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Deciphering the route to cyclic monoterpenes in *Chrysomelina* leaf beetles: source of new biocatalysts for industrial application?

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Abstract: The drastic growth of the population on our planet requires the efficient and sustainable use of our natural resources. Enzymes are indispensable tools for a wide range of industries producing food, pharmaceuticals, pesticides, or biofuels. Because insects constitute one of the most species-rich classes of organisms colonizing almost every ecological niche on earth, they have developed extraordinary metabolic abilities to survive in various and sometimes extreme habitats. Despite this metabolic diversity, insect enzymes have only recently generated interest in industrial applications because only a few metabolic pathways have been sufficiently characterized. Here, we address the biosynthetic route to iridoids (cyclic monoterpenes), a group of secondary metabolites used by some members of the leaf beetle subtribe *Chrysomelina* as defensive compounds against their enemies. The ability to produce iridoids *de novo* has also convergently evolved in plants. From plant sources, numerous pharmacologically relevant structures have already been described. In addition, in plants, iridoids serve as building blocks for monoterpenoid indole alkaloids with broad therapeutic applications. As the commercial synthesis of iridoid-based drugs often relies on a semisynthetic approach involving biocatalysts, the discovery of enzymes from the insect iridoid route can account for a valuable resource and economic alternative to the previously used enzymes from the metabolism of plants. Hence, this review illustrates the recent discoveries made on the steps of the iridoid pathway in *Chrysomelina* leaf beetles. The findings are also placed in the context of the studied counterparts in plants and are further discussed regarding their use in technological approaches.

1 Introduction

Iridoids comprise a large family of biologically active molecules that have thus far been found in plants and insects. Structurally, they are known as cyclopentan-[c]-pyran with a hydroxyl (iridoid aglucones) or glucosyl group (iridoid glucosides) at the C-1 position of the pyran ring. In particular, plants produce manifold structures subgrouped according to, for example, substituents, linkage to other molecules, or modifications of the ring structures (summarized, e.g. in Refs. [1–4]). Among the 2500–3000 identified plant iridoids/secoiridoids are the secologanins, which serve as key building blocks in the synthesis of thousands of monoterpenoid indole alkaloids, including vinblastine/vincristine or camptothecin that are widely used as anticancer agents [5, 6]. Besides anticancer activity, iridoids have additional pharmaceutical potentials that provide valuable resources for the development of novel drugs and therapeutic strategies against diverse diseases [7–9]. In the natural environment, iridoids benefit plants by preventing microbial invasions [10–14], and by repelling herbivorous vertebrates and invertebrates [10, 15, 16].

The protective effect of iridoids can be based on their bitter taste, making these phytochemicals in particular distasteful to mammals [17], and on their physiological toxicity, which also affects invertebrates and pathogens in a dose-dependent manner [2]. To date, it has been shown that iridoid toxicity can be attributed to the highly reactive aglycones that are released from the corresponding nontoxic iridoid glucosides that are often safely stored in plant organelles [18, 19]. Glycoside hydrolysis can be achieved nonenzymatically or enzymatically by β -glucosidases (hydrolases, EC 3.2.1.21) produced by the plants themselves [20–22] or by their enemies [12, 13, 23, 24]. If the resulting compound is a reactive aldehyde, it has the ability to link irreversibly and nonselectively essential cellular components including proteins. This

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may affect the physiological processes of invasive organisms directly and/or decrease the nutritive value of dietary proteins considerably [25–28]. Although a universal target of iridoids has not been defined, these rather nonspecific effects contribute to an increased mortality of, for example, nonadapted herbivores feeding on iridoid glycoside-containing diets [24].

The biosynthesis of iridoids in plants proceeds through C_5 -isopentenyl diphosphate (C_5 -IDP) and C_5 -dimethylallyl diphosphate (C_5 -DMADP) – the universal building blocks for all terpenoids. C_5 -IDP and C_5 -DMADP are biosynthesized in two pathways existing side by side in higher plants – namely, the cytosolic mevalonic acid (MVA) pathway and the plastid 2-methyl-D-erythritol 4-phosphate pathway. The 2-methyl-D-erythritol 4-phosphate route was found to be the main route for the synthesis of iridoid precursors in plants [7]. The iridoid pathway starts with its key intermediate, geraniol diphosphate, and comprises a number of oxidation, reduction, glycosylation, and methylation reactions [5, 29]. The biosynthesis of secologanin through the intermediates iridodial and iridotrial in the Madagascar periwinkle, *Catharanthus roseus*, has been best understood [30–35]. The pathway in *C. roseus* is organized in a complex manner, with the enzymes localized in different cell types and subcellular compartments [36, 37].

Although iridoids are typically encountered in the plant kingdom, these secondary metabolites can also be identified in insects. In fact, the name iridoid is a generic term derived from iridomyrmecin, a component of defensive secretions identified from species of the ant genus *Iridomyrmex* [38] (Figure 1). Insects use iridoids frequently as chemical stimuli for communication or defense [41–47]. Although many insects profit from the sequestration of iridoid glucosides from their host plants [16, 48–50],

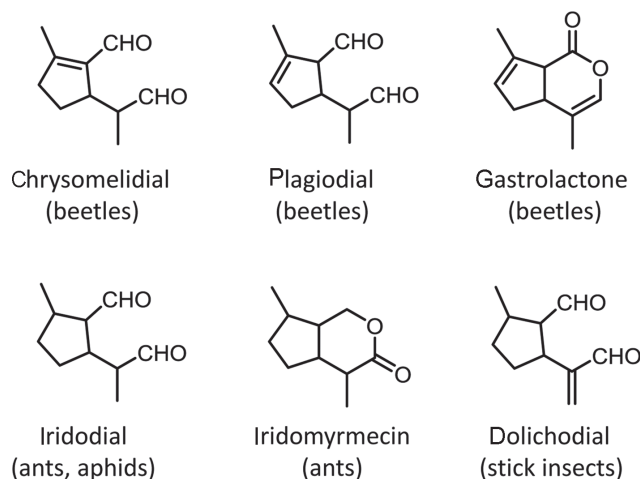


Figure 1: Iridoid defense molecules identified from insects (according to Kunert et al. [39] and Weibel et al. [40]).

others, such as stick insects [51], rove beetles [40], or leaf beetles [52–56] are able to produce iridoids de novo.

The biosynthetic steps are thought to proceed in a similar way to the known pathway in plants. However, as in plants, in insects, the iridoid pathway has also been characterized in only a few species and, even in these pioneering examples, the pathway is not yet fully resolved. The best investigated insect species regarding iridoid synthesis thus far belong to the family of leaf beetles (Chrysomelidae, subtribe Chrysomelina) (Table 1). In particular, the juveniles of Chrysomelina beetles evolved specialized pair-wise exocrine glands (composed of a reservoir with adhered glandular cells) on their dorsal segments to release iridoids in droplets as defensive secretions. Considering the evolutionary aspects of Chrysomelina beetles, it has been shown that iridoid de novo synthesis precedes the sequestration of plant-derived secondary metabolites

Table 1: Iridoid compounds in defensive secretions from the larval stages of selected Chrysomelina (Chrysomelidae) species specialized to different host plants.

Species	Compounds	Configuration	de (%)	Host plant
<i>Phaedon cochleariae</i>	Chrysomelidial	(5R,8R)	94	<i>Brassica rapa</i> subsp. <i>chinensis</i>
<i>Hydrothassa marginella</i>	Chrysomelidial, plagiolactone	(5S,8S)	95	<i>Ranunculus acris</i>
<i>Phratora vulgatissima</i>	Chrysomelidial, plagiodial	(5S,8S)	93	<i>Salix caprea</i>
<i>Gastrophysa viridula</i>	Chrysomelidial	(5R,8R)	96	<i>Rumex obtusifolius</i>
<i>Gastrophysa polygoni</i>	Chrysomelidial	(5R,8R)	92	<i>Polygonum aviculare</i>
<i>Gastrophysa cyanea</i>	Chrysomelidial, gastrolactone	(5R,8R)	97	<i>Rumex obtusifolia</i>
<i>Gastrophysa atrocyanea</i>	Chrysomelidial	(5R,8R)	91	<i>Rumex obtusifolia</i>
<i>Plagiodera versicolora</i>	Plagiodial, plagiolactone			<i>Salix fragilis</i>
<i>Linea aenea</i>	Plagiodial, plagiolactone			<i>Alnus glutinosa</i>
<i>Prasocuris phellandrii</i>	Plagiodial			<i>Caltha palustris</i>
<i>Phratora laticollis</i>	Plagiodial			<i>Populus canadensis</i>

The absolute configuration of chrysomelidial has been measured by gas chromatography–mass spectrometry (GC-MS) on a chiral column (adapted from Kunert et al. [39]). de, diastereomeric excess.

for the production of defensive compounds [57]. To achieve the exploitation of phytochemicals, the larvae use transport and metabolic mechanisms already present in the iridoid de novo-producing species. These mechanisms were adapted according to host plant affiliations during the evolutionary sequence of *Chrysomelina* species.

Based on the fact that iridoid/secoiridoid-derived compounds have tremendous pharmacological potential, the plant enzymes of this pathway are the focus of the current research as biocatalysts in commercial drug production [58, 59]. Additionally, because iridoids are also used as sex pheromones by aphids, which are agriculturally relevant pest species, they are exploited for the development of innovative and integrated pest management strategies [60, 61]. Hence, a molecular understanding of the iridoid metabolism, not only in plants but also in insects, has the potential to expand the repertoire of enzymatic workhorses available for different industrial approaches in the field of human health or nutrition. Due to the ecological and socioeconomic relevance of the iridoid pathway, we highlight in this review the most recent developments in our understanding of the iridoid biosynthesis and its enzymatic machinery in *Chrysomelina* beetles.

2 Iridoid de novo synthesis: early steps

The iridoid de novo biosynthesis in the larvae of *Chrysomelina* species starts with the formation of the isoprene units C_5 -DMADP and C_5 -IDP, which are derived from the MVA pathway [62]. To date, two enzymes have been studied in this early part of the pathway: the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR, EC 1.1.1.34) and the short-chain isoprenyl diphosphate synthase (scIDS) whose properties are described below in more detail.

HMGR catalyzes the rate-limiting step in the MVA pathway [63]. The enzyme utilizes two molecules of NADPH to mediate the four-electron reduction of 3-hydroxy-3-methyl-glutaryl-coenzyme A to the carboxylic acid mevalonate. Because HMGR is one of the most regulated enzymes known [64], its regulatory features may also be important for the biosynthesis of iridoids in chrysomelids. Consistently, analyses of different larval tissues from the iridoid synthesizing species *Phaedon cochleariae* and *Gastrophysa viridula* revealed high HMGR mRNA levels, high HMGR activity, and accumulation of the iridoid intermediate, 8-hydroxygeraniol-8-*O*- β -D-glucoside, in the fat body tissue of the iridoid de novo producers [65]. Hence, the fat body – the most prominent tissue in the larvae performing

myriad metabolic functions throughout the insects' development [66] – is implicated in de novo production of the glucosidically bound iridoid precursor. It is further reasonable to assume that iridoid biosynthesis is spatially distributed and the glucosidically bound intermediate is released from fat body tissue into the hemolymph followed by transport into the defensive glands for further conversion.

HMGR is regulated on very different levels, pre- and posttranslationally [64]. In insects, for example, it is known that HMGR transcription is affected by juvenile hormones [67, 68]. From *Chrysomelina* beetles, we reported that HMGR is negatively regulated by 8-hydroxygeraniol, another intermediate of iridoid biosynthesis [69]. Purification of the catalytic HMGR domain revealed that inhibition by 8-hydroxygeraniol is subject to the catalytic domain, which was corroborated by docking analyses on the modeled HMGR catalytic portion. De novo producing larvae possess the potential to sequester glucosidically bound 8-hydroxygeraniol if present in the diet [70–73]. After cleavage of the sugar moiety, the aglucon may interfere with HMGR and, consequently, the enzyme may represent a key regulator to maintain homeostasis of endo- and exogenous metabolites of the iridoid synthesis. Inhibition was also observed for other insect HMGRs including *Drosophila melanogaster*.

The second characterized enzyme of the early steps in the iridoid pathway is a member of the scIDS. Generally, catalysis by scIDSs follows a sequential mechanism called “head-to-tail alkylation”. During chain elongation, the allylic cosubstrate, e.g. C_5 -DMADP or C_{10} -geranyl diphosphate (C_{10} -GDP), undergoes coupling with homoallylic C_5 -IDP through electrophilic alkylation at its carbon-carbon double bond. For the scIDS in the iridoid pathway, it is expected that the enzyme produces C_{10} -GDP, the ubiquitous C_{10} -building block of many monoterpenes [74–77]. The reaction mechanism depends on the activation on a trinuclear metal cluster usually containing Mg^{2+} or Mn^{2+} [78].

Compared with plants, only a few GDPs have thus far been characterized in insects [75]. Strikingly, most of them have the ability to form multiple products. For example, an enzyme studied from the bark beetle, *Ips pini*, displayed prenyltransferase and terpene synthase activity [79–81], resulting in the formation of precursors for the de novo synthesis of monoterpenoid aggregation pheromones such as ipsdienol, which coordinates the colonization of coniferous trees [82]. Another scIDS from *Dendroctonus* spp. bark beetles produced C_{10} -GDP and C_{15} -farnesyl diphosphate (C_{15} -FDP) depending on the C_5 -IDP/ C_5 -DMADP substrate ratio [79–81]. Bifunctionality was also observed from the scIDSs characterized from

different aphid species [83–87]. Here, the recombinant proteins generated both GDP and FDP in parallel, and hence may be involved in the biosynthesis of either aphid sex pheromones or the sesquiterpene (*E*)- β -farnesene, the most common component of alarm pheromones.

Based on earlier studies describing the role of metal cofactors for scIDS catalysis, the product composition of a scIDS discovered from juvenile *P. cochleariae* has been tested in the presence of different metal ions [88]. Surprisingly, we found the enzyme isoprenyl diphosphate synthase 1 (*PcIDS1*) from *P. cochleariae* possessing an unusual product regulation mechanism not previously described for scIDSs. It alters the chain length of its products depending on the cofactor: the recombinant *PcIDS1* yielded 96% C_{10} -GDP and only 4% C_{15} -FDP in the presence of Co^{2+} or Mn^{2+} as a cofactor, whereas it yielded only 18% C_{10} -GDP but 82% C_{15} -FDP in the presence of Mg^{2+} . Kinetic studies further reinforced their assertion that *PcIDS1* has an energetic preference for Co^{2+} with C_5 -DMADP as an allylic cosubstrate for C_{10} -GDP production but showed that C_{15} -FPP production was favored when Mg^{2+} was the cofactor. Cation quantification studies in *P. cochleariae* larval tissues strengthened the physiological plausibility that the flux of carbon into separate metabolic pathways (C_{10} - vs. C_{15} -isoprenoids) could be accomplished by these ions *in vivo*.

Inspired by our work, the functional characterization of a farnesyl diphosphate synthase from the yellow fever mosquito, *Aedes aegyptii*, for example, revealed a similar dependency from the divalent cation of product condensation as observed for *PcIDS1* [89]. Given that plants possess a number of genes encoding IDS's (e.g. at least 10 in *Arabidopsis thaliana*), whereas insects possess only a few (e.g. 3 in *Bombyx mori*), insects may compensate for this disparity by generating different chain-length products in other ways. Instead of “inventing” a new IDS, insects seem to use different cofactors to add products to an enzyme's repertoire, thereby lowering metabolic costs. This type of “adjustable” enzyme may afford insects an efficient mechanism for the generation of chemical diversity that is critical for adaptation to ever-changing ecological contexts. Compared with plants, the functions of the many predicted isoprenyl diphosphate synthases in insects are much less understood. For example, putative trans-isoprenyl diphosphate synthases recently characterized from the flea beetle, *Phyllotreta striolata*, displayed terpene synthase activity [90]. Hence, the few functionally characterized isoprenyl diphosphate synthases from insects have already shown the potential of these enzymes or of chimeric insect-plant/microbe proteins [91] for use in a biotechnological context, such as in the optimization

of carbon fluxes during the production processes of pharmaceuticals.

Later in the iridoid pathway, geranyl diphosphate is converted into 8-hydroxygeraniol through geraniol, thereby removing the diphosphate moiety (Figure 2). The enzymes responsible for these reactions still remain to be elucidated in iridoid *de novo*-producing leaf beetles. In the plant *C. roseus*, the conversion of GDP into geraniol is catalyzed by a terpene synthase (geraniol synthase), but in beetles, a homologous sequence has not been identified. It is conceivable that a phosphatase is involved in the PPi group cleavage followed by a cytochrome P450 mediated ω -hydroxylation to obtain 8-hydroxy geraniol [92, 93]. An alternative would be the implication of a so-called “moonlighting P450” enzyme that possesses two catalytic centers exerting monooxygenase and terpene synthase activity [94]. Such an enzyme could cleave the diphosphate and oxidize geraniol to form 8-hydroxygeraniol. In *C. roseus*, it is known that the P450 enzyme CYP76B6 (G8O) oxidizes geraniol to 8-hydroxygeraniol [95]. Following oxidation, a glucose unit is transferred onto 8-hydroxygeraniol to enable the translocation of the precursor from the hemolymph into the defensive glands. The responsible glycosyltransferase, however, remains elusive.

3 Iridoid *de novo* synthesis: late steps

In *P. cochleariae*, the iridoid biosynthesis proceeds not entirely in the glands but it is most likely compartmented within the larval body [69]. After translocation of 8-hydroxygeraniol-*O*- β -D-glucoside into the defensive glands, its final transformation into iridoids occurs in the secretions. This transformation involves the hydrolysis of the glucoside and oxidation of the two primary hydroxy groups to produce the dialdehyde 8-oxogeraniol, which is followed by a cyclization [52, 54, 39].

In general, hydrolysis of glycosides occurs in the metabolism of all organisms. Enzymes that catalyze such reactions often belong to the glycosyl hydrolase family 1 (GH1) according to CAZy [96, 97]. Whereas in plants, GH1s play an important role in the activation of glucosides for defense purpose [98–101], insects use those enzymes mainly for digestion, either in the gut or in the salivary glands [24, 102, 103]. In addition to this, GH1s may also be involved in the production of chemical defenses widely distributed in insects [104]. However, because only a few metabolic pathways have been characterized to date, it is not surprising that only a few insect β -glucosidases are known to be

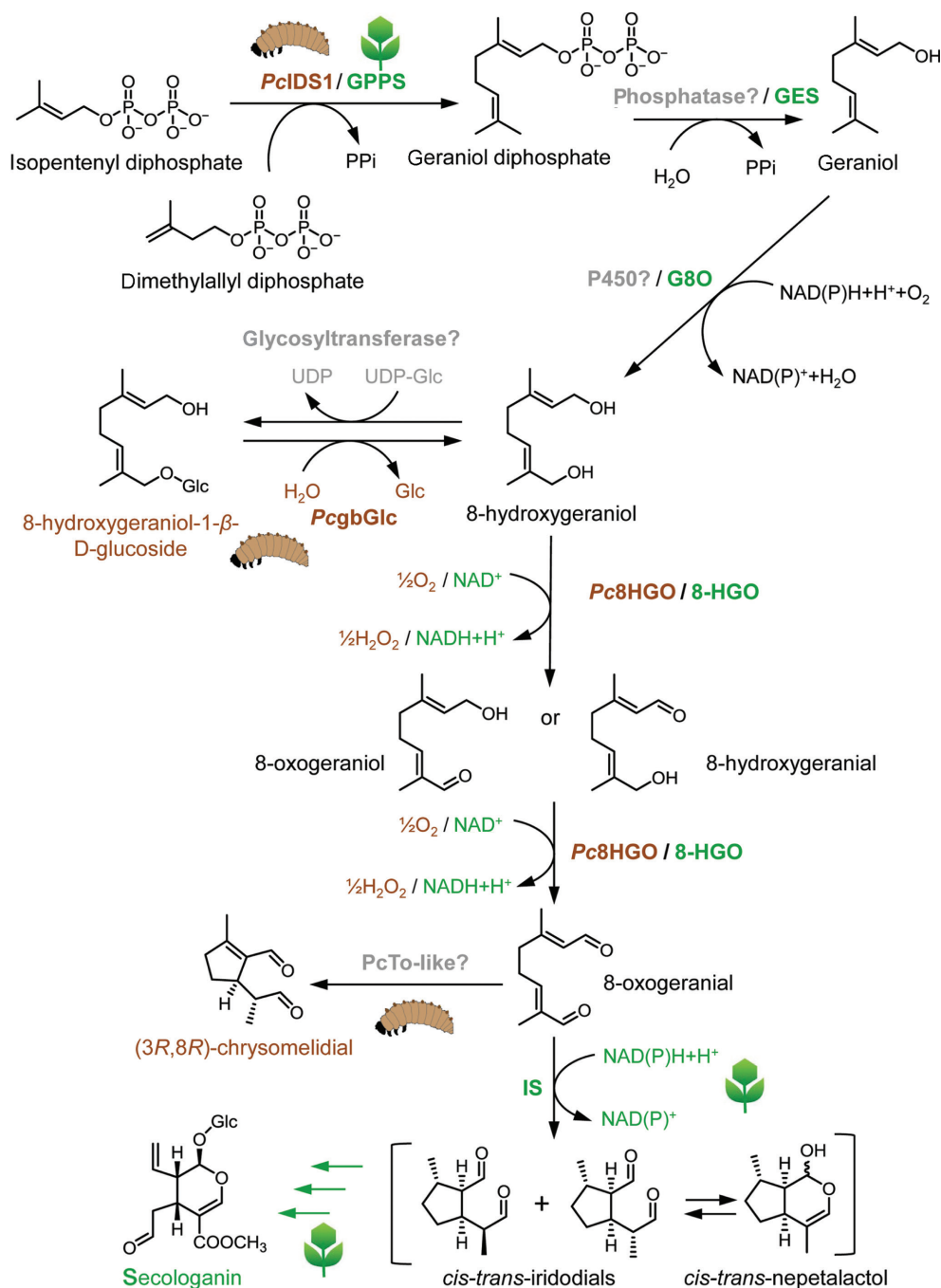


Figure 2: Comparative illustration of key steps in the iridoid/secoidoid biosynthesis from mustard leaf beetle, *P. cochleariae* (*Pc*), and Madagascar periwinkle, *C. roseus*. GPPS, geranyl diphosphate synthase; GES, geraniol synthase; G8O, geraniol 8-oxidase; 8-HGO, 8-hydroxygeraniol oxidoreductase; IS, iridoid synthase; *PcIDS1*, isoprenyl diphosphate synthase; *PcgbGlc*, glandular β-glucosidase; *Pc8HGO*, 8-hydroxygeraniol oxidoreductase (GMC superfamily); *PcTo-like*, takeout-like protein.

utilized for the synthesis of deterrents. Examples include the β-glucosidase linamarase from the caterpillars of the six-spot burnet moth, *Zygaena filipendulae* [105, 106], the ascorbate-dependent β-thioglucosidases (called myrosinases) from the cabbage aphid, *Brevicoryne brassicae* [107–109], and from the flea beetle, *P. striolata* [110].

To assess their importance in deterrent biosynthesis, we identified and functionally characterized GH1 glucosidases from different Chrysomelina species [111]. Determination of the kinetic parameters of heterologously expressed enzyme from *P. cochleariae* revealed hydrolase activity in the presence of physiological precursors

from different deterrent pathways found in *Chrysomelina* beetles, i.e. activity was determined in the presence of 8-hydroxygeraniol-*O*- β -D-glucoside (intermediate in the iridoid metabolism of *P. cochleariae*), salicin (intermediate in the salicyl aldehyde synthesis of *Chrysomela populi*), and 2-phenylethyl- β -D-glucoside (intermediate in the ester production found in *Chrysomela lapponica*). Evidently, the intrinsic broad substrate selectivity of the enzyme does not require changes in the catalytic center to allow the conversion of different plant-derived compounds.

β -glucosidases play an important role in complex biomass hydrolysis from renewable sources for the production of biofuels, food, or food additives [112, 113]. However, product inhibition impairing yields, thermal inactivation of enzymes, and the high cost of enzyme production are still the main obstacles to commercial hydrolysis processes. Hence, the demand for alternatives to the currently available enzyme preparations has prompted researchers to further characterize enzymes from the most diverse organisms. The hydrolysis reactions from insects represent one of these sources that have not yet been sufficiently exploited. For example, the glucosidases from *Chrysomelina* leaf beetles exhibited an optimal hydrolytic activity at particularly low pH (optimal range 4.5–6), a property that might be of interest for specific technological processes [111].

The following reaction, flavin adenine dinucleotide (FAD)-dependent oxidation, is known to be catalyzed from members of the multigene family of glucose-methanol-choline (GMC) oxidoreductases [114]. Interestingly, plants use a completely different enzyme family for the oxidation of 8-hydroxygeraniol. In *C. roseus*, an NAD(P)-binding Rossmann fold domain-type oxidoreductase (8-hydroxygeraniol oxidoreductase, 8-HGO) contributes to the formation of 8-oxogeraniol by the catalysis of two successive and reversible oxidation steps [30].

The GMC protein from the larvae of *P. cochleariae* (Pc8HGO) has been identified in defensive secretions by proteomic analyses [115]. The importance of this enzyme for the formation of chrysomelidial has been further verified by RNAi in vivo. Functional characterization, including the substrate specificity of this enzyme after heterologous expression, revealed the selective oxidation of 8-hydroxygeraniol to 8-oxogeraniol. In addition, the substrate specificity of Pc8HGO was tested by incubating the oxidase with salicyl alcohol, the substrate of *Chrysomela* spp. salicyl alcohol oxidase (SAO). No enzyme-based conversion to salicyl aldehyde could be detected, indicating that this particular enzyme does not react with salicyl alcohol. Also, the recently tested SAO is selective only

for the genuine substrate of the salicyl aldehyde pathway [116–118]. Thus, unlike the glandular β -glucosidases, the GMC oxidases have a narrow substrate spectrum.

In comparison to other oxidoreductases, the members of the GMC oxidoreductase family share a conserved sequence motif, the β - α - β dinucleotide binding motif (GxGxxG(x)₁₈E) responsible for the binding of cofactor FAD. Despite the diversity of substrates that can be converted by the members of this protein family, the majority of GMC oxidoreductases seem to share a catalysis mechanism involving a hydride transfer from the substrate to FAD that is promoted by a conserved histidine residue. Subsequently, molecular oxygen is utilized as the acceptor for the hydride and is further reduced to hydrogen peroxide. In insects, the GMC oxidoreductase multigene family has undergone a massive expansion. For comparison, in vertebrates, only one to two GMC genes are known, whereas, in insects, 15–43 genes are known. It is believed that these GMC genes are involved in developmental, immune, or defensive processes in insects. It seems that the substrate diversity in redox reactions potentially supplied by this multigene family equips insects with a toolbox that allows them to adjust to the particular biotic and abiotic conditions that may result, for example, when shifting host plants [119, 120]. Although the exact functions of many of these GMC genes have not been elucidated, it can already be suggested that GMC members have the potential to catalyze reactions that are valuable from a biotechnological perspective.

Whereas deglucosylation and oxidation reactions are also found in sequestering species, the final cyclization of acyclic dialdehydes to generate iridoids proceeds exclusively in iridoid de novo producing species. Interestingly, with the same precursor, isotopic tracing studies have shown that there are two mechanistically different cyclization modes in different leaf beetle groups [39]. When deuterium atom labeled [²H₅]Ger-8-OH was used for the feeding experiments, the precursor lost a single deuterium atom from C(4) in *P. cochleariae*, *Hydrothassa marginella*, and *Phratora vulgatissima*. In contrast, in *Gastrophysa cyanea*, *Gastrophysa polygoni*, *Gastrophysa atrocyanea*, and *G. viridula*, [²H₅]Ger-8-OH was observed to exchange all three deuterium atoms from the methyl group at C(3) (Figure 3). Based on these isotopic labeling studies, two different cyclization mechanisms have been proposed proceeding through either a “transoid” orientation of the dienamine intermediate (*Phaedon*-type cyclization) or a “cisoid” orientation of the dienamine (*Gastrophysa*-type cyclization).

Moreover, the absolute configuration and optical purity of chrysomelidial secreted by different families

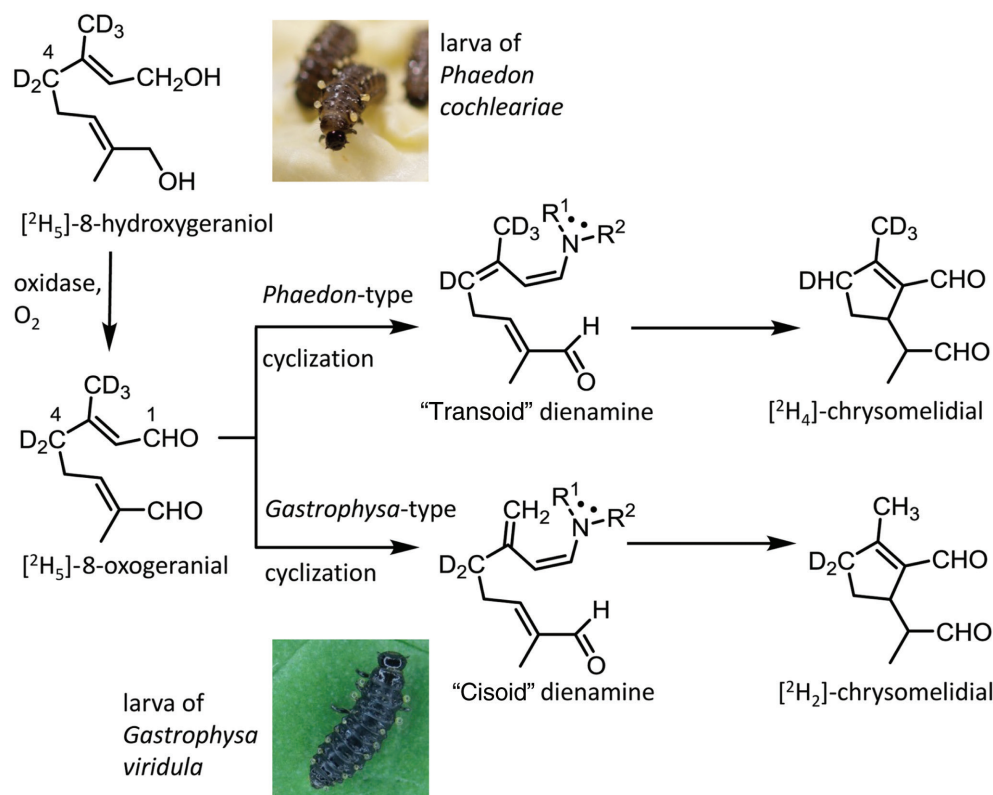


Figure 3: Metabolism of $[^2\text{H}_5]$ -8-hydroxygeraniol toward chrysomelidial in different leaf beetle larvae. The proposed mechanism for iridoid cyclization includes the formation of a dienamine. The dienamine intermediate is "transoid" for *Phaeton* and "cisoid" for *Gastrophysa* (adapted from Kunert et al. [39]).

was determined by GC-MS. Curiously, except for those in *H. marginella* and *P. vulgatissima*, which are (5*S*,8*S*)-chrysomelidial, secretions in *P. cochleariae* and all investigated members of the genus *Gastrophysa* contain (5*R*,8*R*)-chrysomelidial. To date, however, the only enzyme capable of performing the reductive cyclization step of 8-oxogeraniol has been identified in *C. roseus*. The iridoid synthase is a member of the Rossmann-fold NAD(P)⁺-binding protein superfamily. It cyclizes 8-oxogeraniol to *cis-trans*-iridodials and *cis-trans*-nepetalactol under the consumption of NAD(P)H [32]. In a proteomic analysis, however, no protein similar to the iridoid synthase from *C. roseus* could be detected in the secretions of iridoid-producing Chrysomelina larvae. Chemical analysis of the cyclization reaction predicted a NAD(P)H-independent reaction leading to the assumption that a different class of enzymes is catalyzing the reaction in insects compared with plants.

The most promising candidate to be involved in cyclization is a takeout-like protein, a member of the juvenile hormone-binding protein superfamily, which comprises ligand-binding proteins for juvenile hormones or similar hydrophobic terpenoids [121]. The

detailed biochemical characterization of this protein in vitro, however, is still in progress. As the enzymes from Chrysomelina leaf beetles are known to differ in their stereoselectivity [39], a variety of cyclic products can be produced that could serve as building blocks for new metabolites of pharmacological relevance. Hence, deciphering the reaction mechanism would make the enzyme prospectively a candidate for application in drug development.

4 Conclusion and future aspects

Important gaps in our understanding of insect metabolism have been filled in recent years. However, we are still only scratching the surface of the metabolic complexity present in the approximately one million insect species on our planet [122]. Every newly discovered and functional studied protein contributes to the growing set of biocatalysts that can render chemical synthesis more efficient and sustainable. Iridoid biosynthesis in leaf beetles definitely offers special features that could be

attractive for industrial applications. Because the iridoid pathway in beetles diverges from that in plants, its catalytic proteins can come into consideration as valuable tools for alternative synthesis strategies for iridoid-derived drugs. Our mechanistic studies on the insect iridoid route hence provide a framework for further enzyme engineering.

The intrinsic catalytic activity itself as well as the physicochemical properties of the enzymes can be exploited for industrial applications. For example, the secretory proteins localized in the defensive secretions of *Chrysomelina* larvae have to function in an extracellular milieu not comparable with the normal cellular interior. Defensive secretions possess nonphysiologic pH values and contain the enzymes together with the end products. Often, these metabolic end products are lipophilic and form separated phases or a kind of oil-water emulsion in the secretions [123]. Furthermore, the deterrents can have damaging effects on the proteins, e.g. iridoids can cause a nonspecific cross-linking of proteins. Hence, the enzymes in emulsions or on the interface between hydrophilic and hydrophobic phases have to meet special requirements to fulfill their function. Often, special decorations of proteins or interactions with chaperone-like proteins improve stability in nonphysiological environments.

In particular, protein modifications by *N*- or *O*-glycans modulate the physicochemical properties of proteins including thermodynamics, kinetics, chemical stability, or 3D architecture [124]. Because protein-based pharmaceuticals such as antibodies or enzymes require long-term stability, much emphasis has been placed on optimal glycosylation parameters by researchers in the field of glycoprotein engineering [125, 126]. An understanding of the sugar composition of secretory proteins in defensive secretions could thus contribute alternative glycosylation patterns that might benefit protein design in modern medicine.

Because secretory enzymes have to ensure deterrent production in a closed reservoir “outside” the insect body, they are exposed to a fluctuating surrounding temperature and should be able to operate in a varying range of temperatures. As technological processes often have to proceed under nonphysiological conditions simply to be profitable, an understanding of the secretory proteins from a thermodynamic perspective might promote these processes. Our current understanding of the secretory proteins in insect iridoid producers provides a platform to build on future research on defensive secretions from insects with regard to biochemical as well as technological aspects.

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