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Beetles Do It Differently: Two Stereodivergent Cyclisation Modes in Iridoid-Producing Leaf-Beetle Larvae

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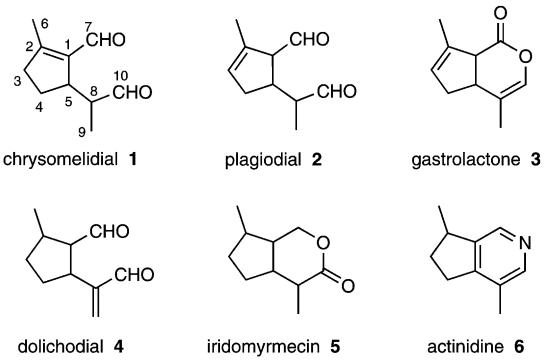
Larvae of the Chrysomelina species *Phaedon cochleariae*, *Hydrothassa marginella*, *Phratora vulgatissima*, *Gastrophysa viridula*, *Gastrophysa atrocyanea*, *Gastrophysa cyanea* and *Gastrophysa polygoni* produce the iridoid chrysomelidial (**1**) to defend themselves against predators. Feeding experiments with a deuterated precursor ([$^2H_5$]8-hydroxygeraniol **9**) and in vitro isotope exchange experiments with defensive secretion in ^2H_2O revealed differences in the cyclisation of the ultimate precursor 8-oxogeranial (**8**) to **1**, between members of the genus *Gastrophysa* and all other species. In *P. cochleariae*, *H. marginella* and *P. vulgatissima* **1** is most likely produced by a Rauhut–Currier-type cyclisation via a "transoid dienamine", with loss of a single deuterium atom from C(4) of the precursor. In contrast, members of the genus *Gastrophysa* cyclise **8** via a "cisoid dienamine" intermediate, with exchange of all three deuterium atoms from the methyl group at C(3). To study whether the different cyclisation modes influence the stereochemistry of **1**, the absolute configuration of **1** of the larvae was determined by GC-MS on a chiral column. In accordance with literature (J. Meinwald, T. H. Jones, *J. Am. Chem. Soc.* **1978**, *100*, 1883 and N. Shimizu, R. Yakumaru, T. Sakata, S. Shimano, Y. Kuwahara, *J. Chem. Ecol.* **2012**, *38*, 29), we found (5*S*,8*S*)-chrysomelidial (**1**) in *H. marginella* and *P. vulgatissima*, but *P. cochleariae* and all investigated members of the genus Gastrophysa synthesise (5*R*,8*R*)-chrysomelidial (**1**).

Introduction

Defence mechanisms, such as mechanical, behavioural and chemical responses,[1] have evolved in Chrysomelidae for protection against predation. Chrysomelina larvae respond to disturbance and attack by everting dorsal glandular reservoirs to release defensive secretions. Methylcyclopentanoid monoterpenes with an iridane skeleton are widespread constituents of the defensive secretions of phytophagous leaf-beetle larvae of the subtribe Chrysomelina. The principal low-molecular-weight compound of the defensive secretion of the larvae of *Phaedon cochleariae, Gastrophysa polygoni, Gastrophysa atrocyanea* and *Gastrophysa viridula* is chrysomelidial (**1**; Scheme 1, Table 1).

In addition to **1**, larval secretions of *Gastrophysa cyanea* contain gastrolactone (**3**), and those of *Hydrothassa marginella* contain plagiolactone (Table 1). Plagiodial (**2**) and plagiolactone are typical for the larvae of *Plagiodera versicolora* and *Linaeidea aenea*, and **2** was also found in *Prasocuris phellandrii, Phratora laticollis* and *Phratora vulgatissima*. Larvae of *P. vulgatissima* show, in addition to **2**, a low amount of **1** (Table 1). Dolichodial (**4**) and iridomyrmecin (**5**) are components in the blend of the Argentine ant *Iridomyrmex*, which suppress the necrotrophoretic behaviour of nestmates.[2] Actinidin (**6**) is produced by *Iridomyrmex nitidiceps*,[3] and this has a powerful repelling effect on their predators. All these compounds share a common biosynthetic origin, and their biosyntheses proceeds in plants and insects along the same principal pathway, outlined in Scheme 2.



Scheme 1. Iridoid defence molecules in insects.

In juvenile leaf beetles, the early biosynthetic steps to the iridoid monoterpenes take place in the fat body.[4] There, the terpenoid backbone of geranyl diphosphate is assembled from precursors derived from the mevalonic acid pathway. Hydroxylation and conjugation with glucose generate 8-hydroxygeraniol-8-*O*-β-D-glucoside (**7**),[4–7] which is transported in the hemolymph to the glandular reservoir.[8–10] The final transformations involve the hydrolysis of the glucoside and oxidation of

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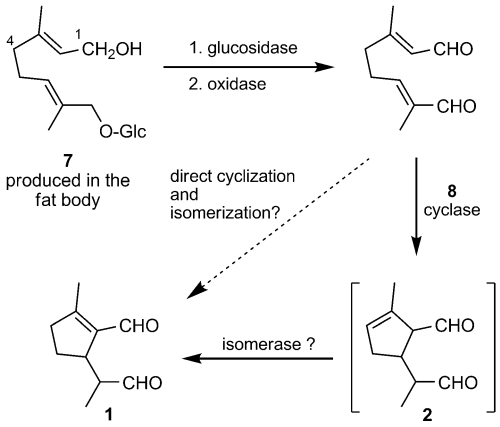
Table 1. Survey of all investigated species and their host plants, and the main low-molecular-weight compounds in insect secretions

Species	Compounds	Host plant (family)
P. cochleariae	chrysomelidial	*Brassica rapa* subsp. *chinensis* (Brassicaceae)
H. marginella	plagiolactone, chrysomelidial	*Ranunculus acris* (Ranunculaceae)
P. vulgatissima	plagiodial, chrysomelidial	*Salix caprea* (Salicaceae)
G. viridula	chrysomelidial	*Rumex obtusifolius* (Polygonaceae)
G. polygoni	chrysomelidial	*Polygonum aviculare* (Polygonaceae)
G. cyanea	chrysomelidial gastrolactone	*Rumex obtusifolia* (Polygonaceae)
G. atrocyanea	chrysomelidial	*Rumex obtusifolia* (Polygonaceae)
P. versicolora	plagiodial, plagiolactone	*Salix fragilis* (Salicaceae)
L. aenea	plagiodial, plagiolactone	*Alnus glutinosa* (Betulaceae)
P. phellandrii	plagiodial	*Caltha palustris* (Ranunculaceae)
P. laticollis	plagiodial	*Populus canadensis* (Salicaceae)



Scheme 2. Principal pathway of the biosynthesis of **1**.

the two primary hydroxy groups to produce dialdehyde **8**.[11,12] Finally, cyclisation of the acyclic dialdehyde **8** generates the iridoid **1**, most likely via a transient plagiodial-type intermediate that is not observed in the secretion. Whether this cyclisation and isomerisation sequence requires two discrete enzymes (Scheme 2) or only a single cyclase (catalysing both steps) remains unknown.[6,7]

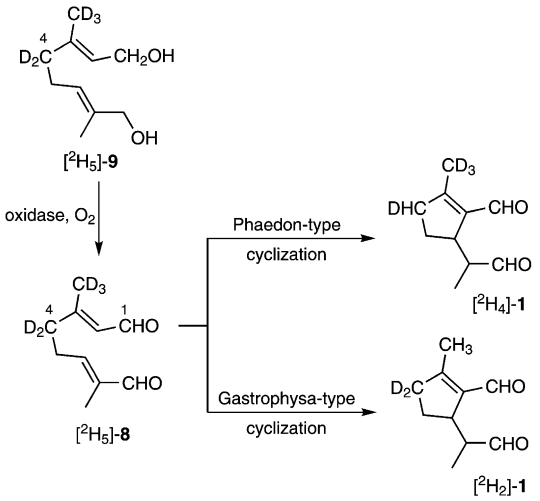
Lorenz et al. and Veith et al.[6,7] studied the biosynthesis of **1** in *P. versicolora*, *G. viridula* and *P. cochleariae*, and used synthetic (2*E*,6*E*)-[5,5-$^2H_5$]2-methylocta-2,6-diene-1,8-diol as a precursor. They observed loss of a single deuterium atom from C(4) in nor-chrysomelidial of *P. versicolora* and *P. cochleariae,* but not in the product of *G. viridula.* This obvious difference in the mode of cyclisation of the same precursor in two different leaf-beetle species prompted us to reinvestigate the cyclisation of 8-oxogeranial (**8**) in more detail, by using natural precursors labelled with deuterium atoms in mechanistically relevant positions. Moreover, the influence of the larval enzymes on the stereochemistry and configuration of the resulting **1** was studied by gas chromatography on chiral stationary phases. (5*R*,8*S*,)-Chrysomelidial (**1**) from the essential oil of *Actinidia polygama* Miq.[13] served for comparison with the compounds from the larval secretion. The results demonstrate that these leaf-beetle larvae are able to produce different stereoisomers.

Results and Discussion

Feeding and metabolism of [$^2H_5$]Ger-8-OH by leaf-beetle larvae

Insects either synthesise **7** de novo or sequester it from plant material[14] as a precursor for the biosynthesis of **1**.[4–7] The glucoside is transported in the circulating hemolymph from the fat body to the glandular reservoir, where the final transformations take place.[8–10] As insects glucosylate the free aglucon to **7**,[14] free (2*E*,6*E*)-[3-trideuteromethyl,4,4-$^2H_5$]-3,7-dimethylocta-2,6-diene-1,8-diol ([$^2H_5$]Ger-8-OH; **9**) could also be used for the feeding experiments.[6]

Consistent with the previously studied stereochemical course of iridoid biosynthesis,[7,12] loss of a single deuterium atom from C(4) of the precursor was observed in **1** produced by feeding larvae of *P. cochleariae* (Scheme 3), *H. marginella*



Scheme 3. Metabolism of [$^2H_5$]-**9** towards **1** in different leaf-beetle larvae.

and *P. vulgatissima*. The same observation was made for **2**, generated from [$^2H_5$]-**9** by the larvae of *P. versicolora, P. laticollis, L. aenea, P. vulgatissima* and *P. phellandrii*, as well as for plagiolactone produced by *P. versicolora, L. aenea* and *H. marginella*, and for **3** produced by *G. cyanea.* The deuterium atoms at the trideuteromethyl group along with the second deuterium atom from C(4) of administered [$^2H_5$]-**9** remained untouched, as evidenced by mass spectroscopy (Figure 1). Under electron impact conditions, the iridoid skeleton (*m/z* 166) sequentially lost the two substituents, thereby leading to a first fragment at *m/z* 138 (–CO), and further decomposed with the loss of the three-carbon unit (–$C_3H_5O^{\cdot}$) to the cyclopentanoid core fragment at *m/z* 81. As evidenced by the shifts from *m/z* 81 to 85, from *m/z* 109 to 113 and from *m/z* 138 to 142, the four remain-

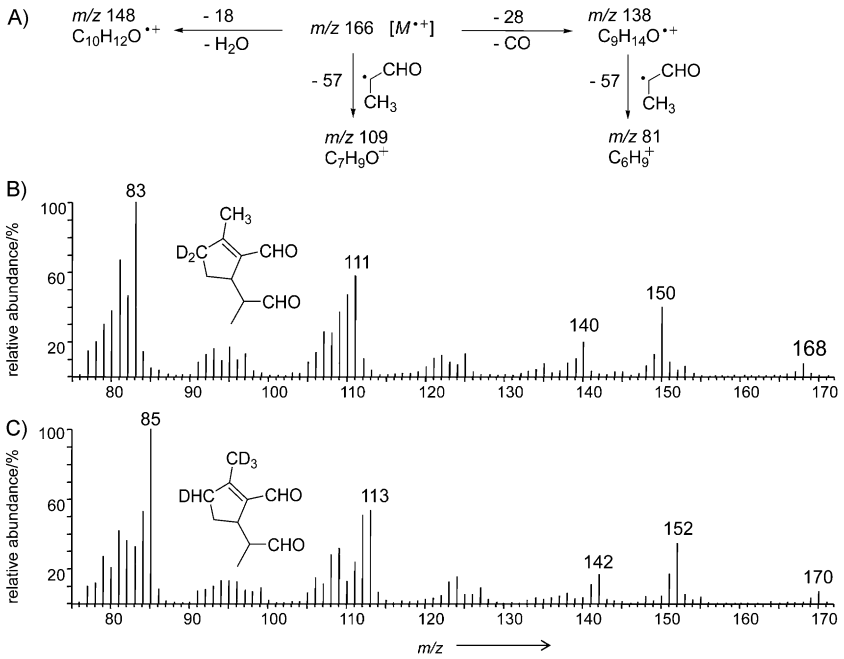


Figure 1. A) Mass fragmentation pattern of unlabelled **1** according to [14]. Mass spectra of B) [$^2H_2$]-**1** from *G. viridula* and C) [$^2H_4$]-**1** from *P. cochleariae*, feeding for 24 h on leaves treated with [$^2H_5$]-**9**.

ing deuterium atoms were present in these fragments. The core fragment at *m/z* 85 comprises the trideuteromethyl group and an additional deuterium atom at the ring, consistent with the removal of the C(4)-H_S from **9**, as shown previously with *P. cochleariae*.[7]

In contrast, feeding of [$^2H_5$]-**9** to larvae of *G. viridula, G. polygoni, G. atrocyanea* and *G. cyanea* resulted in an entirely different pattern of **1** (Scheme 3). The molecular ion at *m/z* 168 (Figure 1) is consistent with the loss of three deuterium atoms from the precursor. The two remaining deuterium atoms clearly resided at the core methylcyclopentane moiety, as indicated by the fragment shifts from *m/z* 81 to 83 and from *m/z* 109 to 111. The very small fragment at *m/z* 153, which corresponds to the loss of a non-deuterated methyl group, suggests that the exchange of the hydrogen atoms has occurred at this site, while the two deuterium atoms from C(4) of the precursor remained in the molecule. Consequently, the cyclisation of **8** in larvae of the genus *Gastrophysa* does not involve transient formation of a plagiodial-type intermediate (Schemes 2 and 3), which would have required the removal of a hydrogen atom from C(4) of the precursor.

Next, we performed isotope-exchange experiments with larval secretions and deuterium oxide. As the larval secretions contain all the necessary enzymes to convert 8-hydroxygeraniol (**9**) via 8-oxogeranial (**8**) to chrysomelidial (**1**),[7] we used in vitro experiments with larval secretions and unlabelled **9** in deuterium oxide to gain more information about the mode of cyclisation. Chrysomelidial (**1**) from *P. cochleariae* displayed the presence of a single deuterium atom after 24 h; longer incubation periods led to the incorporation of a very low amount of a second deuterium atom. One deuterium atom was incorporated into **1** when secretions from the *Phaedon*-type cyclisation were shaken with ^2H_2O without previous addition of unlabelled **9**.

Interestingly, **1** produced by shaking secretions of all *Gastrophysa* species in ^2H_2O (with and without addition of unlabelled **9**) displayed the presence of five deuterium atoms (*m/z* 171 instead of *m/z* 166). After 10 min, 1% of the peak area belonged to *m/z* 171; this rose to 4, 7, 9, 27, 61 and 92% after 30, 60, 90, 270 min, 24 h and 4 days, respectively (calculated from the intensity of the ion peaks at *m/z* 166 and 171). The impact of the pH on the isotope exchange was determined after 24 h in ^2H_2O, adjusted to pH 5, 6, 8 and 9. At pH 7 we obtained an isotopomer distribution of the molecular ion: *m/z* 167 (2%), 168 (3%), 169 (14%), 170 (18%), and 171 (63%). Upon changing the pH, almost the same distribution was observed. When boiled insect secretion (30 min at 100 °C) in ^2H_2O was used, only the acidic α-proton of the C_3 side chain was exchanged after long incubation periods (~72 h).

The position of the five deuterium atoms in the secretion of *G. viridula* in ^2H_2O was also assessed by ^{1}H NMR (Figure 2). After 24 h the protons of the exocyclic methyl group (resonating as a singlet at 2.1 ppm) were clearly exchanged. Moreover, the doublet for the protons of the methyl group (0.8 ppm) next to the aldehyde in the C_3 side chain collapsed to a singlet, thus indicating that the acidic α-proton H(8) was exchanged.

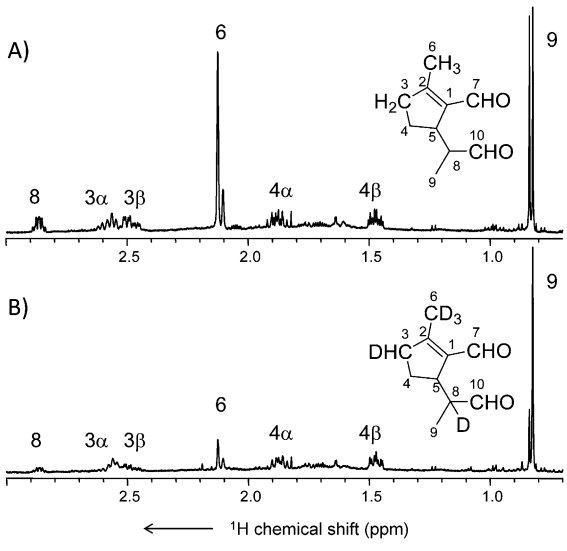


Figure 2. Partial ^{1}H NMR spectra of insect secretion of *G. viridula* in ^2H_2O: A) recorded directly after adding ^2H_2O, and B) after 24 h.

The diastereotopic protons of the allylic ring methylene at C(3) appeared as two complex multiplets assignable to the hydrogen atoms below (α) or above (β) the ring. The β-proton was exchanged selectively from these hydrogen atoms. The measurement was repeated after five days, and showed almost complete exchange of the assigned protons.

Absolute configuration of chrysomelidial from leaf-beetle larvae

Because of the obvious differences in the labelling pattern of **1** produced from [$^2H_5$]-**9** by the different leaf-beetle larvae, we also addressed the absolute configuration of **1** in the different species. Chrysomelidial (**1**) has two asymmetric carbon atoms and four stereoisomers. The elution order of the four stereoisomers was determined by comparison with freshly prepared hexane extracts from leaves of *A. polygama* Miq. (Botanical garden, Tübingen, Germany), which is known to produce (5*R*,8*S*)-chrysomelidial (**1**).[13] The absolute configuration of (5*R*,8*S*)-chrysomelidial from *A. polygama* Miq. was independently determined by synthesis of (5*R*,8*S*)-chrysomelidial from (*S*)-limonene.[15] Enders and Kaiser[16] synthesised (5*R*,8*S*)-chrysomelidial **1** by using the SAMP hydrazone method, and assigned the absolute configuration by polarimetric data and X-ray structures of related compounds prepared by Michael-initiated ring closure (MIRC) reactions.[16]

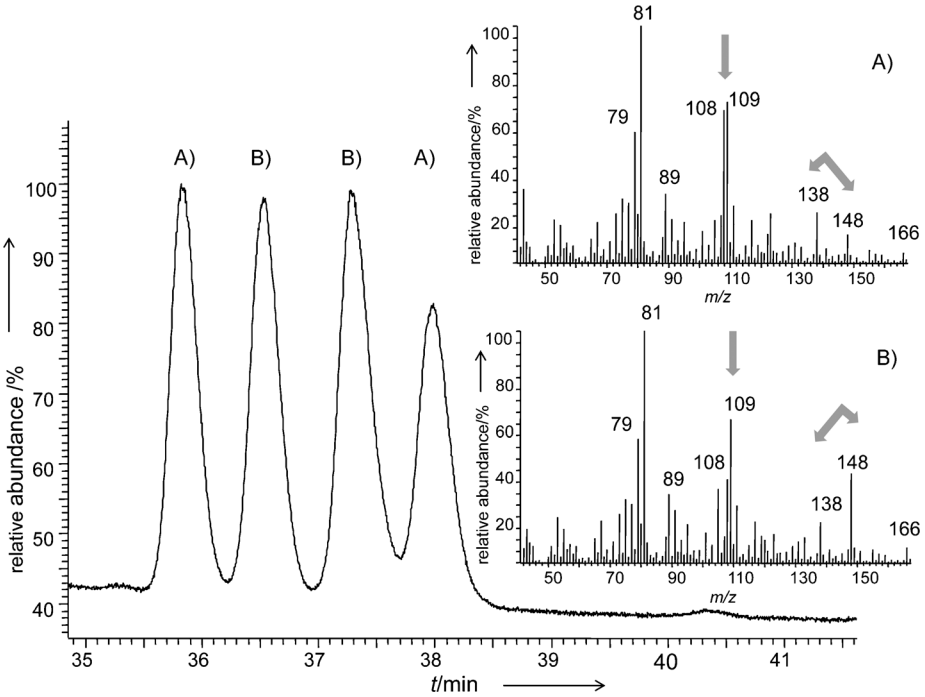


Figure 3. GC separation of *rac*-chrysomelidial (**1**) on a chiral Hydrodex-β-6TBDM column along with the mass spectra of the two diastereomeric pairs (5*R*,8*S*),(5*S*,8*R*) and (5*R*,8*R*),(5*S*,8*S*). The two diastereomeric pairs display different, but significant relative intensities of the ions at *m/z* 108 and 109, or at *m/z* 148 and 138. Elution order: A) (5*R*,8*S*), 35.8 min, (5*S*,8*R*) 38.0 min. B) (5*R*,8*R*) 36.5 min, (5*S*,8*S*) 37.3 min.

Gas chromatographic analysis of racemic **1** resulted in four completely separated peaks on a Hydrodex-β-6TBDM column as a chiral stationary phase (Figure 3). Racemic **1** was synthesised in a biomimetic fashion by using imidazol as a catalyst. Upon treatment with a saturated solution of K_2CO_3, (5*R*,8*S*)-chrysomelidial (**1**) from *A. polygama* Miq. (95% *de*, peak A) was slowly converted into the second eluting peak (B, 57% *de* after four days), namely (5*R*,8*R*)-chrysomelidial, identical to the natural compound of *P. cochleariae*. Hence the elution order of the first two peaks was established as shown in Figure 3: first (5*R*,8*S*), second (5*R*,8*R*).

Moreover, the diastereomeric pairs (5*S*,8*S*), (5*R*,8*R*) and (5*R*,8*S*), (5*S*,8*R*) showed differences in the relative intensities of the fragments at *m/z* 108 and 109 [$M^+ - C_3H_5O^\cdot$], *m/z* 148 [$M^+ - H_2O$] and *m/z* 138 [$M^+ - CO$];[17] this allowed us to assign the elution order of the two other peaks. The spectra of the first- and the last-eluting peaks, as well as of the second and third peaks were identical, and we characterised them as enantiomers. As the third peak (measured in insect secretion of *H. marginella*) was partly converted into the subsequently eluting epimer (fourth peak) upon base treatment (saturated K_2CO_3), the elution order was 1: (5*R*,8*S*), 2: (5*R*,8*R*), 3: (5*S*,8*S*), 4: (5*S*,8*R*).

The secretions of all investigated leaf-beetle larvae (freshly collected and immediately diluted with pentane) were analysed together with a small amount of racemic **1**. Coelution of the racemate with the secretion allowed direct correlation of the configuration of the insect metabolites with the configuration of the four stereoisomers. Chrysomelidial from *P. cochleariae*, *G. viridula, G. polygoni, G. cyanea* and *G. atrocyanea* had the same (5*R*,8*R*)-configuration, but the *de* of their metabolites slightly differed (Table 2). The major enantiomer in *H. marginella* and *P. vulgatissima* was (5*S*,8*S*)-chrysomelidial (**1**). Despite the differences in the modes of cyclisation of **8** to **1** in *P. cochleariae* and *G. viridula, G. polygoni, G. atrocyanea* and *G. cyanea*, the absolute configuration of **1** was the same.

The absolute configuration of **1** extracted from the oribatid mite *Austrotritia dentata* was recently determined as (5*S*,8*S*).[15] According to the study by Meinwald et al.[18] this defensive compound from leaf-beetle larvae is assumed to be (5*S*,8*S*)-chrysomelidial (**1**), but no further experiments to confirm the assignment have been reported. To the best of our knowledge, the absolute configuration of **1** from leaf-beetle larvae was not rigorously addressed before. In addition to the already described[18–20] (5*S*,8*S*)-chrysomelidial, we could also identify (5*R*,8*R*)-chrysomelidial in five species (Table 2).

Table 2. Absolute configuration and *de* of chrysomelidial

Species	Configuration	*de*
Actinidia polygama	(5*R*,8*S*)	95
Phaedon cochleariae	(5*R*,8*R*)	94
Hydrothassa marginella	(5*S*,8*S*)	95
Phratora vulgatissima	(5*S*,8*S*)	93
Gastrophysa viridula	(5*R*,8*R*)	96
Gastrophysa polygoni	(5*R*,8*R*)	92
Gastrophysa cyanea	(5*R*,8*R*)	97
Gastrophysa atrocyanea	(5*R*,8*R*)	91

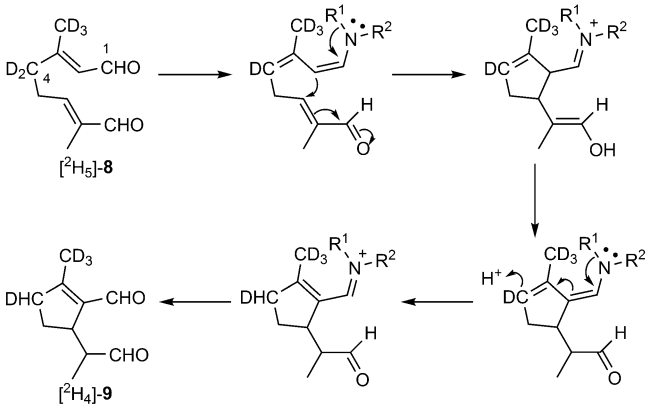
Mechanistic aspects of iridoid cyclisation

The current study clearly demonstrates that two different modes of cyclisation of **8** to **1** exist in leaf-beetle species. Lorenz et al.[6] already observed differences in the biosynthesis of **1** when using juvenile *P. cochleariae* or *G. viridula* leaf beetles. In the current study we used a deuterated probe ([$^2H_5$]Ger-8-OH; **9**) that is oxidised in the defensive system to the ultimate precursor [$^2H_5$]2,6-dimethylocta-2,6-dienedial ([$^2H_5$]8-oxogeranial; **8**).

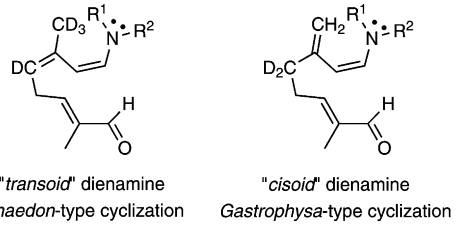
In agreement with Lorenz et al. and Veith et al.[6,7] this precursor is cyclised in *P. cochleariae* and other leaf-beetle larvae with the loss of a single deuterium atom from C(4) of the acyclic precursor **8**: C(4)-H_S[7] in the case of *P. cochleariae* (Scheme 3), and C(4)-H_R in the carnivorous *Philonthus* spp.[12] The results of the current and previous experiments with labelled precursors such as [$^2H_5$]-**9** or [$^2H_5$]-**8** are consistent with an intramolecular Rauhut–Currier reaction, which has been well studied and employed for the synthesis of iridoids.[21] Accordingly, [$^2H_5$]-**8** might be first converted into a dienamine (e.g. by involvement of a proline moiety); this implies the loss of a single hydrogen atom from C(4) of the precursor (Scheme 4). The electron-rich dienamine forms a new bond to the α,β-unsaturated aldehyde moiety. Protonation of the resulting enol generates the chiral centre (8*R*) and regenerates the aldehyde moiety. Next, the intermediate iminium salt loses a proton and regenerates the dienamine. Protonation of the dienamine terminates the catalytic cycle and releases **1** without the need for an additional isomerisation step (Scheme 4). NMR studies with model systems have shown that iminium ions do indeed exist in an equilibrium with the electron-rich dienamines.[22] The whole sequence proceeds with the loss of a single deuterium atom from the precursor [$^2H_5$]-**9**.

The isotopic labelling of **1** in species of the genus *Gastrophysa* is different, and proceeds with the complete exchange of all three deuterium atoms from the trideuteromethyl group of [$^2H_5$]-**9** or [$^2H_5$]-**8** as the immediate precursor. Most likely, their cyclase possess a basic centre that deprotonates the exocyclic methyl group during formation of the dienamine. The ring closure appears to be slow, as because of free rotation of the methyl group all the three deuterium atoms are lost prior to formation of **1**. No other deuterium atom (e.g., from C(4) of precursor [$^2H_5$]-**9**) is lost during cyclisation.

If both enzymes (one leading to the *Phaedon*-type cyclisation, the other catalysing the *Gastrophysa*-type ring closure) generate a dienamine intermediate, a mechanism using different geometric arrangements of the intermediate is consistent with all the experimental results. A "transoid" dienamine (*Phaedon*-type cyclisation) and a "cisoid" dienamine (*Gastrophysa*-type cyclisation), as outlined in Scheme 5, are fully consistent with the feeding experiments with [$^2H_5$]-**9** or the isotope exchange of the natural secretion in ^2H_2O.



Scheme 4. Proposed mechanism for the biosynthesis of **1** in *Phaedon*-type insects. The sequence implies the formation of a dienamine. The electron-rich dienamine forms a new bond to the α,β-unsaturated aldehyde moiety. Protolysis of the dienamine terminates the catalytic cycle and releases **1** without the need for an additional isomerisation step.



Scheme 5. Proposed mechanism for iridoid cyclisation in leaf-beetle larvae. The configuration of the dienamine intermediate is "transoid" for *Phaedon* and "cisoid" for *Gastrophysa*.

Thus, the enzyme supporting the *Phaedon*-type cyclisation by a "transoid" orientation of the dienamine requires loss of a deuterium atom from C(4) of the precursors [$^2H_5$]-**9** or [$^2H_5$]-**8**. In contrast, the *Gastrophysa*-type cyclisation proceeds via the "cisoid" dienamine and involves loss of all deuterium atoms from the trideuteromethyl group. As the process of dienamine formation is reversible, all three deuterium atoms can be exchanged with the medium (H_2O or ^2H_2O) prior to cyclisation, because of the free rotation of the methyl group. Both enzymes support the exchange of the acidic α-proton next to the aldehyde moiety of the C_3 side chain. An additional proton from C(4) of the acyclic precursor [$^2H_5$]-**9** or [$^2H_5$]-**8** is slowly exchanged (Figure 2) and appears to be identical to the one that is involved in the *Phaedon*-type cyclisation. Moreover, we observed cases that suggest that even combinations of these enzymes might occur within a single insect. Larvae of *G. cyanea* produce both **3** and **1**. After feeding [$^2H_5$]-**9** to the

larvae, the resulting **1** had lost the expected three deuterium atoms from the methyl group of the precursor (*Gastrophysa*-type cyclisation); but in the corresponding **3** only a single deuterium atom was lost, thus suggesting *Phaedon*-type cyclisation for this particular metabolite.

Apparently, the configuration of the products is not linked to the cyclisation mechanism, as members of both the *Phaedon* and *Gastrophysa* groups produce (5*R*,8*R*)-chrysomelidial (**1**). On the other hand, **1** from *H. marginella* and *P. vulgatissima* results from the *Phaedon*-type cyclisation, but both species produce (5*S*,8*S*)-chrysomelidial of high *de*. As we were able to epimerise the chiral centre next to the aldehyde by base treatment, the use of only fresh secretions stored in neutral solvents is highly recommended for determination of configurations and *de* of natural iridoids.

Conclusions

Several insects secrete iridoid monoterpenes.[6,18,19,23] In juvenile leaf beetles they are synthesised de novo.[6,7,11] The early steps of terpenoid biosynthesis along the mevalonic acid pathway occur within the fat body.[4] This tissue produces **9**, which is glucosylated to **7** and transported in the haemolymph to the defensive system. Following hydrolysis and oxidation, the resulting precursor **8** is cyclised to **1** by hitherto unknown enzyme(s). In addition, we observed labelling patterns consistent with combinations of the *Phaedon*- and *Gastrophysa*-type enzymes within the defensive system of single insects. The different stereochemical modes of iridoid cyclisation in herbivores and carnivores,[7,12] together with combination of *Phaedon*- and *Gastrophysa*-type cyclisation in a single insect species, point towards a complex evolutionary history of the enzyme(s). In particular, both their selective allocation to discrete biosynthetic pathways and their generation of the different iridoids **1**–**6** (Scheme 1), suggests precise, pathway-dependent regulation. Moreover, the different configurations of **1** in the various leaf-beetle larvae raise questions about the modes of action of these compounds. Currently no information is available to connect the configurations with the biological activities of the compounds against the typical enemies of the leaf-beetle larvae, such as ants, syrphids and parasitic wasps.

Experimental Section

Leaf beetles: Leaf beetles were reared in the laboratory (18–20 °C, 16:8 h light/dark period). A continuous culture of *P. cochleariae* was maintained on *Brassica rapa* subsp. *chinensis*. *H. marginella* was reared on *Ranunculus acris*; *P. phellandrii* was reared on *Caltha palustris*; larvae of *P. versicolora* were reared on *Salix fragilis* and *Salix tristis*; larvae of *P. laticollis* were reared on *Populus canadensis*; larvae of *P. vulgatissima* were reared on *Salix caprea*; larvae of *L. aenea* were reared on *Alnus glutinosa;* larvae of *G. viridula, G. atrocyanea and G. cyanea* were reared on *Rumex obtusifolius*; larvae of *G. polygoni* were reared on *Polygonum aviculare*; larvae of *P. versicolora, P. laticollis, G. polygoni, H. marginella, L. aenea* and *P. vulgatissima* were collected near Brussels (Belgium); larvae of *P. phellandrii* were collected in the surroundings of Bayreuth (Germany); larvae of *G. cyanea* were collected in Chico (CA, USA); larvae of *G. atrocyanea* were collected in Kyoto (Japan); larvae of *G. viridula* were collected in Jena (Germany). The glandular systems of the larvae were emptied prior to feeding to stimulate biosynthetic activity. For all experiments, 9–12 day-old larvae (third instar) were used.

Collection of defensive secretion: Defensive secretions (~0.2 mg) were collected with thin capillaries (diameter 0.2 mm; Hilgenberg, Malsfeld, Germany) from the larvae. The larvae were placed under a stereomicroscope and gently squeezed with a pair of tweezers to provoke extrusion of the dorsal glands. The secretion was then dissolved in dichloromethane (20 μL) or buffer (see below), by forcing the highly viscous liquid with slight positive pressure (attached syringe) from the capillary into the solvent. The secretion was put in a 0.2 mL PCR tube (Nerbe plus, Winsen/Luhe, Germany).

Synthesis of labelled precursors: All reactions were performed under argon; dry solvents were used. Silica gel Si 60 (0.200–0.063 mm; Merck, Darmstadt, Germany) was used for chromatography. A DRX 500 spectrometer (Bruker, Rheinstetten, Germany) was used for recording ^{1}H NMR (500 MHz) and ^{13}C NMR spectra (125 MHz). For GC-MS a TRACE MS (Thermo–Finnigan) device equipped with a ZB5 column (15 m × 0.25 mm × 0.25 μm) was used with a 10 m Guardian End column (Phenomenex, Aschaffenburg, Germany), with helium (1.5 mL min^{-1}) as the carrier gas. Mass spectra were measured in electron impact (EI) mode at 70 eV. Products were eluted under programmed conditions: 50 °C (2 min), increase (10 °C min^{-1}) to 200 °C, increase (30 °C min^{-1}) to 280 °C. The GC injector (split ratio 1:7), transfer line and ion source were set at 220, 280 and 200 °C, respectively.

[1,1,1,3,3-^{2}H$_5$]6-Methylhept-5-en-2-one: 1,5-Diazabicyclo[4.3.0]non-5-ene (DBN, 796 mg, 6.36 mmol) was added to a solution of 6-methylhept-5-en-2-one (10 g, 79.4 mmol) in deuterated methanol (CH_3OD, 99.5 %, 200 mL), and the mixture was stirred under argon for 24 h at room temperature. After removal of solvent in vacuo, the crude product was purified by filtration through silica gel with diethyl ether for elution. Yield: 9.0 g (71.5 mmol, 90 %). ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 5.06$ (m, 1 H), 2.24 (d, $J = 6.99$ Hz, 2 H), 1.67 (s, 3 H), 1.61 ppm (s, 3 H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 209.1$, 132.7, 122.6, 42.9, 28.8, 25.6, 22.3, 17.5 ppm. Isotopic purity: 98 % d_5 (GC-MS).

(*E*)-[3-Trideuteromethyl,4,4-^{2}H$_5$]ethyl 3,7-dimethylocta-2,6-dienoate: Butyllithium (0.05 mol) in hexane (9.4 mL) was slowly added with stirring to a chilled solution of triethyl phosphonoacetate (9.4 mL, 0.05 mol) in dry tetrahydrofuran (200 mL). After 20 min, [1,1,1,3,3-^{2}H$_5$]6-methylhept-5-en-2-one (5.3 g, 0.04 mol) in dry tetrahydrofuran (20 mL) was added, and the mixture was stirred for 24 h at room temperature. After addition of water (200 mL), the ester was extracted with diethyl ether (3 × 150 mL). The combined ether extracts were washed with brine and dried over Na_2SO_4. The crude product was purified by chromatography on silica gel with petroleum ether/diethyl ether (40:1, *v*/*v*) for elution. Yield: 4.4 g (0.022 mol, 55 %). ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 5.66$ (s, 1 H), 5.07 (m, 1 H), 4.14 (q, 2 H), 2.14 (d, $J = 6.9$ Hz, 2 H), 1.68 (s, 3 H), 1.60 (s, 3 H), 1.27 ppm (t, 3 H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 166.9$, 159.6, 132.4, 123.0, 115.7, 59.4, 25.9, 25.6, 17.6, 14.3 ppm. Isotopic purity: 58 % d_5, 23 % d_4, 10 % d_3 (GC-MS).

(2*E*,6*E*)-[3-Trideuteromethyl,4,4-^{2}H$_5$]ethyl 8-hydroxy-3,7-dimethylocta-2,6-dienoate: A solution of *tert*-butylhydroperoxide in nonane (5.5 M, 3 mL) was added to a chilled suspension of SeO_2 (375 mg, 3.4 mmol) in $CHCl_3$ (50 mL) and stirred for 30 min. Deuterated ethyl 3,7-dimethylocta-2,6-dienoate (4.4 g, 0.022 mol) was added, and stirring was continued for 1 h at 0 °C. After 2.5 h at

room temperature, the insoluble SeO_2 was removed by filtration. For work-up, diethyl ether was added, and the solution was washed with brine and dried over Na_2SO_4. The crude product was purified by chromatography on silica gel with petroleum ether/diethyl ether (1:1, *v/v*) for elution. Yield: 1.6 g (0.0076 mol, 34%); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 5.66$ (s, 1H), 5.38 (m, 1H), 4.15 (q, 2H), 4.00 (s, 2H), 2.21 (d, $J = 7.0$ Hz, 2H), 1.67 (s, 3H), 1.28 ppm (t, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 166.8$, 159.0, 135.8, 124.3, 115.9, 68.6, 59.5, 25.4, 14.3, 13.6 ppm. Isotopic purity: 31% d_5, 32% d_4, 18% d_3 (GC-MS).

(2*E*,6*E*)-[3-Trideuteromethyl,4,4-$^2H_5$]3,7-dimethylocta-2,6-diene-1,8-diol ([$^2H_5$]Ger-8-OH; 9): $AlCl_3$ (357 mg, 2.7 mmol) was slowly added to a chilled suspension of $LiAlH_4$ (447 mg, 11.8 mmol) in dry diethyl ether (40 mL), The mixture was stirred for 30 min at room temperature, and a solution of deuterated ethyl 8-hydroxy-3,7-dimethylocta-2,6-dienoate (400 mg, 1.8 mmol) in dry diethyl ether (10 mL) was added dropwise. The reaction was allowed to complete for 1 h at room temperature. For hydrolysis, NaOH (2 N) was added, and the resulting solid was removed by filtration and washed with water and chloroform. The organic layer was dried over Na_2SO_4 and purified by chromatography on silica gel with petroleum ether/diethyl ether (1:1, *v/v*) for elution. Yield: 0.25 g (0.0015 mol, 78%). ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 5.32$–5.44 (m, 2H), 4.13 (d, $J = 4.6$ Hz, 2H), 3.98 (s, 2H), 2.15 (d, $J = 7.2$ Hz, 2H), 1.65 ppm (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 135.1$, 125.3, 68.7, 59.1, 25.4, 18.8, 13.7 ppm. Isotopic purity: 38% d_5, 32% d_4, 17% d_3 (GC-MS).

Administration of labelled precursors to leaf-beetle larvae: Leaves from the different food plants were painted on both sides (3–10 cm^2) with a solution of [$^2H_5$]Ger-8-OH in deionised water (0.75 μmol cm^{-2}). The glandular reservoirs of ten larvae were emptied, and the larvae were placed onto the air-dried leaves. After feeding for 24 h, their defensive secretion was collected (vide supra) and analysed.

In vitro incubations with defensive secretions: All in vitro experiments were performed with freshly collected secretions from 10–20 insects. The secretion was placed in a 0.2 mL PCR tube (Nerbe plus). For control experiments the secretion was mixed with phosphate buffer (20 μL, K_2HPO_4 and KH_2PO_4 (0.1 M, pH 7)) or with ^2H_2O (20 μL, 99.9%; Deutero, Kastellaun, Germany). The impact of the pH was investigated by phosphate buffers prepared from K_2HPO_4 and KH_2PO_4 (0.1 M) adjusted to pH 5, 6, 8 and 9. To start the reaction, [$^2H_5$]Ger-8-OH (20 μL, 0.5 mg mL^{-1}) in phosphate buffer (pH 7) or Ger-8-OH (0.5 mg mL^{-1}) in ^2H_2O was added to the secretion. The sample was shaken with free access to air. Denaturated secretions of *G. viridula* and *P. cochleariae* were obtained by heating the freshly collected secretions at 100 °C for 30 min. In vitro incubation experiments were run for 20–24 h. The products were extracted with dichloromethane (30 μL) and analysed by GC-MS.

Mass spectroscopic analysis of defensive secretions: An aliquot (1 μL) of the respective stock solution in dichloromethane was analysed on a Finnigan TRACE Instrument. The instrumental parameters are described above (under "Synthesis of labelled precursors").

NMR measurements of insect secretion: An Avance 500 spectrometer (Bruker) equipped with a TCI cryoprobe was used for recording ^{1}H NMR spectra (500 MHz). The PURGE sequence was used to suppress the water signal in ^{1}H NMR spectra. Insect secretion (0.5–0.7 μL) was added to ^2H_2O (60 μL). Each sample was placed into a 2 mm capillary NMR tube without purification. Each spectrum was collected with 512 scans; between the measurements the sample was stored at 30 °C.

Racemic chrysomelidial: 8-Hydroxygeraniol (180 mg, 1.06 mmol) was oxidised with iodoxybenzoic acid (889 mg, 3.18 mmol) as previously described.[24] Following work-up, the dialdehyde was purified by chromatography on silica gel with petroleum ether/diethyl ether (9:1, *v/v*) for elution. The cyclisation of 8-oxogeranial (6 mg, 0.036 mmol) was achieved with a solution of imidazole in water (1 mL, 500 mmol) and stirred for 24 h. The product was then extracted with pentane (yield: 1.65 mg, 0.01 mmol, 28%), diluted and used directly for gas chromatographic analysis.

Analysis of chrysomelidial on a Hydrodex-β-6TBDM for separation of the stereoisomers: An aliquot (1 μL) of the defensive secretion in pentane was analysed on a Finnigan Trace instrument equipped with a Hydrodex-β-6TBDM column (25 m × 0.25 mm; Macherey–Nagel, Düren, Germany) with helium (1.5 mL min^{-1}) as the carrier gas. The GC injector (splitless), transfer line and ion source were set at 220, 250 and 200 °C, respectively. Racemic chrysomelidial (**1**) was eluted under programmed conditions: 60 °C (2 min), increase (15 °C min^{-1}) to 120 °C (60 min), increase (30 °C min^{-1}) to 200 °C (2 min) and separated into four baseline-separated peaks. In addition, small amounts of the insect secretion were added to the racemic chrysomelidial to establish peak identity by coelution.

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