved are modest.
- Most ecdysteroids are not very active. Only muristerone A and ponasterone A are effective.
- Ecdysteroids are not orally available
- Ecdysteroids or ecdysteroid analogues are not likely to get approval for human therapeutic use.

Prospects

There is little doubt that ecdysteroid-regulated transgenic systems have considerable potential for *in vitro* work. The applied potential is somewhat more questionable at present, owing to the following current limitations: i) genetic complexity, ii) altered affinity and selectivity of VgEcR for ligands and iii) potential problems in the registration of ecdysteroids and BAHs for human therapeutic uses or with plant transgenic systems. However, significant progress is being made in designing chimaeric receptors which would allow only one *trans*-acting factor to be transfected. It is only a matter of time until the reasons for the altered affinity and selectivity of ecdysteroid receptors in mammalian and plant cells are elucidated and more efficient systems are developed either by identifying more effective ligands or site-directed mutagenesis of EcR to enhance affinity for currently used ligands. Although

ecdysteroids and BAHs are nontoxic to humans, general public resistance to steroids and insecticides may hamper their registration.

Ecdysteroid metabolism in mammals, including humans

Although the question of mammalian metabolism is certainly of importance for the practicability of the *in vivo* use of ecdysteroid-inducible gene expression systems (with the aim of using them for gene therapy), it is not well documented at the present time. Ecdysteroids have a very low toxicity in mammals: in the mouse, the LD₅₀ of 20E is 6.4 g/kg (for intra-peritoneal injection) and it is >9 g/kg when given orally (Matsuda *et al.*, 1970; Ogawa *et al.*, 1974). Up to now, studies have concerned mice, rats, lambs and humans, and all have shown that these molecules are short-lived in mammals. Several strategies have been used to analyse the metabolic fate of ecdysteroids.

Ecdysteroids are rapidly eliminated

In the case of humans, two different studies have been performed. Simon and Koolman (1989) analysed the pharmacokinetics of E and 20E (given orally, 0.2 mg/kg b.w.) to a male volunteer, by monitoring with a radioimmunoassay the subsequent plasma and urine titres. This gave an effective half-time (EHT) of elimination of 4 hours for E and 9 hours for 20E. In lambs, EHT for 20E was shown to depend strongly on the mode of administration, with values of 0.4, 0.2 and 2 hours after oral, intravenous and intramuscular administration, respectively (Simon and Koolman, 1989). The method used did not allow the detection of metabolites, if present. The half-life seems shorter in smaller mammals, with reported values of 8.15 min for 20E in mice (Dzukharova *et al.*, 1987). More recently, Albanese *et al.* (2000) found a plasma half-life of 48 min for ponA in mice after intra-peritoneal injection of 750 µg of this compound.

Both urinary and faecal routes seem to be used for the elimination of the administered molecules. In mice, the faecal route was found to be the major one by Hikino *et al.* (1972a&b) and Lafont *et al.* (1988), although Dzukharova *et al.* (1987) found that faecal and urinary routes were equally important. Such a question can be easily assessed only by the use of radiolabelled molecules, but no data are available for humans. Kinetic studies in mice showed that ecdysteroids were taken up by the liver and then excreted into the gut via the bile (Hikino *et al.*, 1972a&b; Lafont *et al.*, 1988).

Metabolic conversions

Another question concerns whether ecdysteroids undergo metabolic conversions in mammals. The presently available data are not fully consistent. In mice, Girault *et al.* (1988) analysed the faecal metabolites of injected E and isolated unchanged E, a major metabolite identified by MS and proton NMR as 14-deoxyecdysone together with molecules with a fully reduced B-ring and, additionally, epimerized in position 3 (Figure 6A). Such a metabolism is reminiscent of the hepatic reduction of the 4-en-3-one on ring-A of vertebrate steroid hormones, whereas dehydroxylation resembles that of bile acids and could result from the actions of anaerobic intestinal bacteria.

More recent studies were performed on ingested 20E in rats (Ramazanov *et al.*, 1996) and humans (Tsitsimpikou *et al.*,

2001). In these cases only urine was analyzed. Ramazanov *et al.* administered 20E to 40 rats (50 mg/kg) directly in stomach with a special probe, and they collected urine (3.5 L) over the following 10 days. After several chromatographic steps, they isolated unchanged 20E and three new metabolites, which were analyzed by IR and mass spectrometry. The IR spectra showed the disappearance of the signal at 650 cm⁻¹ (7-en-6-one) and the structures were deduced from MS data (Figure 6B).

Tsitsimpikou *et al.* (2001) analysed the urine of a volunteer having ingested 20 mg of “Ecdysten™” (a commercial preparation containing 20E – see section 6); they collected urine over 5 days and analysed ecdysteroids by GC-MS after derivatization. They found, together with 20E, two less hydroxylated metabolites, which they tentatively identified as 2d20E and 2dE by comparison with available reference molecules.

Mass spectrometry does not provide sufficient information, and only NMR can allow an unambiguous determination of structures. Anyway, it seems reasonable to assume that modification of the B-ring and dehydroxylation are general features of ecdysteroid metabolism in mammals.

Conclusions/prospects

There is rapid catabolism/elimination of ecdysteroids, which means that large amounts would have to be used in order to maintain circulating levels above the concentration required for gene switches systems to be activated. Alternatively, slow-delivery systems like subcutaneous implants represent another way to maintain sustained ecdysteroid levels for several days (Albanese *et al.*, 2000). Another remaining question concerns the metabolism in peripheral tissues. As we have seen with mice, the observed conversions are most probably performed by hepatocytes and intestinal bacteria. It would be of interest to determine whether other mammalian tissues are able to metabolise ecdysteroids, and the nature of the reactions they can perform.

Whether side-chain cleavage between C-20 and C-22 (and possibly also between C-17 and C-20) can take place is a very important question which remains to be investigated by using ecdysteroids labelled on the steroid nucleus, as labelling on the side-chain would be lost if such a reaction would occur. This question seems particularly important for several reasons: (1) cleavage between C-20 and C-22 would result in the formation of 21C steroids that would share some resemblance with vertebrate neurosteroids (Lafont and Sláma, 1995), and (2) in some pharmacological studies rubrosterone (2β,3β,14α-trihydroxy-5β-androst-7-ene-6,17-dione) was as active as 20E (Otaka *et al.*, 1968).

Pharmacological effects of ecdysteroids on vertebrates

The pharmacological actions of ecdysteroids on vertebrates have been reviewed in several previous articles (Burdette, 1962, 1972; Ogawa *et al.*, 1974; Syrov, 1984, 1994; Sláma and Lafont, 1995; Xu *et al.*, 1997; Syrov, 2000; Kholodova, 2001; Báthori, 2002). We will therefore focus on some aspects only, especially on those where recent developments have occurred. The most important data are summarised in Table 1.

Ecdysteroids and growth (Table 2)

The anabolic effects of several phytoecdysteroids (20E,

cyasterone, turkesterone, viticosterone E – see structures on Ecdybase) on mice or rats were reported long ago (see e.g. Okui *et al.*, 1968; Syrov and Kurmukov, 1975a&b; 1976a-c, Syrov *et al.*, 1978, 1981a; Stopka *et al.*, 1999). Growth-promoting effects have also been more recently reported for pigs (Kratky *et al.*, 1997) and Japanese quails (Koudela *et al.*, 1995; Sláma *et al.*, 1996). In many instances however, these effects are not spectacular when considering the growth (weight) curves as they are observed during certain phases of growth or for one sex only and, in many cases, adequate statistical analyses are lacking. Nevertheless, even small effects (i.e. <5 % increase) on growth could be of economical interest for nutritionists, but their firm establishment requires the use of large numbers of animals, which is hardly feasible with large mammals. The addition of E to sheep food increases body growth rate and also wool growth (Purser and Baker, 1994). Surprisingly, these effects were obtained with minute amounts of ecdysone (0.02 µg/kg per day!), and were more evident when animals were fed on a poor quality diet, which indicates that E improves food utilization. In this case, it has been suggested that the effect results from the toxicity of E towards rumen protozoa, but this has not been fully established. In fact, through a stimulation of protein synthesis (and/or a reduction of protein catabolism), ecdysteroids would increase

the lean body mass. In pigs, doses of 0.2-0.4 mg/kg/day resulted in better nitrogen retention and a body weight increase of 112-116% relative to controls, while food consumption was lowered by 11-17% (Kratky *et al.*, 1997). Other experiments used diets supplemented with ecdysteroid-containing plants (e.g. *Rhaponticum carthamoides*) and reported similar growth-promoting effects on pigs over a 30-day period (Selepcova *et al.*, 1993b). In quails, 20E in the diet promoted increased growth (115% of controls), but this was associated with a decreased index of food conversion (Sláma *et al.*, 1996). From these data, it appears difficult to draw general conclusions.

Ecdysteroids and physical performance

20E is claimed to have tonic properties (Abubakirov *et al.*, 1988). Indeed it stimulates muscle growth, provided that protein supply is adequate. Such anabolic effects result in increased physical performance without training (Chermnykh *et al.*, 1988). This was for instance demonstrated using the forced swimming test with rats: animals given ecdysteroids for one week were able to swim for significantly longer times (Azizov and Seifulla, 1998). These effects are similar to those of anabolic steroids. 20E is also able to increase muscle ATP content in vitamin D-deprived rats (Kholodova *et al.*, 1997).

Ecdysteroids: effects on cellular proliferation and differentiation

Wound-healing effects of ecdysteroids have been described (Syrov and Khushbatkova, 1996; Darmograi *et al.*, 1998). 20E (applied at 0.1% w/w in liposomes) shortens the duration of skin repair after superficial wounding and 20E (2 x 10⁻⁴M) stimulates keratinocyte differentiation *in vitro* (Detmar *et al.*, 1994), an effect measured by the increase of the activity of transglutaminase (an enzyme involved in protein connection through isopeptidic bond formation). Accordingly, ecdysteroids show psoriasis-inhibiting effects (Inaoka *et al.* 1997). These results have led to many patents concerning the use of ecdysteroids in cosmetics (Lin and Lin, 1989; Meybeck and Bonté, 1990, 1993; Meybeck *et al.*, 1994; Tsuji *et al.*, 1995a&b; Darmograi *et al.* 1998; Meybeck 1999a&b). In this context, the incorporation of 20E or its acyl ester (2,3,22-tripalmitate) into liposomes has been tested as a slow-release form (Politova *et al.*, 2001).

20E administered orally to rats (5 mg/kg) accelerates the healing process after an experimental bone fracture (Syrov *et al.*, 1986a), and the same molecule (10-100 ng/ml) can stimulate the *in vitro* proliferation of rat osteosarcoma UMR106 cells (osteoblasts) by 41% (Gao and Wang, 2000). Similarly, 20E stimulates proliferation of human umbilical vein endothelial cells (Lin *et al.*, 1997; Wu *et al.*, 1998b), and several phytoecdysteroids can stimulate erythropoiesis in rats (Syrov *et al.*, 1997b).

The effects of ecdysteroids on tumorous cell proliferation are somewhat conflicting: Lagova and Valueva (1981) reported that 20E (0.1-300 mg/kg, subcutaneous injections for 5 days) was mainly ineffective on tumour growth in mice, but it stimulated the growth of mammary gland carcinomas in mice and rats. El-Mofti (1987, 1994) reported that E was able to induce neoplastic lesions in toads and mice; other authors reported inhibitory effects on tumor cell proliferation (Hirono *et al.*, 1969; Burdette, 1974; Shibatani *et al.*, 1996). More recently, Konovalova *et al.* (2002) showed that injected

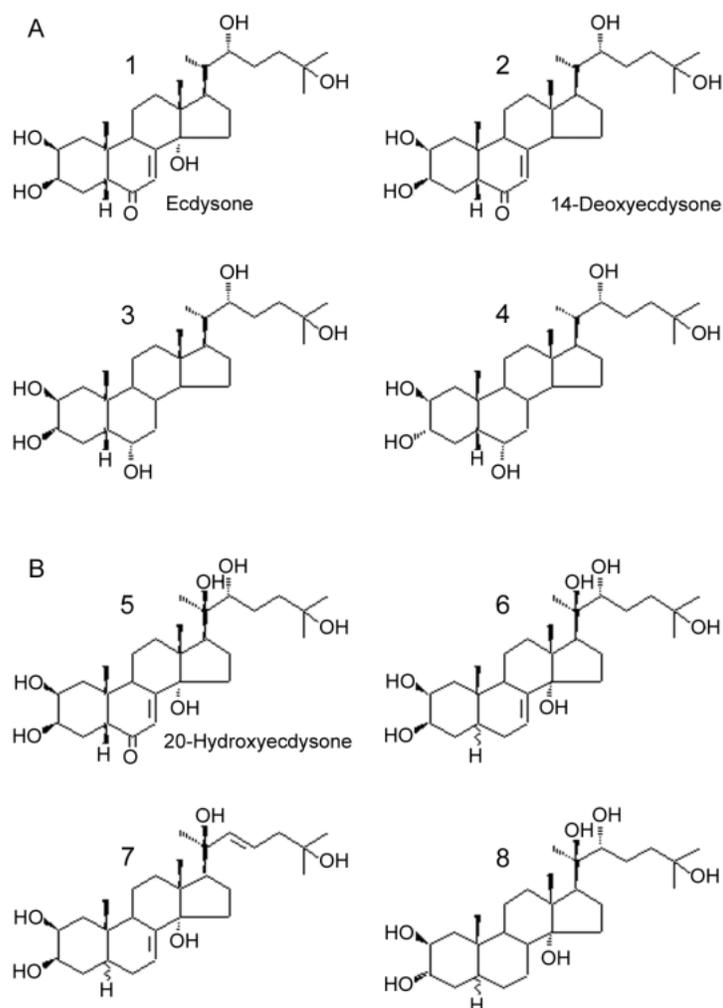


Figure 6. Major E and 20E metabolites in Mammals (see text for details). **A:** E metabolites (2-4) isolated from murine faeces (Girault *et al.*, 1988); **B:** 20E metabolites (6-8) isolated from rat urine (Ramazanov *et al.*, 1996).

Table 1. Pharmacological effects of ecdysteroids on mammals or humans (see also Sláma and Lafont, 1995 for additional references – in red : references to patents)

Biological area	Ecdysteroid Effects	References
Growth	<i>Slight stimulation of growth in mice (by dietary 20E or cyasterone)</i> <i>No effect of dietary ponA , 20E and inokosterone on rat growth</i> <i>Stimulation of growth in rats (by dietary viticosterone E , 20E , turkesterone and turkesterone tetraacetate)</i> <i>Stimulation of growth in sheep by E</i> <i>Simulation of growth in quails by dietary 20E</i> <i>Stimulation of growth in pigs</i> <i>Stimulation of growth in mice (b y 20E in jections)</i>	Hikino <i>et al.</i> , 1969 Matsuda <i>et al.</i> , 1970 Syrov and Kurmukov, 1975a, 1976a,b Purser and Baker, 1994 Koudela <i>et al.</i> , 1995; Sláma <i>et al.</i> , 1996 Krátky <i>et al.</i> , 1997 Stopka <i>et al.</i> , 1999
Cell proliferation and differentia-tion	<i>Dietary 20E (5mg/kg) a ccelerates bone fracture healing process in rats</i> <i>20E possesses wound healing and skin regenerating properties</i> <i>20E (100 µg/ml) promotes keratinocyte differentiation in vitro</i> <i>E induces breast and lung neoplastic lesions in mice</i> <i>20E as skin metabolism-activating and anti-wrinkling agent</i> <i>PonA in a hair tonic preparation</i> <i>Dietary ecdysteroids (20E , cyasterone , turkesterone , 5 mg/kg) accelerate wound healing in rats</i> <i>20E and some analogues inhibit psoriasis</i> <i>20E (200 µg/ml) stimulates proliferation of human umbilical vein endothelial cells</i> <i>Dietary ecdysteroids (20E , turkesterone,...) enhance erythro-poiesis in rats</i> <i>Phytoecdysteroids use for the treatment of burns and wounds</i> <i>20E promotes proliferation of osteoblast-like cells</i>	Syrov <i>et al.</i> , 1986a Lin and Lin, 1989; Meybeck and Bonté, 1990, 1993; Meybeck <i>et al.</i> , 1994 Detmar <i>et al.</i> , 1994 El-Mofty <i>et al.</i> , 1994 Tsuji <i>et al.</i> 1995a Tsuji <i>et al.</i> , 1995b Syrov and Khushbaktova, 1996 Inaoka <i>et al.</i> , 1997 Lin <i>et al.</i> , 1997 Syrov <i>et al.</i> , 1997b Darmograi <i>et al.</i> , 1998 Gao <i>et al.</i> , 2000
Reproduction, development	<i>20E and polypodine B show embr yotoxicity</i> <i>Ecdysteroids might increase milk production in mammals</i> <i>Dietary 20E (5-10 mg/kg) enhances sexual function in rats</i>	Kosar <i>et al.</i> , 1997 Khalitova and Syrov, 1998 Mirzaev <i>et al.</i> , 1992, 2000
Protein metabolism	<i>Injected ecdysteroids (20E , 5 mg/kg) stimulate protein synthesis in mouse liver; this effect corresponds to a stimulation of translation</i> <i>20E stimulates protein synthesis in mouse or gans</i>	Okui <i>et al.</i> , 1968; Otaka <i>et al.</i> , 1968, 1969 a,b ; Todorov <i>et al.</i> , 2000
Carbohydrate metabolism	<i>Hypoglycaemic formulation containing 20E</i> <i>Injection of 20E (0.1–10 mg/kg) in mice reduces hyperglycaemia provoked by alloxan or glucagon , and increases glucose incorporation into glycogen and proteins</i> <i>Dietary 20E (5 mg/kg) for 25 days increases glycogen content in heart , liver and muscles of rats</i> <i>Antidiabetic agents containing 20E or inokosterone</i> <i>Dietary administration of 20E (5 mg/kg) or other ecdysteroids reduces alloxan-induced hyperglycaemia in rats</i> <i>20E in oral antidiabetic preparations</i>	Uchiyama and O gawa, 1970 Yoshida <i>et al.</i> , 1971 Syrov <i>et al.</i> , 1975a; Aizikov <i>et al.</i> , 1978 Takahashi and Nishimoto, 1992 Syrov <i>et al.</i> , 1997a Yang <i>et al.</i> , 2001
Lipid metabolism	<i>Injections of E (10-50 µg/kg) reduce de novo cholesterol biosynthesis in rats</i> <i>Dietary 20E (0.1 mg/kg/day) for 30 days prevents (= has antiradical properties) free-radical lipid peroxidation of membranes in tissues from vitamin D-deficient rats</i> <i>20E in vitro prevents lipid peroxidation in liposomes micelles</i>	Lupien <i>et al.</i> , 1969 Kuzmenko <i>et al.</i> , 1997 Osynskaya <i>et al.</i> , 1992 ; Kuzmenko <i>et al.</i> , 2001
Nervous system	<i>Analgesics containing 20E</i> <i>20E exerts a neuromodulatory action on GABA_A receptor of rat cortical neurons</i> <i>Cerebral neuron protective effects of 20E</i> <i>20E has antie pileptic effects on rats</i> <i>20E has protective effects on amnesia induced by diazepam and alcohol</i>	Takemoto <i>et al.</i> , 1988 Tsujiyama <i>et al.</i> , 1995; Sasa <i>et al.</i> , 1996 ; Okada <i>et al.</i> , 1998 Aikake <i>et al.</i> , 1996 Hanaya <i>et al.</i> , 1997 Xu <i>et al.</i> , 1999

Table 1. Continued on next page

Table 1. Continued from previous page

Heart and circulatory system	<i>Phytoecdysteroids have antiatherosclerotic effects</i> <i>20E can eliminate arrhythmia induced by occlusion of the left coronary descending branch or by aconitine or calcium chloride</i> <i>20E can eliminate arrhythmia induced by aconitine</i> <i>20E (0.25-2.5 mg/kg) can restore normal rheologic indices (fibrinogen concentration and viscosity) of blood from rats with a "high blood viscosity syndrome" induced by cerebral ischemia</i> <i>20E can counteract the damages induced by TNF α on human umbilical vein endothelial cells</i> <i>20E can be used in preparing medicine for angiocardopathy</i>	Syrov <i>et al.</i> , 1983 Kurmukov and Yermishina, 1991 Yang <i>et al.</i> , 1996 Plotnikov <i>et al.</i> , 1998 Wu <i>et al.</i> , 1998b Wu, 2001
Liver function and detoxification mechanisms	<i>Dietary 20E improves liver regeneration after chemically induced damage</i> <i>Dietary 20E or cyasterone (5-50 mg/kg) stimulate bile secretion in rats</i>	Syrov <i>et al.</i> , 1981b, 1992 ; Badal'yants <i>et al.</i> , 1996 Syrov <i>et al.</i> , 1986b
Kidney	<i>Dietary 20E (5 mg/kg) restores normal glomerular filtration rate and suppresses albuminuria in rats treated with a nephrotoxic mixture</i>	Syrov and Khushbatkova, 2001
Defence systems	<i>Dietary 20E (10-20 mg/kg/day) has anti-inflammatory properties in mice and rats</i> <i>Dietary 20E protects gastric mucosa against ulcerogenic chemicals</i> <i>Dietary 20E stimulates primary immune reaction</i> <i>20E (1 pM – 10 μM) and other ecdysteroids enhance DNA synthesis in mitogen-stimulated lymphocytes in vitro</i> <i>20E (10⁻⁹ – 10⁻⁵ M) inhibits histamine release by mast cells</i> <i>Dietary 20E promotes thymus weight increase in rats</i> <i>20E (7.5.10⁻¹³ – 7.5.10⁻⁸ M) activate human lymphocytes in vitro (E-rosette formation, agar migration test)</i> <i>Dietary 20E (5 mg/kg) has no effect on experimentally induced inflammatory processes (pleuresia, arthritis) in rats</i> <i>20E (1 μM) stimulates T-cell CD2 presentation in vitro</i>	Kurmukov and Syrov, 1988 Syrov <i>et al.</i> , 1989 Kuzmitsky <i>et al.</i> , 1990 Fomovska <i>et al.</i> , 1992 Takei <i>et al.</i> , 1991 Gizatullina <i>et al.</i> , 1994 Trenin <i>et al.</i> , 1996 Taniguchi <i>et al.</i> , 1997 Trenin and Volodin, 1999
Antibiotic activity	<i>20E can be used for the treatment of herpes zoster</i> <i>Administration of 20E to rabbits reduces infection by protozoa (Lambliia duodenalis, Fla gellates)</i> <i>20E (200 μg/ml) possesses antibacterial and antifungal activities</i> <i>20E and its acetates have antimicrobial activities</i>	Vargas Gonzalez, 1986 Syrov <i>et al.</i> , 1990 Ahmad <i>et al.</i> , 1996 Volodin <i>et al.</i> , 1999

20E had a synergistic effect with low doses of an antitumour drug (cis-platin). Most probably, the results may differ according with the cell types, the nature and concentration of ecdysteroids used, and this clearly requires more extensive studies. In addition, genoprotective effects of ecdysteroids have been reported (Gubskii *et al.*, 1993; Levitskii *et al.*, 1993a&b, 1996; Chabanny *et al.*, 1994); ecdysteroids can prevent chromatin damages induced by various chemicals.

Ecdysteroids and protein synthesis

Stimulatory effects of ecdysone on protein synthesis were reported as early as 1963 (Burdette and Coda, 1963), and the discovery of phytoecdysteroids made these molecules available in large amounts for pharmacological assays. It was rapidly shown that ecdysteroids were able to stimulate protein synthesis in mouse liver (Okui *et al.*, 1968; Otaka *et al.*, 1968, 1969a&b). In fact, it was shown that 20E stimulates the incorporation of [¹⁴C]leucine in

a cell-free translation system (rat liver polysomes), i.e. it increases the efficiency of the translational machinery (Syrov *et al.*, 1978). Such conclusions have been confirmed and extended to other tissues, especially heart and muscles (Syrov *et al.*, 1975a; Aizikov *et al.*, 1978; Khimiko *et al.*, 2000). Recent structure-activity studies (Syrov *et al.*, 2001) as measured by a stimulation of [¹⁴C] aminoacid incorporation into proteins showed that among the compounds tested turkesterone was the most active, followed by cyasterone and 20E.

Ecdysteroids and glucose metabolism

It was shown early on (Table 3) that 20E given *per os* to rats reduces hyperglycaemia induced either by glucagon or by alloxan treatment (Matsuda *et al.*, 1970; Uchiyama and Ogawa, 1970; Yoshida *et al.*, 1971, Uchiyama and Yoshida, 1974). In fact, 20E stimulates the incorporation of glucose into glycogen and protein in mouse liver (Yoshida *et al.*, 1971) and more generally it enhances glucose utilization by tissues (Syrov *et al.*, 1997a). The

mechanism involved seems to be an increase of tissue sensitivity to insulin (Kosovsky *et al.*, 1989) and preparations containing phytoecdysteroids have been proposed as oral antidiabetics (Takahashi and Nishimoto, 1992; Yang *et al.*, 2001). Depending on the extent of hyperglycaemia, phytoecdysteroid effects may be more or less pronounced than those of manilil, a widely used pharmacological molecule (Kutepova *et al.*, 2001).

Ecdysteroids and lipid metabolism

Ecdysteroids display hypocholesterolaemic effects (Lupien *et al.*, 1969; Mironova *et al.*, 1982; Syrov *et al.*, 1983), through a reduction of cholesterol biosynthesis and an increase of its catabolism (Uchiyama and Yoshida, 1974). 20E (5 mg/kg *per os*) stimulates the conversion of cholesterol into bile acids in rats (Syrov *et al.*, 1986b), and such an effect is reminiscent of some oxysterols (Schroepfer, 2000). In connection with these effects, ecdysteroids may also have antiatherosclerotic actions (Matsuda *et al.*, 1974; Syrov *et al.*, 1983). Intraperitoneally injected 20E (0.5 mg/kg in rats) also enhances [¹⁴C]acetate incorporation into liver triglycerides and reduces triglyceride lipase activity (Catalán *et al.*, 1985).

Ecdysteroids: a “universal medicine”?

An impressive number of papers dealing with ecdysteroid effects are available in the literature. They concern almost every physiological function, and we will give below a brief insight of

the published data. It must be noted, however, that in many instances that, in addition to the difficulties caused by language barriers, the experiments are not always described with all the desirable details.

Ecdysteroids improve nervous function: in early studies, it was shown that 20E induced glutamic decarboxylase (an enzyme involved in GABA biosynthesis) in rat brain (Chaudhary *et al.*, 1969), and that E was able to induce acetylcholinesterase in rat brain too (Catalán *et al.*, 1984). More recently, ecdysteroids were shown to represent neuron-protective agents; they reduce glutamate-induced cell death in cortex neurons of rat foetuses and they are proposed as a therapy against mental and behavioural disorders (Aikake *et al.*, 1996). In addition, they may protect against amnesia induced by diazepam or alcohol (Xu *et al.*, 1999). Similar neuroprotective effects have been described for progesterone and oestradiol mixtures in animal models of neurodegeneration (Vongher and Frye, 1999).

Ecdysteroids stimulate hepatic functions: 20E accelerates recovery after hepatitis induced by heliotrine treatment (Syrov *et al.*, 1981b). 20E and other ecdysteroids (turkesterone, cyasterone) administered (10 mg/kg) to rats with hepatitis induced by subcutaneous injection of carbon tetrachloride prevent its hepatotoxic action (Syrov *et al.*, 1992). Moreover, a pretreatment with 20E (5 mg/kg) for one week will reduce the effects of a subsequent heliotrine treatment (Badal'yants *et al.*, 1996).

Ecdysteroids improve heart and lung function: 20E has

Table 2. Effects of ingested or injected ecdysteroids on growth of various vertebrate species (in red, reference to patents).

Species	Ecdysteroids	Daily dose	Mode of administration	Duration of treatments (days)	Effects	References
Mice	20E, Cyas	0.005-0.1 mg	per os	90	Increased protein synthesis in liver and kidney	Hikino <i>et al.</i> , 1969
Mice	Turk	5 mg/kg	i.p.		Increased liver protein synthesis <i>in vivo</i> and <i>in vitro</i>	Syrov <i>et al.</i> , 1978
Mice (juveniles and adults)	20E	0.1, 0.5, 1 mg	i.p.	30	Increased growth of juvenile females and of adults of both sexes	Stopka <i>et al.</i> , 1999
Rats (29-day old)	E, 20E, PonA	2, 10, 50 mg	per os	5	No effect on growth rate	Matsuda <i>et al.</i> , 1970
Rats (castrated juveniles)	VitE, Turk	0.5-10 mg/kg	i.p.	10	Increased muscle and liver weight No androgenic effect	Syrov and Kurmukov, 1975a,b Syrov <i>et al.</i> , 1975b
Rats (juveniles and adults)	20E	5 mg/kg	per os	7	Increased muscle and liver weight No androgenic effect	Syrov and Kurmukov, 1976c
Rats (juveniles, adults and ovariectomized juveniles)	20E	5 mg/kg	i.p.	7	Increased body weight and growth of liver, kidneys, muscles	Syrov <i>et al.</i> , 1981a
Sheep	E	0.02 µg/kg	per os, or i.v.	35-150	Increased growth rate of body and of wool	Purser and Baker, 1994
Pigs	20E	0.2-0.4 mg/kg	per os	30	Increased growth and higher nitrogen retention	Krátky <i>et al.</i> , 1997
Japanese quails	20E	20, 100, 500 mg/kg of food	per os	28	Increased growth	Sláma <i>et al.</i> , 1996

Cyas: cyasterone; E: ecdysone; 20E: 20-hydroxyecdysone; PonA: ponasterone A; Turk: turkesterone; VitE: viticosterone E
i.p.: intraperitoneal injection; i.v.: intravenous injection.

been recommended for the prevention of myocardial ischaemia, arrhythmia and is described as enhancing VEGF expression (Wu, 2001). An antiarrhythmic effect of 20E was also reported by Kurmukov and Yermishina (1991) and Yang et al. (1996), and an extract of *Leuzea carthamoides* containing high amounts of 20E also showed a similar effect (Maimeskulova and Malslov, 2000). In rabbits experimentally rendered atherosclerotic (by a high cholesterol diet), 20E (10 mg/kg/day *per os*) given for 28 days was able to increase Na⁺/K⁺ ATPase in myocardium (Khushbaktova et al., 1987). Intravenous injection of 20E showed also a therapeutic effect after lung contusion (Wu et al., 1997, 1998a).

Ecdysteroids improve renal function: when rats are given a nephrotoxic mixture (uranyl acetate + glycerol), 20E (5 mg/kg) seems thereafter able to restore a normal glomerular filtration rate and to suppress albuminuria (Saatov et al., 1999; Syrov and Khushbaktova, 2001).

Ecdysteroids and the immune system: various immunomodulatory effects of ecdysteroids have been described. Single intraperitoneal injections of various ecdysteroids (20E, 2dE, 2d20E, polB, turkesterone, 1-5 mg/kg) increase the concentration of antibody-forming cells in the spleen of mice immunised with sheep red blood cells (Sakhibov et al., 1989). Low (7.5x10⁻¹²-7.5x10⁻⁸ M) concentrations of 20E induce the activation (E-rosette formation test) of human lymphocytes (Trenin et al., 1996; Trenin and Volodin, 1999). Low to moderate (10⁻¹²-10⁻⁵ M) concentrations of 20E or other ecdysteroids stimulate, whereas higher (10⁻⁴M) concentrations eventually inhibit, DNA synthesis in concanavalin A – activated lymphocytes (Kuzmitsky et al., 1990; Fomovska et al., 1992; Chiang et al., 1992).

20E (10-20 mg/kg/day *per os*) has antiinflammatory properties similar to cortisone acetate in rats and mice (Kurmukov and Syrov, 1988; Fomovska et al., 1992) and turkesterone improves lung defence mechanisms in diabetic rats (Najmutdinova and Saatov, 1999). 20E was shown to inhibit in a dose-dependent fashion (10⁻⁹-10⁻⁴ M) histamine release from rat peritoneal mast cells induced by anti-IgE or concanavainin A (Takei et al., 1991). Taniguchi et al. (1997), however, could not observe any antiinflammatory effect of 20E given orally to rats (5 mg/kg/day for 7 days).

Ecdysteroids have antioxidant properties: 20E has

antioxidative and anti-free radical properties (Osynska et al., 1992) and it can thus reduce lipid peroxidation (Kuzmenko et al., 1997, 2001). Several models were used in these studies, as the chemiluminescence of blood serum induced by H₂O₂ using rats receiving a vitamin D-deficient diet eventually supplemented with 0.1 mg 20E/kg per day, or the uptake of oxygen by methyl linoleate micelles in the presence or absence of 20E.

Are ecdysteroids toxic to microorganisms?: there are a few reports about antimicrobial activity of ecdysteroids. However, Ahmad et al. (1996) reported antifungal and antibacterial activity of 20E at rather high concentrations (between 100 and 400 µg/ml, i.e. 2-8 x 10⁻⁴ M). An antimicrobial activity of 20E and its acetates was also observed by Volodin et al. (1999). Toxic effects on protozoa have also been reported; rabbits receiving 20E *per os* (5 mg/g/day for 3 months) showed a reduced infection with *Lamblia duodenalis* (Syrov et al., 1990), and the improvement of ruminant productivity by ecdysone was also interpreted by its toxicity towards rumen protozoa (Purser and Baker, 1994).

Ecdysteroids are not toxic to vertebrates: ecdysteroids have a very low toxicity (LD50 > 6g/kg), they are not hypertensive and, in spite of their anabolic action, they would have neither androgenic nor oestrogenic (or antioestrogenic) effects; they induce no virilisation and they do not induce significant changes in castrated animals (e.g. Prabhu and Nayar, 1974). All together this suggests that ecdysteroids are attractive compounds for a wide array of uses, which have been proposed, and of course it would be of particular interest to understand more precisely their mode(s) of action in mammals.

Genomic and/or non-genomic effects of ecdysteroids?

Do ecdysteroids have genomic effects on vertebrates?

In insects, ecdysteroids have well-known genomic effects which involve nuclear receptors (see Section 2). When considering the molecules in 3-dimensions, it is clear that they show striking differences to vertebrate sex or adrenal steroids, and their full cholesterol side-chain most probably prevents any binding to the receptors of these vertebrate hormones. Recently, however, it has been found that some previously “orphan” nuclear receptors (e.g.

Table 3. Effects of ingested or injected ecdysteroids on carbohydrate metabolism (in red, reference to patents).

Species	Ecdysteroids	Dose	Mode of administration	Duration of treatments (days)	Effects	References
Rats or Mice	20E	0.5 mg/kg	i.p.		Hypoglycaemic	Yoshida et al. , 1971; Uchiyama and Yoshida, 1974
Rats	20E	5 mg/kg	per os	15	Recovery of liver mito-chondrial function after alloxan treatment	Tashmukhamedova et al. , 1985 Syrov et al. , 1992
Rats	20E				Increase sensitivity to insulin treatment in experimental diabetes	Kosovsky et al. , 1989
Humans	20E, Ino		per os		Antidiabetic effects	Takahashi and Nishimoto, 1992
Rats	20E, Cyas, Turk		per os		Decrease of glycaemia in diabetic animals	Syrov et al. , 1997a
Humans	20E + 20E2Ac	-	per os	-	Antidiabetic effects	Yang et al. , 2001

Cyas: cyasterone; E: ecdysone; 20E: 20-hydroxyecdysone; Ino : inokosterone A; Turk: turkesterone.

LXR, PXR) bind endogenously produced oxysterols (Janowski *et al.*, 1999; Schroepffer, 2000) or have a broad specificity and may bind a wide array of xenobiotics including several steroids (Jones *et al.*, 2000). Given this broad ligand specificity, these proteins might rather function as “endocrine sensors” rather than “true receptors” (Evans, 2002). So, until ecdysteroids are directly tested for binding to such receptors, it remains conceivable that they may have transcriptional effects through binding to some nuclear receptor(s); indeed early studies showed a rapid *in vivo* stimulation by 20E of the incorporation of [¹⁴C]orotic acid into RNA in mouse liver (Uchiyama and Otaka, 1974).

Ecdysteroids: membrane effects?

Membrane effects of steroids are nowadays well documented and they may proceed through three different pathways (Figure 7). According to Brann *et al.* (1995), these effects may either involve: (1) the dissolution of ecdysteroids in the membrane bilayer and a change in the environment of some membrane proteins (and hence of their activity), (2) their interaction with a specific membrane receptor, which will activate some transduction mechanism, or (3) their binding to a modulatory site of the receptor for another molecule. These different effects are not mutually exclusive. Very recently, a membrane progesterin receptor involved in fish oocyte meiotic reinitiation was cloned and its physiological relevance was fully established (Zhu *et al.*, 2003).

Dissolution of edysteroids in the membrane lipid bilayer:

In order to test for the first hypothesis, Tuganova and Kotsyuruba (1996) developed experiments designed to analyse the dissolution of ecdysteroids in human erythrocyte membranes. They did not perform direct experiments, i.e. by measuring the incorporation of radiolabelled ecdysteroids. In a first set of experiments, erythrocytes were first incubated with various steroids (10⁻⁶ M) and then with [³H]cholesterol; both 2d20E and 20E pretreatments reduced the radioactivity associated with membrane fractions. In a second set of experiments, the authors first incubated erythrocytes with various concentrations of 20E (10⁻¹⁴ to 10⁻¹⁰ M) then with either radiolabelled cholesterol, cholecalciferol or calcitriol; 20E reduced mainly calcitriol incorporation. Such experiments support the idea that ecdysteroids can be incorporated into membrane bilayers, although they do not constitute an absolute proof. It is tempting to make a relation between these results and the rapid effect of 20E on Na⁺/K⁺ ATPase activity in *D. melanogaster* salivary gland cells (Schneider *et al.*, 1996).

Rapid membrane effects: A rapid increase of cGMP and a decrease of cAMP levels in mouse plasma, together with a decrease of PKA activity in liver were described 40 min after an intraperitoneal injection of 10 µg 20E (Catalán *et al.*, 1979a&b, 1982). More recently, it was shown that 20E evokes rapid (1-2 min) and transitory effects on membranes (Kotsyuruba *et al.*, 1995a-c, 1998a&b, 1999); 20E increases the pool of free arachidonic acid and the synthesis of leukotrienes and prostaglandins. Such responses were observed with different cell types (hepatocytes, erythrocytes, lymphocytes, macrophages etc.). In many instances the effects of 20E resemble those evoked by calcitriol (1,25OH-D₃), a molecule often used for comparison by those working on ecdysteroid

pharmacology (Barsony and Marx, 1988). The same effects were also produced by 20E bound to magnetite nanoparticles (Mykhaylyk *et al.*, 1999; 2001), a formulation which should prevent 20E diffusion into target cells, and thus restricting its possible action(s) to the plasma membrane level.

We should emphasise here that ecdysteroids can be recognised by membrane receptors in arthropods: ecdysteroids can be detected by taste cell receptors both in Crustacea (Tomaschko, 1999) and insects (Tanaka *et al.*, 1994; Descoins and Marion-Poll, 1999), and in vertebrates too steroids can work as pheromones (see e.g. Sorensen *et al.*, 1990). Thus, such a mode of action is conceivable.

Neuromodulatory actions: Such effects are well documented for vertebrate neurosteroids, which may modulate the response of neurotransmitter receptors to their cognate ligands. The binding of the steroid alone has no apparent effect. Thus, the GABA_A receptor possesses (in a domain separate for the neurotransmitter binding site) a binding site for steroids, which may therefore modify the response to GABA. In a similar way, 20-hydroxyecdysone showed a neuromodulatory effect on GABA_A receptor of rat cortical neurons (Tsujiyama *et al.*, 1995; Sasa *et al.*, 1996), although this was observed for rather high concentrations (10-100 µM). In connection with this effect, 20E showed an antiepileptic activity in rats (Hanaya *et al.*, 1997); when 20E was given orally (100-200 mg/kg) to spontaneous epileptic rats, it was able to reduce tonic convulsions.

Some recent data

Recently, Constantino *et al.* (2001) fortuitously made a crucial observation. Using the Invitrogen® ecdysteroid-inducible expression system to analyse the transduction mechanisms of interleukin-3 (IL-3) in a pro-B lymphocyte cell line, they found in control experiments that murA and ponA were able to potentiate the IL-3-dependent activation of PI 3-kinase/Akt pathway in non-transformed cells.

Given the central role of the Akt/PKB pathway in mammalian cell metabolism (e.g. Brazil and Hemmings, 2001; Whiteman *et al.*, 2002), such results provide an interesting basis for explaining in a single way many effects of ecdysteroids on mammals, as concerns their hypoglycaemic, antiapoptotic and anabolic actions (Figure 8). The available data do not allow to decide whether edysteroids act on the IL-3 receptor itself or on a downstream step.

Where is 20-hydroxyecdysone found?

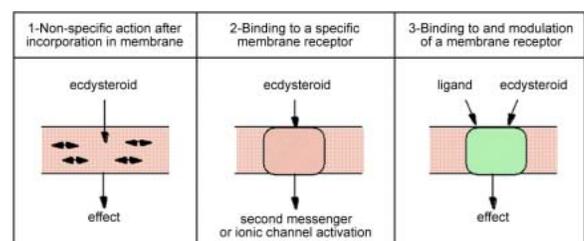


Figure 7. Three possible ways for a membrane effect of ecdysteroids (adapted from Brann *et al.*, 1995).

Phytoecdysteroids are found in many plant species, where they can reach concentrations above 1-2% of the plant dry weight (e.g. Lafont, 1998, Dinan, 2001). Ecdysteroid-rich species are found among ferns and angiosperms and some of these species are either very common (e.g. the fern *Polypodium vulgare*) or they are cultivated on a large scale for their pharmacological properties (e.g. *Leuzea*, *Pfaffia*, *Cyanotis*). Given the still limited market at the moment, a few plant species only (Table 4) are currently used as a source of phytoecdysteroids: (1) *Leuzea* (= *Rhaponticum carthamoides*) (Asteraceae) from Eastern Europe countries, where it is cultivated as a remedy in traditional medicine, (2) *Pfaffia* (in fact a group of related species) = Brazilian ginseng (= Suma), again a plant used in traditional medicine and (3) *Cyanotis vaga* or *C. arachnoides*, a monocotyledonous plant, extracts of which are used on a large scale also for the synchronization of spinning in silkworm larvae (Guo, 1989; Chandrakala *et al.*, 1998).

Over 140 different preparations containing ecdysteroids for oral use can be found on the market (Table 5). We may distinguish several categories among them: (1) those containing crude or semi-purified plant extracts (plant powders, or alcoholic extracts - elixirs) and (2) those containing "pure" 20E or a defined ecdysteroid mixture. Most of them are proposed for use by bodybuilders, but some have been designed for more specific users (e.g. golfers), or for animals (dogs, horses). In addition, ecdysteroids are also present in at least two cosmetic preparations (Hydrastar and Phenomen A from Christian Dior).

The impressive development of preparations containing ecdysteroids suggests that this class of molecule has indeed at least some of the claimed effects. The scientific justification for such commercial developments relies, however, on just a few references (ca. 10), often with the same ones being cited to support quite different effects.

Conclusion

Ecdysteroids are probably the most abundant steroids in

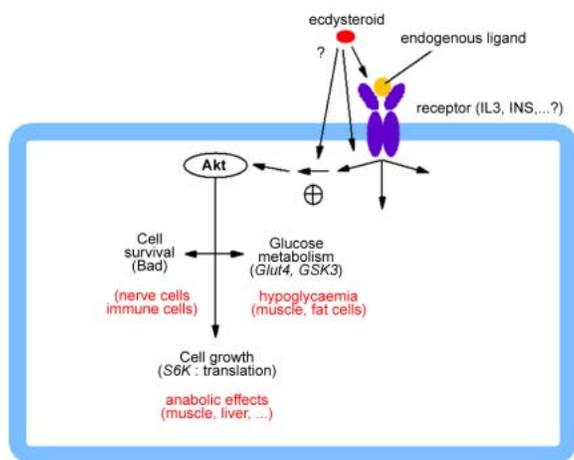


Figure 8. A working hypothesis for ecdysteroid action on mammalian cells: a stimulation of the Akt/PKB pathway would explain a large set of the described effects of 20E on mammals (see text for details).

Bad: a proapoptotic factor, which is inhibited upon phosphorylation by Akt; Glut4: glucose transporter type 4; GSK3: glycogen synthase kinase-3; IL3: interleukin-3; INS: insulin; S6K: ribosomal protein S6-kinase.

nature because they are produced not only by arthropods, but also by many plant species. They seem to display a wide array of pharmacological effects on vertebrates, many of which are beneficial. However, these claims require more thorough validation and clinical testing. Ecdysteroids are used by an increasing number of humans as anabolic compounds, and it may well be that in the near future they will also be used on domesticated animals. This is the reason why new methods of detection and quantification have been recently proposed (Tsitsimpikou, 2001; Le Bizec, 2002) and further developments in this area are required. Whether ecdysteroid use will become controlled (e.g. for high-performance sportsmen or domestic animals [e.g. race horses]) is still open.

Ecdysteroids have also been successfully developed as effective inducers for gene switch control systems, several of which are presently in use. Ecdysteroids and/or bisacylhydrazines fulfil many of the required criteria, but not all. There are still problems which need to be overcome (e.g. the need for highly potent ligands for modified ecdysteroid receptors in transformed mammalian or plant cells). However, there is clearly great potential in this area. The future of ecdysteroid-regulated gene switches as an experimental tool is assured, but the prospects as *in vivo* systems is more debatable; the numerous pharmacological effects of ecdysteroids may preclude the development of their use in humans for gene therapy systems. This can only be resolved if more effort is invested into examining the biochemical fate and pharmacological consequences of ecdysteroids in mammals, especially humans.

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Table 4. Plants currently used to obtain the ecdysteroids used for various preparations

Species	Family	Local name	Part of the plant used
<i>Ajuga turkestanica</i>	Labiatae		Whole plants ?
<i>Cyanotis vaga</i>	Commelinaceae		Whole plants and/or roots
<i>Cyathula capitata</i>	Amaranthaceae	Chuan Niu Hsi	Roots
<i>Leuzea (= Rhaponticum) carthamoides</i>	Asteraceae	Maral	Roots, seeds
<i>Pfaffia iresinoides</i>	Amaranthaceae	Suma, Para Toda	Roots
<i>Pfaffia paniculata</i>	Amaranthaceae	Suma	Roots
<i>Pfaffia stenophylla</i>	Amaranthaceae	Suma	Roots
<i>Polypodium aureum</i>	Ferns	Calaguala	Rhizomes
<i>Polypodium lepidopteris</i>	Ferns	Samambaia	Rhizomes, leaves
<i>Polypodium vulgare</i>	Ferns	common polypody	Rhizomes ?

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Table 5. Preparations based on purified ecdysteroids or on ecdysteroid-containing plant powders/extracts.

Nr	Product	Supplier	Web address	Plant source(s)
1	Active	Velocity	http://www.fastathlete.com/velocity/active.html	<i>Leuzea</i> + <i>Cyanotis</i>
2	ACT-SUM™	7th Millenium Nutrition	http://www.diabetestea.com/actsum.html	<i>Pfaffia</i>
3	Adaptogen N	Muscle And Sport Science (MASS)	http://www.musclemass.com/prohormone.html	<i>Pfaffia</i>
4	Adrena+	Greens+™	http://www.greenspluscanada.com/fr/infonp.htm	<i>Pfaffia</i>
5	Advanced Compound 2	Advanced Labs	http://www.reach4life.com/details.asp?ProdID=4534	<i>Pfaffia</i>
6	ALL-iN1	Sports Nutrition Net	http://www.sports-supplements.co.uk/reviews/sportsnutrition/allin1.shtml	<i>Leuzea</i> + <i>Cyanotis</i>
7	Amazonia Immuno Forte	Nutrisana	http://www.nutrisana.com/html/amazonia_fr.htm	<i>Pfaffia</i>
8	Animal Methoxy Stak	Universal Nutrition	http://www.fitnessfirstusa.com/details.asp?item=7781	?
9	Atomic Muscle	Maximus Nutrition	http://www.maximusnutrition.com/products/MN012.html	?
10	Beta-Builder	Ultimate Nutrition	http://www.onlineprotocols.com/product/beta_product.htm	?
11	β-Ecdysone	NuTrex Nutrition	http://www.fitnessone.com/load_frames.html?anabolic-products/b-ecdysone	?
12	Beta-Ecdysterone	Gennapharm	http://www.gennapharm.com/BetaEcdysteroneFrame.asp	?
13	Beta-Ecdysterone Stack	PEAK Nutrition	http://peaknutrition.com/betstac90tab.html	<i>Pfaffia</i>
14	Beta-Mass	7th Millenium Nutrition	http://www.diabetestea.com/beta1.html	<i>Pfaffia</i>
15	Betaoxytol	BSN	http://www.bodybuilding.com/store/bsn/beta.html	<i>Leuzea</i>
16	Beta-X capsules/liquid	Flexstar Sports Nutrition	http://primenutrition.com/betbet180cap.html	<i>Cyanotis</i>
17	Bicreatol	BSN	http://www.vigorousliving.net/bsnbic12.html	?
18	Bio-Pro Plus	Body Ammo	http://www.nutritionblvd.com/284132.html	<i>Pfaffia</i>
19	BPS	Syntrax	http://store.yahoo.com/xsportsnutrition/syntraxbps.html	<i>Leuzea</i>
20	Brazilian Ginseng	Paradise Herbs	http://www.iherb.com/braziliangin.html	<i>Pfaffia</i>
21	Bug Juice	?	http://www.supervita.com/top12/bugjuice.htm	?
22	Changing Times™	Nature's Plus	http://www.naturesplus.com/products/	<i>Pfaffia</i>
23	Clear Energy	Garden of Life	http://www.iherb.com/clearenergy.html	<i>Leuzea</i>
24	Creabolic fizz	Maximum Human Performance	http://www.fitnessfirstusa.com/details.asp?item=7138	<i>Pfaffia</i>
25	Cyclone	Maximuscle	http://gymratz.co.uk/bodybuilding-supplements/item44.htm	?
26	Desire-X	MasoN natural	http://www.vitaminsalon.com/sexenhancers/hornygoatweed/goatweed.htm	<i>Polypodium</i>

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27	Ecdy Max HP	EAS	http://www.bodybuilding.com/store/eas/ecdymax.html	?
28	Ecdy-20	MRM	http://www.metabolicresponse.com/detail.asp?id=86	Leuzea
29	EcDyBol	Bodyonics Pinnacle	http://www.pinnaclebody.com/ecdybol.htm	Leuzea
30	Ecdy-Bolin	?	http://www.trulyhuge.com/ecdy-bolin.htm	Leuzea
31	Ecdy-Force	PEAK Nutrition	http://www.peaknutrition.com/ec180tab.html	Pfaffia
32	Ecdylean	NuTrex Nutrition	http://www.fitnessone.com/anabolic-products/ecdylean.html	Leuzea ?
33	Ecdy-Mass™	Gaspari Nutrition	http://www.richgaspari.com/products/ecdymass.htm	Leuzea
34	Ecdymax™	A R Nutrition	http://www.bodybuilding.com/store/ar/ecdymax.html	Leuzea
35	Ecdysone	Reflex Nutrition	http://www.creatinestore.co.uk/productsinfo/Ecdysone_x_90_Capsules.asp	Cyanotis
36	Ecdysten	Thermo Life International	http://www.thermolife.com/Products-Ecdysten.html	Leuzea
37	Ecdysterone	ZOE Labs	http://www.tigerfitness.com/Ecdysterone.htm	?
38	EcdyVone	Prolab	http://www.fitnessfirstusa.com/details.asp?item=7701	Leuzea + Cyanotis
39	EcdyVone Plasma	Prolab	http://www.bodyworks-nutrition.com/ecdyvone-plasma.html	Cyanotis
40	Ekdisten	BodybuilidnG Estonia	http://www.bodybuilding.ee/est/texts/toit/ekdisten.htm	Leuzea
41	Elite Athlete™	MD Healthline	http://mdhealthline.com/cgi/sgx/store/web_store.cgi?cart_id=134.157.163.233&page=elite.html&afnum=1897	Cyanotis
42	Enact+	Greens+™	http://www.masantesexuelle.com/nouvelle6.htm	Pfaffia
43	Equine Gold	Equine Gold ?	http://natural-stress-management.com/equine_gold.htm	Leuzea
44	Eroto 2 Caps™	7th Millenium Nutrition	http://www.diabetestea.com/erotojoy.html	Pfaffia
45	Especially Yours	Nature's Plus	http://www.naturesplus.com/products/	Pfaffia
46	Excite	Dynamitize Nutrition	http://www.affordablesupplements.com/excite.asp	Polypodium
47	Fem Actin	Herbal Actives	http://www.healthclub.ru/natures/femactin.php	Pfaffia
48	FirmEase	Gero Vita International	http://www.gvistore.com/gbd/fxa.html	Leuzea + Pfaffia
49	Fitnessky	Slovakofarma/Intercaps	http://www.ishop.purus.cz/start.php?menu=E%7CX9&soubor=podkat.php	Leuzea
50	20-H	TKE (The Kutting Edge)	http://www.tkefitness.com/20h.asp	Leuzea + Cyanotis
51	Horny Goat Weed	Bodyonics Pinnacle	http://www.marigarden.com/hgw/index.html	Polypodium
52	Horny Goat Weed	Nature's A.....	http://www.horny-goat-weed-maca-pure.com/ingredients.htm	Polypodium
53	Horny Goat Weed	Herbal Remedies USA™	http://www.herbalremedies.com/horgoatweed6.html	Polypodium
54	Horny Goat Weed Plus	Natural Men's Health ?	http://www.onlynaturalinc.com/store/hornygoat.html	Polypodium
55	HumanoPro	GEN® Human Performance Nutrition	http://www.pacific-nutrition.com/whey-humanopro.htm	Leuzea
56	Hydra-Star	Christian Dior	http://www.auravita.com/factfile.asp?pCode=PACH11050&pType=5	Cyanotis
57	IMH-Ecdysterone	Supplements Research & Advancements (SRA)	http://www.healthnutwarehouse.com/imhec90cap.html	?
58	Immunectar®	Nature's Plus	http://www.naturesplus.com/products/	Pfaffia
59	IsoStak AFC	Universal Nutrition	http://www.sportvoeding.nl/isostak.html	?
60	IT-Ideal Transformation Formula	Miada Sports Nutrition (NZ)	http://www.miadaspport.com/	Cyanotis
61	Lean 65 MRP	Iron-Tek	http://www.fitnessone.com/load_frames.html?mrp-products/lean_65_mrp	Cyanotis
62	Leuzea drops	Slovakofarma	http://www.aha.ru/~slovakof/products/leuzea.html	Leuzea
63	Liquid Dynamite	Beast Sports	http://www.buyjustnatural.com/liquiddynamite.html	?
64	Maralan	Firma Kr`en	http://http://www.kren.cz/maralan.htm	Leuzea
65	Maralan Super	Firma Kr`en	http://http://www.kren.cz/maralan.htm	Leuzea
66	Maxabol II	Maxam Nutraceutics	http://ssl.ipns.com/maxamssl/MAXAM_ASP_ViewProduct_LongDescription.asp?ProductIDX=35	Pfaffia ?
67	Medicinal Cyathula Root	Sinoking	http://sinoherbking.com/sk3/41052.html	Cyathula
68	Methoxerone Plus	MaxMuscle	http://www.maxmuscle.com/PressRelease/methoxerone/methoxerone.htm	Pfaffia + Cyanotis
69	Methoxy ECD™ Xtreme	EAS ?	http://www.usadiamondnutrition.com/MethoxyECDxtreme.html	?
70	Methoxy Factor HP	EAS	http://www.musclesurf.com/metfachp50so.html	?
71	Methoxy Gro™	Myvitanet Nutritional Products	http://myvitanet.com/vitanet/5met100mg60c.html	Cyanotis
72	Methoxy Pure	Sports Science Research	http://www.sportsscience-research.com/pages/mythoxy_pure.asp	?
73	Methoxy+Ecdysterone Sublingual Stack	Supplements Research &	http://www.gotsupplements.com/methoxy-ecdysterone-sublingual-stack.htm#69	Cyanotis
74	Methoxybol-7	Scientific Advanced Nutrition (SAN)	http://store.yahoo.com/ampharma/metbysan.html	?

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75	Methoxybolic X	Total Nutritional Technologies (TNT)	http://www11.netrition.com/tnt_methoxybolic_x_page.html	<i>Cyanotis</i>
76	Methoxy-sterone	Muscle Science	http://www.jkdu.co.za/JKDU_new/jkd_supp/methoxy.htm	?
77	Methoxy-Tek™	Iron-Tek	http://www.advantagesupplements.com/birmet60cap.html	<i>Cyanotis</i>
78	Methoxy Whey	Ultimate Nutrition	http://www.fitnessfirstusa.com/details.asp?item=7709	<i>Cyanotis</i>
79	MSB	Syntrax	http://www.totalhealthsource.com/SY013.asp	?
80	Muscle Drive HP	EAS	http://www.sportsupplementsource.com/sportsource/easmusdrivhp.html	<i>Cyanotis</i>
81	Muscle Drive HP Bars	EAS	http://www10.netrition.com/eas_muscle_drive_bars_page.html	<i>Cyanotis</i>
82	MX-7 Extreme	GEN® Human Performance Nutrition	http://www11.netrition.com/gen_mx7_extreme_page.html	<i>Leuzea</i>
83	Myoblast II	Body Life Science	http://www.pinsonsfitness.com/body_life_sciences.html	<i>Pfaffia</i>
84	Myo-Blast™	?	http://www.muskelpiraten.com/mpshop/shop.asp?action=visaen&art=CD200	<i>Leuzea</i>
85	MyoMeth	Scitech Nutrition	http://www.healthcenterplus.net/Myometh.htm	<i>Leuzea</i> ?
86	Natural Sterol Extreme	Universal Nutrition	http://www.b-fit.com/natstere90.html	?
87	New Power	Anew ?	http://www.anew.com.br/02_new_power.asp	<i>Pfaffia</i>
88	Norateen II	LA™ Muscle	http://www.norateen.com/docs/norateen2.php3	?
89	Nu-Nitro-8™	Nutec	http://www.nutecperformance.com/productinfo.asp	?
90	Nutrejuva	Microlight	http://www.microlight.net/nutrejuva2.htm	<i>Pfaffia</i>
91	NutriZAC	Nature's Plus	http://www.naturesplus.com/products/	<i>Pfaffia</i>
92	Nutrizen	Nature's Plus	http://www.natural-distribution.com/produits/natureplus/nutrizen.htm	<i>Pfaffia</i>
93	Organic Germanium	Nature Most	http://www.totaldiscountvitamins.com/Merchant/germaniumframe.htm	<i>Pfaffia</i>
94	Peak Performance-2	K-9 Power Products LLC	http://www.dogzone.com/april/ingredie.htm ; http://k9power.com/peak.htm	??
95	<i>Pfaffia paniculata</i>	?	http://www.derivatamedicus.se/produktinfo.asp?artikelnr=1	<i>Pfaffia</i>
96	<i>Pfaffia paniculata</i>	New Deal	http://www.newdeal.ch/pfaffia/	<i>Pfaffia</i>
97	<i>Pfaffianico</i>	PA	http://www.anew.com.br/02_pfaffianico.asp	<i>Pfaffia</i>
98	Phenomen-A	Christian Dior	http://www.hyperbelle.com/Tests/phenomenA.html	<i>Cyanotis</i>
99	Phytobol	ASN	http://www.musclesurf.com/phytobol.html	<i>Pfaffia</i>
100	Power Meal	Advanced Pharmaceutical Research	http://shop.store.yahoo.com/sports-nutrition/powermeal35cho1.html	?
101	Primavar Xtreme	American Research Labs	http://www.pinsonsfitness.com/primavar_xtreme.html	?
102	Prime One	Prime Quest™ International	http://www.whatskillingyou.com/ingredients.html	<i>Leuzea</i>
103	Prime Plus	Prime Quest™ International	http://www.primequest.co.za/primeplus.htm	<i>Leuzea</i>
104	Pro Complex PM	Optimum Nutrition	http://shop.store.yahoo.com/sports-nutrition/procompmhoc.html	<i>Cyanotis</i>
105	Pro-Eroto™	7th Millenium Nutrition	http://www.diabetestea.com/erotojoy.html	<i>Pfaffia</i>
106	Promax Xtreme	Maximuscle	http://affordablesupplements.co.uk/ProductDetail.asp?ProductID=443	?
107	Protein PM™	Interactive Nutrition	http://www.interactivenutrition.com/products/proteinpm.php	?
108	REAP	Phyto-longevity, Inc.	http://www.phytolongevity.com/PRODUCTS/PERFORMANCE/performance.html	<i>Leuzea + Ajuga</i>
109	Retibol®	Eiselt Research	http://www.eiselt.se/produkter/Retibol.htm	<i>Leuzea</i>
110	Robofit tinktura	Bio System BT	http://www.vardanet.hu/biosystem/r.html	<i>Leuzea</i>
111	Rus-Olympic	Nutri-Tech International AS	http://www.adaptogeno.com/rus_olympic.htm	<i>Leuzea</i>
112	Russian Secret	Power Health, Inc.	http://powerhealth.com/detail.cfm?id=365	<i>Leuzea</i> ?
113	SMP	Syntrax	http://www.bodybuilding.com/store/syn/smp.html	?
114	Stenandiol	German American Technologies	http://www.pinsonsfitness.com/stenandiol.html	?
115	Stimara™	Virobky ?	http://www.energy.sk/sk/vyrobky/stimara.htm	<i>Leuzea</i>
116	Suma	Nature's Way	http://www.n101.com/Static/Products/suma_root_1740051275.html ; http://www.iherb.com/sumbrazginna.html	<i>Pfaffia</i>
117	Suma Root	Frontier	http://www.taoherbfarm.com/herbs/herbs/tonic.htm	<i>Pfaffia</i>
118	SuMaca™ Plus	Native Essence Herb Company	http://www.norx.com/smxc.html	<i>Pfaffia</i>
119	Sumacazon™	Rainforest Bio-Energetics	http://www.pipeline.com/~dan_glick/Rainforest/macac.html	<i>Pfaffia</i>
120	SumaCeps™ Plus	Native Essence Herb Company	http://www.norx.com/smxc.html	<i>Pfaffia</i>
121	SumaFlex™ Plus	Native Essence Herb Company	http://www.norx.com/smxc.html	<i>Pfaffia</i>
122	Sumaforme	Laboratoire Holonorm	http://www.clherbs.com/SUMAFORM.htm	<i>Pfaffia</i>
123	Sumah-5	The Millenium Nutrition	http://www.diabetestea.com/sumah.html	<i>Pfaffia</i>
124	Sumax	Ultimate Nutrition	http://www.bodybuilding.com/store/un/sumax.html	<i>Pfaffia</i>
125	Syn-R-Gy	Amerinden	http://www.netricuticals.com/listing.asp?id=70	<i>Leuzea</i>
126	Syntrabol	Syntrax	http://www.pacific-nutrition.com/ecdy-syntrabol.htm	?
127	Ten Lives	Higher Ideals	http://www.touchalife.com/tenlives.htm	<i>Pfaffia</i>
128	Testo Intelligence Stack	Ripfast	http://www.ripfast.co.uk/ENGLISH/PAGES/TESTO.HTM	?

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129	Testo Kick	Maximuscle	http://gymratz.co.uk/bodybuilding-supplements/item9.htm	?
130	TODA	Wolf	http://www.doctormuscle.com/todoa.htm	Pfaffia
131	Tri-Beta™	7th Millenium Nutrition	http://www.diabetestea.com/erotojoy.html	Pfaffia
132	Triboxin	Atletica Sport International	http://www.newremedies.com/ssi/html/triboxin2.shtml	Pfaffia + ?
133	Ultra3 Growth Fuel	TwinLab	http://fitnessfirstusa.com/details.asp?item=8001	Pfaffia + Leuzea + Cyanotis
134	Vyo-Var	Vyo-Tech Nutritional	http://www.fitnessone.com/anabolic-products/vyo-var.html	Leuzea
135	Xtrashot	Linkswalker	http://www.mpdirect.com/sportsfitness/xtrashot/index.html	Leuzea
136	Xtreme Methoxy Rx	Phoenix Athletic	http://www.phoenixathletic.com/SNI/Xtreme%20Methoxy%20Rx.html	Leuzea
137	Zebuto™	ZOE Labs	http://www.zoelabs.com/shop/ZEBUTOL.html	Pfaffia
138	Z-Force	Dynamitize Nutrition	http://www.pacific-nutrition.com/mineral-zforce.htm	Polypodium
139	ZMA PM	Kaizen	http://www11.netrition.com/kaizen_zma_pm_page.html	Cyanotis + Pfaffia
140	Z-Mass	Cytodine	http://www.advantagesupplements.com/cytzmaspm120.html	Polypodium (+Pfaffia ?)
141	Beta-Methoxy Caps	Ultimate Nutrition	http://www.bodybuilding.com/store/un/betam.html	?
142	Ecdy-Meth 600	NFS	http://www.nutritionplus.co.nz/productsother.asp	?
143	Ecdysterone ZMA	Peak Nutrition	http://www.peaknutrition.com/eczma90tab.html	Pfaffia ?
144	Isobol™	Syntrax	http://www.nutrisport.it/php/infoart2.php?art_code=STX7	?
145	Methoxy/Ecdy-Fusion	Protogenex	http://www.protogenex.com/prod1.htm	
146	Methoxyvone	Kaizen	http://www.kaizennutrition.com/specialty/MethoxyVone.htm	Cyanotis
147	Natrex	LA™ Muscle	http://www.natrex.co.uk/docs/main.php3	?
148	SterOne	NFS	http://www.creativeenergy.co.nz/prodnfssterone.htm	?
149	Steronezolin	NFS	http://www.creativeenergy.co.nz/prodnfssteronezolin.htm	?

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