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# Iridoid biosynthesis in staphylinid rove beetles (Coleoptera: Staphylinidae, Philonthinae)

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**Abstract**

The biosynthesis of chrysomelidial and plagiodial was studied in the rove beetle subtribe Philonthina (Staphylinidae). Glandular homogenates were found to convert synthetic (2*E*,6*E*)–[trideuteromethyl–5,5–$^{2}H_5$]octa–2,6–diene–1,8–diol (**10**) into *nor*-chrysomelidial (**14**) and *nor*-plagiodial (**13**). The overall transformation requires; i) oxidation of the substrate at C(1) and C(8), ii) cyclization of the resulting dialdehyde to *nor*-plagiodial followed by iii) isomerization to give *nor*-chrysomelidial. The oxidase requires molecular oxygen as a cofactor and operates with removal of the pro-*R* hydrogen from C(1) and C(8) of synthetic (1*R*,8*R*,2*E*,6*E*)–[1,8–$^{2}H_2$]–2,6–dimethyl–octa–2,6–diene–1,8–diol (**15**), producing a dialdehyde along with $H_2O_2$. Unlike enzymes from iridoid-producing leaf beetle larvae, the *Philonthus* enzyme is able to oxidize saturated substrates such as citronellol. Crude protein extracts prepared from *Philonthus* glands by ammonium sulfate precipitation, were found to produce hydrogen peroxide at a rate of 0.085±0.003 ng $H_2O_2$ (ng protein)$^{-1}$ hr$^{-1}$ with nerol as an oxidase substrate. The cyclase operates with opposite stereochemistry to the enzyme(s) from *Phaedon cochleariae* and other herbivorous leaf beetles, specifically removing the C(5)–$H_R$ hydrogen atom from (4*R*,5*S*,2*E*,6*E*)–[4,5–$^{2}H_2$]–2–methyl–octa–2,6–diene–1,8–diol (**17**). These findings have enabled us to construct a detailed account of iridoid biosynthesis in rove beetles, which resembles the biosynthetic route in leaf beetle larvae, but exhibits distinct stereochemical differences. 

*Keywords:* Rove beetle; Staphylinidae; *Philonthus*; Iridoid; Iridoid biosynthesis; Oxidase; Cyclase; Coleoptera

## 1. Introduction

Methylcyclopentanoid monoterpenes containing an iridane skeleton occur widely as secondary metabolites in both insects (Pasteels et al., 1982) and plants (Inouye and Uesato, 1986). Within the Insecta, iridoids have been found to function as defensive allomones and pheromones. For example, chrysomelidial (**1**), plagiodial (**2**) and actinidine (**3**) serve as defensive compounds in leaf beetle larvae (Blum et al., 1978; Sugawara et al., 1979; Pasteels et al., 1982). Iridodial (**4**) has been found in ants (Cavill et al., 1956) and aphids (Dawson et al., 1987) while anisomorphal (**6**) was isolated from stick insects (Meinwald et al., 1962, 1966) (Scheme 1).

In both plants and leaf beetles, iridoid biosynthesis has been thoroughly investigated and found to proceed along the same principal route. In insects, mevalonic acid is converted into geraniol (**7**) (Oldham et al., 1996) which undergoes subsequent allylic oxidation to produce 8–hydroxygeraniol (**8**). In both organisms the oxidation of geraniol to 8–hydroxygeraniol (**8**) is followed by subsequent oxidation to 8–oxogeranial (**9**). The initial oxidation step (ω–oxidation) has been extensively studied in plants by isolating and cloning the responsible membrane-bound, P450-dependent mixed-function oxidase (Schiel and Witte, 1987; Vetter et al., 1992; Hallahan et al., 1992). In plants, the second oxidation from 8–hydroxygeraniol to 8–oxogeranial has been ascribed to a zinc-containing $NADP^{+}$–dependent oxidoreductase (for *Rauwolfia serpentina*, see Ikeda et al., 1991; for *Nepeta*

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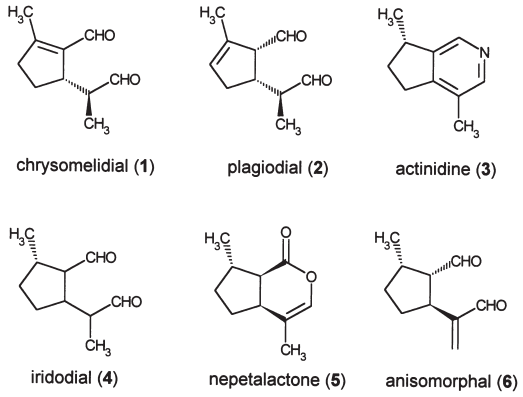



Scheme 1. Selected iridoids from insect defensive secretions.

*racemosa*, Hallahan et al., 1995). The corresponding oxidase in insects, responsible for transforming geraniol (**7**) into 8–oxogeranial (**9**), has been identified in the defensive secretion of several chrysomelid leaf beetle larvae (Lorenz et al., 1993; Veith, 1996) (Scheme 2).

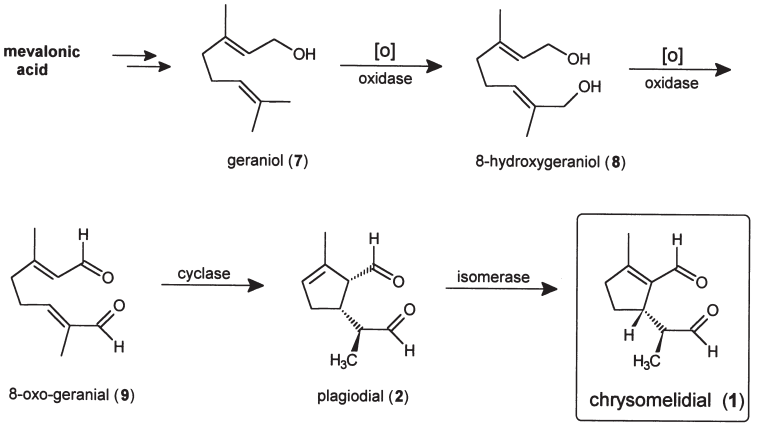
Cyclization of 8–oxogeranial (**9**) to the methylcyclopentanoid nucleus in plants and insects is an enzyme-controlled process (Uesato et al., 1986), substantiated by evidence that iridoid products arising from this pathway are single stereoisomers. Further support of an enzyme-catalysed cyclization is provided by the observation that acid- and base-promoted cyclization of 8–oxocitral leads to a nearly 1:1 mixture of 3*R*,8*R* and 3*S*,8*S* diastereomers (Bellesia et al., 1986; Uesato et al., 1986). Cyclization of 8–oxocitral in leaf beetles has been found to proceed via plagiodial, which undergoes double bond isomerization to yield chrysomelidial (Lorenz et al., 1993; Veith, 1996).

While the biosynthetic intermediates in iridoid biosynthesis among phytophageous leaf beetles are well understood (Lorenz et al., 1993; Veith et al., 1994, 1996; Oldham et al., 1996), little is known about the analogous pathway in carnivorous beetles. Rove beetles of the subtribes Staphylinina and Philonthina within the subfamily Staphylinidae are carnivorous insects containing paired abdominal defensive glands between tergites eight and nine that accommodate a diverse mixture of iridoids (Huth and Dettner, 1990, 1993; Dettner, 1983). Upon mechanical molestation, small aliquots of gland reservoir contents are exuded as droplets and smeared on the aggressor. Within the rove beetle subtribe Philonthina, defensive secretions are dominated by the alkaloid actinidine (**3**) with minor amounts of chrysomelidial (**1**), plagiodial (**2**) and 8–hydroxygeraniol (**8**) (Dettner, 1983). With the exception of the large abundance of actinidine, defensive mixtures secreted by Philonthina display a similar pattern of iridoids to those found in phytophageous leaf beetles. We were interested in investigating the biosynthesis of chrysomelidial (**1**), plagiodial (**2**) and actinidine (**3**) in rove beetles in order to contrast the biosynthetic route in phytophageous and carnivorous beetles. To identify biosynthetic intermediates and compare the stereochemistry of individual transformations within the pathways in carnivorous rove beetles and herbivorous leaf beetles, we carried out mass spectrometry studies to identify labeled products arising from the in vitro metabolism of chiral deuterium-labeled substrates. To investigate oxidase activity, we employed UV spectrometry to probe for requisite enzyme cofactors and an enzyme-based peroxide assay to measure levels of released $H_2O_2$. Herein we report our findings.

## 2. Materials and methods

### 2.1. Chemicals

(2*E*,6*E*)–[Trideuteromethyl–5,5–$^2H_5$]octa–2,6–diene–1,8–diol (**10**) was prepared according to the protocol of Veith et al. (1994). (1*R*,8*R*,2*E*,6*E*)–[1,8–$^2H_2$]–2,6–Dimethyl–octa–2,6–diene–1,8–diol (**15**) was synthesized from unlabeled 8–oxogeranial using a combination of horse liver alcohol dehydrogenase and formiate dehydrogen-




Scheme 2. Proposed biosynthetic pathway to iridoids in insects (Lorenz et al., 1993).

ase. $^{2}$HCOONa served as the source of deuterium (Veith et al., 1996). (4*R*,5*S*,2*E*,6*E*)–[4,5–$^{2}H_2$]–2–Methyl–octa–2,6–diene–1,8–diol (**17**) was also obtained by a chemoenzymatic approach with broken cells of *Clostridium tyrobutyrcum*, DSM 1460, as a biocatalyst (Veith, 1996). All other chemicals were obtained from Sigma–Aldrich or Fluka (SAF Chemicals, Deisenhofen, Germany) and used without further purification.

### 2.2. Insects

*Philonthus sanguinolentus*, *P. rectangulus*, *P. chalceus*, *P. carbonarius*, *P. laminatus*, *P. laevicollis*, *P. nitidus*, *P. politus* and both *Ontholestes* species (*O. tesselatus*, *O. murinus*) were collected from moist green compost in the botanical gardens of Jena and Bayreuth, and stored at 15°C on a diet of mealworms. Insects were typically used for experiments within 72 hours of capture.

### 2.3. Tergal gland excision

Beetles were removed to a holding box and slowly cooled to 0°C in order to prevent alarm and subsequent gland discharge. After incubation for 10 minutes at −10°C, insects were removed from the holding box and equilibrated to room temperature. Excision of paired tergite glands was carried out using forceps under a binocular stereomicroscope (5× magnification). Immediately following removal, glands were transferred to ice cold buffer solution and promptly used for experiments.

### 2.4. In vitro incubation experiments

Substrates were prepared as 1% solutions (w/v) in phosphate buffer ($NaH_2PO_4$, 100 mM, pH 7.1) and incubated in a sonicator bath for 10 min. After gland excision, paired glands were immediately placed in a conical glass vial insert containing 2 μl ice-cold substrate solution. The vial insert was placed inside an Eppendorf tube, and the tube incubated for five minutes in a sonicator bath (0°C). After incubating for 1 min on ice, the sonication cycle was repeated once. Tubes were briefly centrifuged (10,000***g***, 10 s, 4°C) and incubated at 22°C. Organic compounds were extracted from samples by solid phase microextraction (SPME) using a 100 μm polydimethylsiloxane stationary phase (SUPELCO, Sigma–Aldrich) and analysed by gas chromatography/mass spectrometry (GC/MS). Extractions were performed by introducing SPME fibers into incubating solutions for 15 min, followed by direct desorption of analytes into the injection port (250°C) of a GC/MS. Stationary phases were conditioned at 250°C in the injection port of a GC under hydrogen for 1–4 h. Prior to each extraction, phases were thermally cleaned for 15 min at 250°C. Significant levels of substrate conversion could typically be detected by this protocol after 24 h incubation.

### 2.5. Chromatographic methods

A Finnigan GCQ gas chromatograph/mass spectrometer equipped with an Optima5 M3 capillary column (15 m × 0.32 mm, Macherey Nagel, Düren, Germany) was used to analyse splitless injections of SPME extracts. Helium (linear velocity 40 cm $sec^{-1}$) was used as the carrier gas. Compounds were separated and eluted under programmed conditions from 50°C (2 min isothermal) to 200°C (at 10°C $min^{-1}$) to 280°C (at 40°C $min^{-1}$); injector temperature 220°C; GC-interface 275°C; scan range 35–400 $s^{-1}$. The fragmentation pattern of chrysomelidial was analysed on a Micromass MasSpec (Micromass, Manchester, UK) double-focusing magnetic sector mass spectrometer (geometry EBE), connected to a Hewlett Packard HP6890 II gas chromatograph, using linked scans and high resolution measurements.

### 2.6. Protein assay

Freshly excised glands from *Philonthus* spp. were immediately placed in an Eppendorf tube containing 800 μl ice-cold water (Milli–Q, Millipore, Bedford, MA). Tubes were sonicated for 6 min in an ice bath, followed by 5 min incubation on ice. The sonication cycle was repeated once and tubes centrifuged (10,000***g***, 25 s, 4°C). Protein was assayed in triplicate using a Bio–Rad protein assay kit (Bio–Rad Laboratories, Hercules, CA).

### 2.7. Hydrogen peroxide assay

Twenty-seven *Philonthus rectangulus*, *P. sanguinolentus* and *P. nitidus* were divided into three groups of seven beetles, such that each portion contained a nearly identical mass of insects. Within each group, glands from all seven beetles were removed and divided evenly into two fractions; one fraction was used to assay peroxide formation and the other to assay corresponding protein concentration. After dissection, glands used to assay peroxide formation were immediately stored in an Eppendorf tube containing 50 μl ice-cold TCN+buffer (50 mM TRIS buffer, pH 7.0, containing 1.0 M NaCl, 0.01% $NaN_3$ and Complete® protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, FRG). Tubes were sonicated for 6 min (0°C), followed by 5 min incubation on ice. After repeating the sonication cycle once, tubes were centrifuged (20,800***g***, 15 min, 4°C). Total glandular protein was isolated by ammonium sulfate precipitation, dissolved in 40 μl TCN+buffer and stored briefly on ice. Hydrogen peroxide was determined using a modified protocol based on the method of Kreit et al. (1992). 180 μl of nerol substrate solution (4 mM

nerol in PCN6 buffer, consisting of 8.95 mM citrate, 32 mM $Na_2HPO_4$, pH 6.0, 100 mM NaCl, dissolved by sonication) was transferred to the wells of a 96-well microtiter plate. 20 μl of freshly isolated glandular protein in TCN+ buffer was added to the wells. To another set of wells, containing 180 μl of nerol substrate solution, was added a series of hydrogen peroxide standards to give these wells final concentrations of 0.0, 1.0, 2.0, 4.0, 8.0 and 16.0 ng/ml $H_2O_2$, and a total well volume of 200 μl. After incubating the plate for 60 min (22°C), 40 μl of a 1:1 mixture of peroxidase solution (125 U $\mu l^{-1}$ horseradish peroxidase (Roche Molecular Biochemicals, Mannheim, FRG) in PCN6 buffer and freshly prepared ABTS reagent (2 mg $ml^{-1}$ 2,2'–azino–*bis*(3–ethylbenzothiazoline–6–sulfonate)) in PCN6 buffer were rapidly added to wells and mixed using a multi-channel pipette. The plate was incubated for 5 min (22°C) and the absorbance of each well (405 nm) measured with a SPECTRAmax 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The concentration of $H_2O_2$ from wells containing glandular protein was determined against the slope obtained from the $H_2O_2$ standard curve. Glandular protein extracts and $H_2O_2$ standards were assayed in triplicate.

### 2.8. Oxidation kinetics

Freshly excised, paired glands from three *P. laminatus* and five *P. laevicollis* beetles were divided into two equal gland portions. Each portion of glands was placed in 500 μl of ice-cold phosphate buffer ($NaH_2PO_4$, 100 mM, pH 7.1) and sonicated for 5 min (0°C). 500 μl of gland solution was transferred to a cuvette and the oxidation reaction initiated by addition of 1500 μl of a 0.1% 8–hydroxygeraniol (**8**) solution (w/v) in phosphate buffer ($NaH_2PO_4$, 100 mM, pH 7.1). The cuvette was immediately degassed with helium and argon to create an anaerobic atmosphere; the progress of the oxidation reaction was monitored (22°C) on a spectrophotometer at 240 nm against buffer. After 6 min, the cuvette was purged with air to create an aerobic environment. After 40 min, helium and nitrogen were again used to degas the cuvette. After a total of 54 min, the cuvette was purged with air.

## 3. Results

In vitro conversion of (2E,6E)–[trideuteromethyl–5,5–$^2H_5$]octa–2,6–diene–1,8–diol (**10**). In vitro incubations of homogenates of tergite glands from *P. chalceus* with diol **10** led to detection of both labeled *nor*-plagiodial (**13**) and *nor*-chrysomelidial (**14**). Both compounds were resolved by gas chromatography (GC) and could be unequivocally distinguished by their mass spectra. Owing to the lack of the methyl group at C(6), the substrate and its metabolites elute much earlier (lower boiling points) from the GC column and can be analysed without interference from the natural compounds present. The observed relative amounts of **13** and **14** were vastly different; *nor*-chrysomelidial **14** represented the major component (ca. 90%) and **13** the minor compound (ca 10%). The signal for **14** consists of two peaks of approximately equal relative areas, arising from two diastereomers (Fig. 1).

Localization of the deuterium atoms within the labeled metabolites could be achieved on the basis of the fragmentation pattern of natural chrysomedial (**1**) and previous studies with labeled *nor*-chrysomelidial (**14**) and *nor*-plagiodial (**13**) (Veith et al., 1996). According to Fig. 2, major fragmentation pathways arise from the $M^+$ ion ($m/z$=166) with loss of CO to fragment (b) which then eliminates the complete $C_3$-side chain as a radical to give fragment (f) which is indicative of the number of deuterium atoms on the ring of a chrysomelidial-type iridoid. Loss of $C_3$-side chain without preceding elimination of water yields fragment (e). The fragmentation pattern of plagiodial-type iridoids differs from Fig. 2 significantly in the fact that the $C_3$-side chain is lost by McLafferty cleavage ($-C_3H_6O$) leading to fragment (f–H), $C_5H_8$ ($m/z$=80). *Nor*-chrysomelidial and *nor*-plagiodial exhibit fragmentation patterns analogous to those of chrysomelidial and plagiodial, after correcting for the missing methyl group.

Both diastereomers of **14** contain a base peak (f) at $m/z$=68 in their mass spectra, corresponding to ion $C_5H_6{}^2H^+$, in accord with abstraction of one of two diastereotopic deuterium atoms from the C(5) position of precursor **10**. The signal for *nor*-plagiodial (**13**) appears as three peaks constituting one major and two minor diastereomers in an approximately 4:1:1 relative area ratio. All three diastereomers display a base peak at $m/z$=67 corresponding to $C_5H_5{}^2H^{+\bullet}$, further substantiating loss of a single deuterium atom from the C(5) position of **10** during cyclization. In addition to iridoid analogs **13** and **14**, we identified dialdehyde **12** as a major intermediate accompanied by small amounts of starting diol **10**, and a signal for two intermediate semialdehydes, e.g. **11**. Components of the natural defensive secretion, such as actinidine (**3**), chrysomelidial (**1**), and plagiodial (**2**) were also present. Despite very careful analysis, we were unable to detect the presence of a corresponding labeled *nor*-actinidine.

Upon repeating the incubation experiment of diol **10** with glandular secretions from phytophageous leaf beetle *Phaedon cochleariae*, we also detected **13** as three peaks in the GC profile, corresponding to diastereomers with a relative area ratio of approximately 4:1:1. *Nor*-chrysomelidial (**14**) was found as two peaks, pertaining to an approximately 1:1 relative area ratio of diastereomers. The extraordinarily similar ratios of the observed *nor*-plagiodial diastereomers and *nor*-chrysomelidial

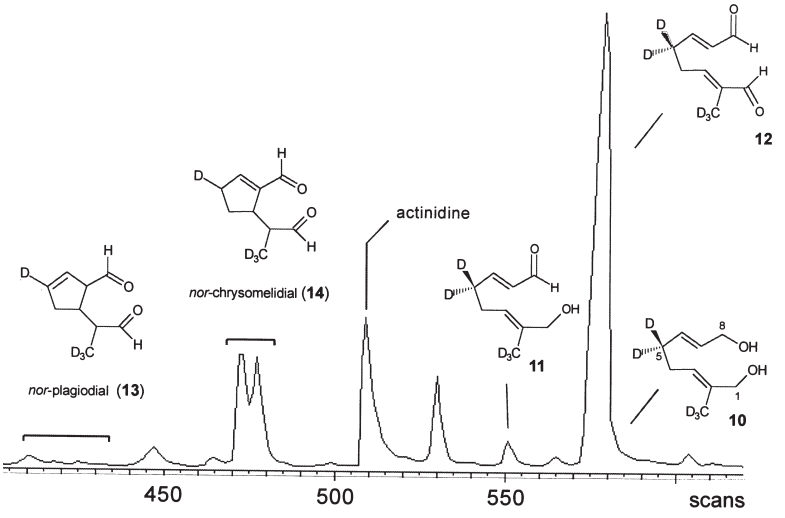



Fig. 1. Gas chromatogram of iridoid metabolites obtained from diol **10**. The diol was converted in vitro by buffered a glandular homogenate from *P. chalceus*. Separation of compounds was achieved on fused silica capillary (Optima M3, 15 m × 0.32 mm) under programmed conditions: 50°C (2 min isothermal) to 200°C (at 10°C $min^{-1}$) to 280°C (at 40°C $min^{-1}$). Peaks were detected by mass spectrometry (for details refer to Materials and methods).

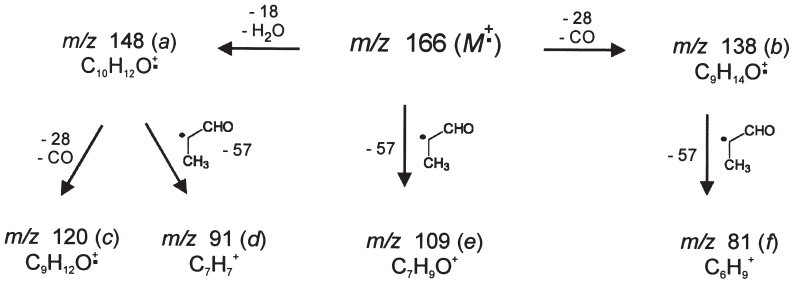



Fig. 2. Mass fragmentation pattern of chrysomelidial (**1**). Indicated are some of the dominant mass fragmentation pathways. The elemental composition of the individual fragments (a) to (f) was determined by high resolution mass spectrometry.

diastereomers in *Phaedon* and *Philonthus*, suggests that the absence of the C(6) methyl group in the unnatural *nor*-substrates has a crucial influence on stereochemical control of the reaction in both insect species. Overall, the successful conversion of **10** in *P. chalceus* and *Phaedon* clearly demonstrates the presence of comparable enzymatic activities in the glandular reservoirs of both species (cf. Fig. 1).

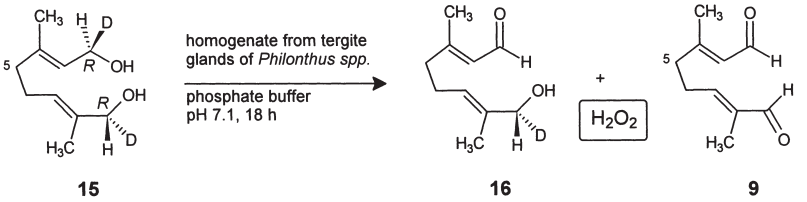
### *3.1. Stereospecificity of the oxidase*

The first enzyme in the biosynthetic sequence from 8–hydroxygeraniol (**8**) to chrysomelidial (**1**) outlined in Scheme 2 is an oxidase that converts **8** into **1** and **2** (via an intermediary ω–hydroxyaldehyde, Scheme 2). In *Phaedon* spp. this enzyme operates stereospecifically by exclusively removing the pro-*R* hydrogen from C(1) and C(8) of chiral precursors (Veith et al., 1996). Interested in comparing the stereochemistry of the oxidation of 8–hydroxygeraniol in herbivorous and carnivorous insects, we used chiral, labeled (1*R*,8*R*)–[1,8–$^2H_2$]–8–hydroxygeraniol **15** as a probe to determine the stereospecificity of the oxidase from *Philonthus* spp. Analysis of incubation experiments by GC/MS illustrated that **15** was almost completely converted into dialdehyde **9** within 18 h, accompanied by minor amounts of two possible intermediary semialdehydes **16** (not separated) (Scheme 3).

Prolonged incubation times (72 h) did not result in further oxidation. Analysis of the spectra of semialdehydes **16** displayed the presence of a single deuterium atom, while dialdehyde **9** had lost both isotopic labels. The spectrum of the resulting dialdehyde was identical with authentic, unlabelled 8–oxogeranial **9**. This finding demonstrates a high degree of site specificity of the enzyme(s) operating at both centres C(1) and C(8), opposing an isotope effect during exclusive removal of the more tightly bound deuterium atom.

### *3.2. Molecular oxygen as a cofactor of the oxidase*

To study the *Philonthus* oxidase, we used 8–hydroxygeraniol as a substrate for monitoring the rate of oxidation by formation of the resulting conjugated alde-




Scheme 3. Stereochemical course of the oxidase from *Philonthus* spp. The stereochemical course follows from the presence of a single deuterium atom in the two possible semialdehydes and the absence of any deuterium label in the product **9**. Only the semialdehyde **16** is shown in the figure.

hyde. After initiating the oxidation reaction under anaerobic conditions by degassing with helium and argon, no significant increase in UV absorption owing to the production of an α,β-unsaturated aldehyde could be detected by spectrophotometry. By purging the cuvette with $O_2$ however, a significant oxidation rate was observed. By repeating this degassing with helium and argon and subsequent flushing with $O_2$, molecular oxygen was confirmed as an imperative oxidase cofactor.

### 3.3. Peroxide formation

After establishing $O_2$ as an oxidase cofactor, we measured the time-dependent generation of $H_2O_2$ using protein isolated from glandular extracts of *Philonthus*. We observed that the oxidase(s) present in isolated protein from gland homogenates of *Philonthus* produce $H_2O_2$ at a rate of 0.085±0.003 ng $H_2O_2$ (ng protein)$^{-1}$hr$^{-1}$, thereby independently confirming that $O_2$ is a necessary cofactor and that the oxidation proceeds with the generation of $H_2O_2$.

### 3.4. In vitro conversion of rac-citronellol

In order to investigate the substrate requirements of the oxidase present in *Philonthus*, we studied the in-vitro oxidation of a substrate such as *rac*–citronellol, possessing an aliphatic alcohol, instead of the allylic substrates **10** and **15**. Paired glands were removed from *P. sanguinolentus* and *P. rectangulus*, divided into two equal portions and one set incubated with *rac*-citronellol. As a control, the other set of glands was incubated with the natural substrate 8–hydroxygeraniol (8). The oxidation of *rac*-citronellol was analysed by GC/MS after 34 h, clearly illustrating the presence of *rac*-citronellal ($M^{+\bullet}$ *m*/*z*=154) along with remaining *rac*-citronellol ($M^{+\bullet}$ *m*/*z*=156) in a relative ratio of 1.6:1, respectively. This result contrasts previous findings with the enzyme(s) from herbivorous *Phaedon* larvae, which only convert allylic and aromatic alcohols into aldehydes (Veith et al., 1997).

### 3.5. Stereochemistry of the cyclization

To establish the stereochemistry of proton abstraction during cyclization to the cyclopentanoid nucleus, we carried out in vitro incubations of *Philonthus* gland extracts with (4*R*,5*S*)–[4,5–$^2H_2$]–3–*nor*-hydroxygeraniol (**17**). GC/MS analysis of incubating solutions identified two diastereomers of deuterated *nor*-chrysomelidial (**18**). The mass spectra of these two stereoisomers displayed base peaks at *m*/*z*=69, corresponding to $C_5H_5D_2^+$ (Fig. 3a) (Veith et al., 1994). The presence of fragment ion $C_5H_5D_2^+$ clearly demonstrates that proton abstraction during cyclization is stereospecific and proceeds with loss of the C(5)-$H_R$ hydrogen atom in precursor **17**. Proton abstraction was additionally confirmed to be stereo-

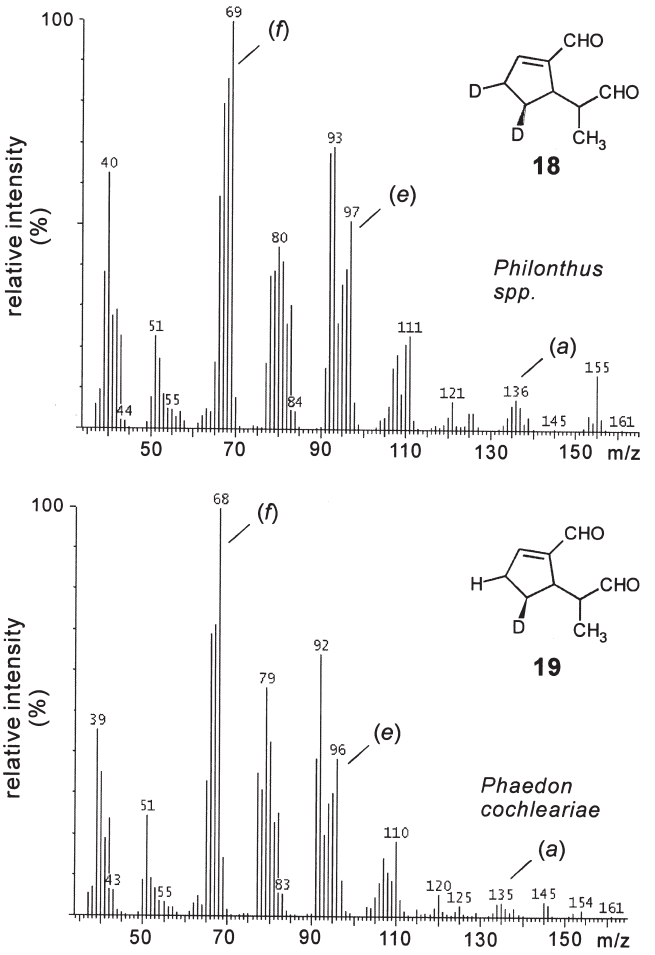



Fig. 3. Mass spectra of isotopomeric nor-chrysomelidials (**18**) and (**19**) obtained from in vitro cyclization of diol **17** by *Philonthus* spp. (upper spectrum) and *P. cochleariae* (lower spectrum). Fragments (a) to (f) refer to Fig. 2.

specific by scrutinizing the mass spectra of deuterated *nor*-plagiodial (**13**), which appears as two diastereomers. Both diastereomers were found to display base peaks at *m*/*z*=68, corresponding to $C_5H_4D_2^{+\bullet}$. The stereospecific cyclization of an acyclic substrate thereby confirms the anticipated enzyme-catalysed step. This experiment was repeated with *Phaedon cochleariae* by collecting glandular secretions via a microcapillary loaded with a solution of **17**.

GC/MS analysis of the incubation solution again identified two diastereomers of *nor*-chrysomelidial **19**. The mass spectra of both diastereomers, however, displayed base peaks at *m*/*z*=68 ($C_5H_6D^+$), unequivocally highlighting the stereoselective removal of the C(5)-$H_S$ proton (Fig. 3b). Two diastereomers of *nor*-plagiodial were also identified and found to contain base peaks at *m*/*z*=67 ($C_5H_5D^{+\bullet}$) in their mass spectra, confirming the loss of a deuterium atom from C(5).

## 4. Discussion

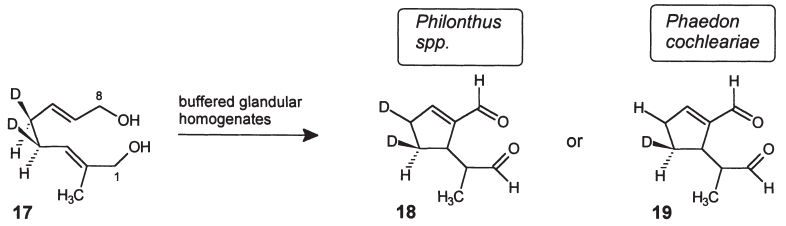
The present work provides initial insight into iridoid biosynthesis in carnivorous beetles. By studying rove beetles, we have found that *Philonthus* spp. utilize the same monoterpenoid precursors and follow a comparable overall sequence (Scheme 2) to previously investigated families of leaf beetles (Veith et al., 1994; Oldham et al., 1996). By studying the in vitro incubation of labeled precursors with homogenates of freshly excised glands in phosphate buffer (pH 7.1), we have observed rapid oxidation of diol **10**, via semialdehyde intermediates (for example **11**), to dialdehyde **12**. Subsequent cyclization provided *nor*-chrysomelidial (**14**) along with small amounts of *nor*-plagiodial (**13**) (Fig. 1). Surprisingly, no *nor*-analogue of the major gland constituent, actinidine (**3**), was produced. The observation that **10** is cyclized to *nor*-chrysomelidial **14**, with loss of a single hydrogen atom from C(5) of the precursor, indicates that *nor*-plagiodial **13** is indeed an intermediate in the biosynthesis of **14**, and is analogous to previous findings with the leaf beetle *Phaedon cochleariae* (Veith et al., 1994). In glands of *P. cochleariae*, 8–oxogeranial (**9**) is first converted into plagiodial (**2**) and then isomerized to the more stable, conjugated cyclopentene ring skeleton of chrysomelidial (**1**). In agreement with the presence of iridoid biosynthetic enzymes in leaf beetles is the observation that the oxidase from carnivorous *Philonthus* spp., catalysing the oxidation of diols and semialdehydes, requires molecular oxygen as a cofactor. The oxidation process is accompanied by simultaneous production of $H_2O_2$, hence using molecular oxygen as the ultimate two-electron acceptor. This requirement clearly differentiates the oxidase(s) present in beetle iridoid biosynthesis from those found in iridoid biosynthesis in plants. Moreover, in contrast to the oxidase from plants, the enzyme from *P. cochleariae* is not dependent on $NADP^+$ and could not be inactivated by complexing agents such as EDTA (Veith et al., 1996). Utilizing chiral diol **15** as a substrate for the *Philonthus* spp. oxidase, we have illustrated that diol oxidation proceeds at both centres (C(1) and C(8)) by removal of $H_R$; in agreement with *Re*-specificity of the enzyme. A preference for the enzymatic removal of the C(1)- or C(8)-$H_R$ proton of diol **15** appears to be general, since the enzymes present in the defensive secretions of several chrysomelid larvae exhibit, without exception, the same *Re*-specificity during in vitro experiments (Veith et al., 1997) (Scheme 4).

Oxidase substrate specificity has been probed using *rac*-citronellol. We have observed that the *Philonthus* oxidase is not solely substrate-specific for the oxidation of α,β-unsaturated and aromatic alcohols, as is the case for enzymes from leaf beetles (Veith et al., 1997), but is capable of efficiently oxidizing saturated alcohols as well.

The stereospecificity of the ring closure was analysed with chiral diol **17**. In vitro incubations of **17** with homogenates from tergal glands of *Philonthus* spp. proceeded with loss of the C(5)-$H_R$ proton while abstraction in *Phaedon* affected the C(5)-$H_S$ proton (Table 1). In previous, unpublished work we have found that incubations of **17** with secretions from larvae of the leaf beetles *Hydrothassa glabra* and *Prasocuris phellandri* lead to abstraction of the C(5)-$H_S$ proton, as determined by GC/MS. Only in another rove beetle genus (*Ontholestes*) did we see removal of C(5)-$H_R$. Overall, therefore there appears to be a correlation between the stereospecificity observed for phytophageous leaf beetles and that observed for carnivorous rove beetles.

To account for the nearly exclusive cyclization of 8–oxogeranial to plagiodial, and considering the ease of nonenzymatic cyclizations of 8–oxo–geranial in the presence of certain primary amines (Schreiber et al., 1986; Unelius and Norin, 1994), we propose the reaction sequence outlined in Scheme 5 as a model for the enzymatic conversion.

In this sequence, 8–oxogeranial is first converted into an imine and then into a dieneamine by base-assisted removal of the C(5)-$H_R$ proton (stereochemistry observed with the enzyme from *Philonthus* spp. and *Ontholestes* sp.). The dieneamine may then readily undergo a concerted [4π+2π]–electrocyclization yielding a 2-aminodihydropyran intermediate that is hydrolysed to yield plagiodial and, after isomerization, chrysomelidial. Such a mechanism accounts for the predominantly *cis*-orientation of the side-chains in plagiodial and iridodial. This observation would be difficult to explain by an intramolecular *Michael*-addition of an enolate onto the double bond C(2)=C(3), which should result in the *trans*-orientation of side-chains. It is interesting to note that with truncated *nor*-precursors, such as **10**, only the first step of the sequence, i.e. generation of the reactive




Scheme 4. Stereochemical aspects of the cyclization of **17** in different insect species. The reaction proceeds via the corresponding dialdehyde. In *Philonthus* spp. both deuterium atoms of the precursor **17** remain in the product **18**. In herbivorous larvae the deuterium label at C(5) of the precursor **17** is lost yielding mono-deuterated **19**.

Table 1
Stereospecificity of iridoid ring closure. The number of deuterium atoms in the resulting *nor*-chrysomelidial (Scheme 4) was determined by mass spectroscopy. All experiments were carried out with buffered glandular homogenates in the presence of diol **17** as the substrate

| Insect species | | Abstracted hydrogen atom from C(5) of **17** |
|---|---|---|
| *Hydrothassa glabra* | herbivore | C(5)–$H_S$ |
| *Ontholestes sp.* | carnivore | C(5)–$H_R$ |
| *Phaedon cochleariae* | herbivore | C(5)–$H_S$ |
| *Prasocuris phellandri* | herbivore | C(5)–$H_S$ |
| *Philonthus spp.* | carnivore | C(5)–$H_R$ |

dieneamine, appears to be precisely controlled by the enzyme(s) involved.

In all organisms (Table 1) an exclusive loss of either the C(5)-$H_R$ or C(5)-$H_S$ proton is observed. The subsequent steps, namely the cyclization to and hydrolysis of the 2-aminodihydropyran moiety proceed without efficient control; two diastereomers are observed for *nor*-chrysomelidial **14** and three diastereomers for *nor*-plagiodial **13** (cf. Fig. 1). The stereochemistry of natural iridoids, such as **1** or **2** originating from **9** is, however, more precisely attained. Unable to detect deuterated *nor*-actinidine metabolites from *Philonthus*, we have been unsuccessful in unravelling information regarding the biosynthesis of this alkaloid. Actinidine is clearly a terpene-derived iridoid, with a structure suggesting its origin from plagiodial or chrysomelidial. During molestation defensive gland reservoirs of *Philonthus* are everted and a droplet of rectal fluid is depleted simultaneously. On the surface of target organisms and at the abdominal tip of the beetle the defensive secretion is mixed/contaminated with evacuated rectal fluid of the same beetle which may contain variable amounts of ammonia. Ammonia either represents an excretory product or is produced by bacteria which colonize the rectal pouch of many staphilinoid beetles (unpublished). A plausible origin of actinidine could be the spontaneous heteroaromatization of chrysomelidial or plagiodial in the presence of an ammonium source. Further investigations of actidinine biosynthesis in *Philonthus* are underway.

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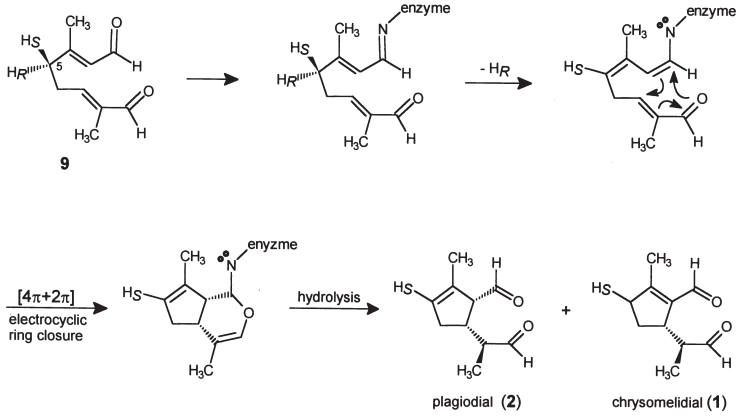


Scheme 5. Mechanistic proposal for the biosynthesis of iridoid monoterpenes in insects involving an electrocyclic [4π+2π] ring closure. The sequence implies the formation of an dieneamine with an appropriate functional group of the enzyme (e.g. the ε-amino group of a lysine) prior to a concerted ring closure generating the *cis*-fused plagiodial skeleton. Hydrolysis and isomerization lead to plagiodial (**2**) and chrysomelidial (**1**), respectively. Removal of the C(5)-$H_R$ from the acyclic precursor corresponds to the stereochemistry of the *Philonthus* enzyme(s).

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