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CHEMICAL DEFENCE IN CHRYSOMELID LARVAE AND ADULTS

J. M. PASTEELS, J. C. BRAEKMAN[1] and D. DALOZE
Collectif de Bio-écologie, Faculté des Sciences, Université Libre de Bruxelles, 1050 Bruxelles, Belgium

and

R. OTTINGER
Service de Chimie Organique EP, Faculté des Sciences Appliquées, Université Libre de Bruxelles, 1050 Bruxelles, Belgium

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Abstract—The defensive secretions of several species of chrysomelid larvae and adults have been analyzed. Salicylaldehyde and some already known methylcyclopentanoid monoterpenes have been identified in the larvae. In the adults of the tribe Phaedonini, two isoxazolin-5-one derivatives (**8** and **9**) were isolated and their structures determined. The chemical defence of chrysomelid beetles is briefly reviewed with emphasis on chemotaxonomy and ecological significance.

Chrysomelids, or leaf beetles, are one of the major families of Coleoptera. Mostly phyllophagous, they are often monophagous or oligophagous, mainly on herbs, on which they form dense aggregates as examplified by some well-known agricultural pests. When discovered by a predator, these colonies offer a rich food source. Thus, not surprisingly, several spectacular defence mechanisms have evolved in the family.[2] These include quick escape by jumping or flying, mechanical defence strengthened by sharp spines or by a glue released during reflex bleeding, crypsis, or, on the contrary, aposematic colorations linked to chemical defence.

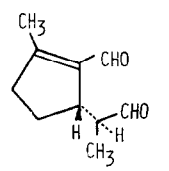
Recently, considerable progress have been made in the chemistry of defensive compounds emitted by chrysomelid larvae and adults. It is the aim of this paper to bring the topic up to date by reviewing the literature, and incorporating original results. Chemical defence will be discussed by considering the phylogeny and taxonomy of the family, as well as possible environmental influences.

Chemical defence in larvae. Defence glands in larvae are only known in some species belonging to the Chrysomelinae, among the 19 recognized subfamilies.

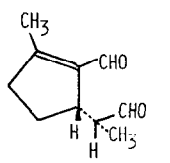
Most species possess 9 pairs of glands located dorsally in the meso- and metathorax, as well as in the first 7 abdominal segments,[3,4] but the Paropsini possess only one pair of glands at the base of the 8th abdominal tergite.[5] The glands are eversible and, when the larvae are disturbed, the secretion appears at the tip of the eversed reservoir.

The principal classes of compounds found until now in the secretion of the larvae are listed in Table 1.

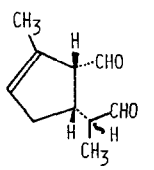
First reported by Meinwald *et al.*[6,7] in the larvae



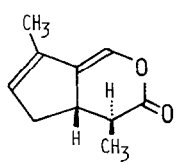
(1) Chrysomelidial



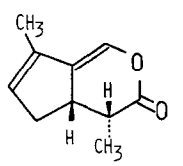
(2) Epichrysomelidial



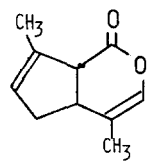
(3) Plagiodial



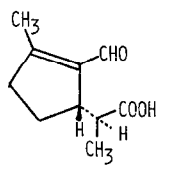
(4) Plagiolactone



(5) Epiplagiolactone



(6) Gastrolactone



(7)

Table 1. Principal classes of compounds found in the larvae secretions of the Chrysomelinae

	Methylcyclopentanoid monoterpenes	Long chain molecules	Salicylaldehyde	Phenylethyl esters	Benzaldehyde	HCN	Juglone	Host plant	References
PHAEDONINI									
Chrysomela (Chrysomela) populi			+					*Populus*	13,17, this paper
Chrysomela (Chrysomela) tremulae			+					*Populus*	this paper
Chrysomela (Microdera) scripta			+					*Salix*	14
Chrysomela (Microdera) 20-punctata costella			+		+			*Salix*	17
Chrysomela (Microdera) 20-punctata			+					*Salix*	this paper
Chrysomela (Microdera) interrupta				+				*Alnus*	16
Chrysomela (Linaeidea) aenea	+	+						*Alnus*	11, this paper
Gastrophysa cyanea	+							*Rumex*	8,9
Gastrophysa atrocyanea	+	+						*Rumex*	10,12
Gastrophysa viridula	+							*Rumex*	this paper
Hydrothassa marginella	+							*Ranunculus*	this paper
Phaedon brassicae	+							*Brassica*	10
Phaedon cochleariae	+							*Nasturtium*	this paper
Plagiodera versicolora	+							*Salix*	6,7,11, this paper
Prasocuris phellandrii	+							*Ranunculus*	this paper
Gastrolina depressa							+	*Juglans*	17
PHRATORINI									
Phratora vitellinae			+					*Salix*	15
PAROPSINI									
Paropsis atomaria					+	+		*Eucalyptus*	5
Chrysophtharta variicollis					+	+		*Eucalyptus*	5
Chrysophtharta amoena					+	+		*Eucalyptus*	5

secretion of *Plagiodera versicolora*, and by Blum *et al.*[8,9] in *Gastrophysa cyanea*, methylcyclopentanoid monoterpenes have been further isolated from 2 other species belonging to the tribe Phaedonini.[10,11] So far, 6 different derivatives have been described (**1–6**). To check if the presence of these compounds in this tribe is a general feature, the secretions of 6 Belgian species were analyzed. The obtained results as well as those reported in the literature, are summarized in Table 2. The identifications are essentially based on the behaviour of the fresh crude secretion in GC and tlc. The reference compounds plagiodial (**3**) and plagiolactone (**4**) were isolated from Belgian *Plagiodera versicolora* whereas chrysomelidial (**1**) was obtained from *Gastrophysa viridula*. They were characterized by comparison of their MS and ^{1}H NMR spectra with those reported for the authentic derivatives.[6,8,11] The ^{1}H NMR spectrum of chrysomelidial showed, besides the expected signals, small signals at 1.09 (d) and 2.83 (m) ppm attributable to the presence of traces of epichrysomelidial (**2**)[11] in the secretion of *Gastrophysa viridula*. Since **1** and **2** cannot be distinguished by their retention time in GC, and since the amount of secretion available to us was too small to record a ^{1}HNMR spectrum, we do not know if the peak having the retention time of chrysomelidial in the chromatogram of *Hydrothassa marginella* and *Phaedon cochleariae* corresponds to **1** and/or **2**. In contrast, no traces of epiplagiolactone (**5**) could be detected by NMR or GC on DEGS in any of the examined secretions. On standing, chrysomelidial is transformed into a more polar derivative, the spectral properties of which suggest that it is the corresponding acid **7**.

Table 2. Proportions of different methylcyclopentanoid monoterpenes in the defensive secretion of Phaedonini Chrysomelid larvae

	Chrysomelidial (1)	Epichrysomelidial (2)	Plagiodial (3)	Plagiolactone (4)	Epiplagiolactone (5)	Gastrolactone (6)	Host plant	Origin
Chrysomela (Linaeidea) aenea	35	-	-	15	50	-	*Alnus*	Japan [11]
C. aenea	-	-	85	15	-	-	*Alnus*	Belgium
Gastrophysa cyanea	50	-	-	-	-	50	*Rumex*	U.S.A. [8]
G. atrocyanea	100	-	-	-	-	-	*Rumex*	Japan [10]
G. viridula	90	10	-	-	-	-	*Rumex*	Belgium
Hydrothassa marginella	—90—		-	10	-	-	*Ranunculus*	Belgium
Phaedon brassicae	100	-	-	-	-	-	*Brassica*	Japan [10]
Ph. cochleariae	—100—		-	-	-	-	*Nasturtium*	Belgium
Plagiodera versicolora	—66—		-	33	-	-	*Salix*	U.S.A. [6]
P. versicolora	-	-	70	30	-	-	*Salix*	Belgium
P. versicolora distincta	-	3	90	3	3	-	*Salix*	Japan [11]
Prasocuris phellandrii	-	-	100	-	-	-	*Ranunculus*	Belgium

From Table 2, it is clear that the monoterpene content of the secretions varies qualitatively and quantitatively from species to species, or even from far distant populations of the same species as examplified by *Plagiodera versicolora* collected in the U.S.A., in Japan or in Belgium, as well as by *Chrysomela (Linaeidea) aenea* collected in Japan or in Belgium. These differences are not due to a sampling effect, since most of the proportions were calculated from pooled secretions of hundreds of third instar larvae. The same proportions were obtained from different samples of *P. versicolora* collected in Belgium during several consecutive years. Besides, interindividual variations seem to be rather small, smaller in any way than most differences observed between species or far distant populations. Two Belgian populations of *C. aenea*, one of only five specimens collected close to Brussels, and the other of 12 specimens collected in Treignes, 100 km south of Brussels, yielded the same proportions of terpenes. Moreover, we have determined the proportions of plagiodial (**3**) and plagiolactone (**4**) for the secretion of 10 different third instar larvae of *P. versicolora* from Belgium. The result (69% ± 7 of plagiolactone) indicates very little individual variation.

Long chain molecules occur sporadically in the defensive secretion of the Phaedonini in mixture with the monoterpenes. Sugawara *et al.*[12] first reported octadecyl acetate and (*Z*)-11-eicosenyl acetate in the secretion of *Gastrophysa atrocyanea* together with an unidentified hydrocarbon. They further detected (*Z*)-11-eicosenyl acetate, hexadecyl acetate, (*Z*)-9-octadecenyl acetate and octadecyl acetate in the secretion of *C. aenea* from Japan.[11] They did not observe such compounds in *Phaedon brassicae* or *Plagiodera versicolora distincta*. Similar compounds were present in the secretion of *C. aenea* from Belgium, but not in the secretion of *Gastrophysa viridula*, *Plagiodera versicolora* and *Chrysomela populi*.

So far, salicylaldehyde was only detected in the secretion of several *Chrysomela* sp. and *Phratora (= Phyllodecta) vitellinae*, all feeding on *Salix* or *Populus*. On the other hand, *Gastrolina depressa* feeding on walnut produces juglone.[17]

β-phenylethyl isobutyrate and β-phenylethyl 2-methylbutyrate (average ratio 1:4) were identified in a single species, *Chrysomela interrupta*, in the secretion of which they constitute more than 90% of the observed volatiles.[16]

Finally, HCN and benzaldehyde characterize the secretion of three Australian Paropsini feeding on *Eucalyptus*. They were found together with glucose, which suggests that they derive from a mandelonitrile glucoside. They are apparently synthesized by the insects, rather than derived directly from the food plant.[5] Benzaldehyde was also found in *Chrysomela vigintipunctata costella* but in admixture with salicylaldehyde.[17]

Chemical defence in adults. In adults, the defence glands are clusters of gland cells opening in files at the surface of the pronotum and elytra.[18] A large survey[2] covering most of the subfamilies demonstrated that the glands are present only in four subfamilies, Criocerinae, Chrysomelinae, Alticinae and Galerucinae which, according to Jolivet[19] belong to a single phyletic line. The precise distribution of the glands varies from species to species, but they are always most developed along the lateral margins. In some species belonging to the Alticinae and Galerucinae, the glands are lost. In these two subfamilies alternative defensive mechanisms have evolved: jumping for the flea-beetles and reflex bleeding for the Galerucinae.

When the beetles are disturbed, the secretion oozes from the glandular openings and covers the integument. The secretion appears to be most abundant in the Chrysomelinae, in which it accumulates in marginal grooves

Table 3. Classes of defensive compounds in adult Chrysomelinae

	Cardenolides •	Amino-acid † derivatives	Saturated † hydrocarbons	Isoxazolin-5-one † glucosides
CHRYSOLININI				
Chrysolina coerulans	++	+	+	-
Leptinotarsa decemlineata	-	+	+	-
PHAEDONINI				
Chrysomela tremulae	-	-	++	++
Chrysomela populi	-	-	++	++
Gastrophysa viridula	-	-	++	++
Prasocuris phellandrii	-	-	NT	++
Hydrotassa marginella	-	-	NT	++
Phaedon brassicae	-	-	NT	++
PHRATORINI				
Phratora laticollis	-	-	NT	++
Phratora tibialis	NT	-	NT	++
Phratora vitellinae	-	-	NT	++
Goniocotena rufipes	-	-	NT	-

NT : not tested

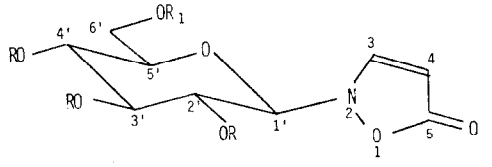
• : for the distribution of cardenolides in the Chrysolinini see ref. 20 and 21

† : this work

where it can easily be "milked". Chemical analyses were only undertaken in species belonging to this subfamily.

The different classes of compounds recognized so far are listed in Table 3.

Cardenolides were detected in the secretion of 12 species of *Chrysolina*, *Chrysochloa* and *Diochrysa* among the Chrysolinini, feeding on a large array of food-plants devoid of cardenolides.[20,21] So far, sarmentogenin, bipendogenin and their corresponding xylosides were identified in the secretions of *Chrysolina coerulans* and *C. herbacea*,[21,22] as well as sarmentogenin in the secretion of *C. didymata*.[21]



(8) R = R_1 = H

(9) R = H ; R_1 = C(=O)H - CH_2 - CH_2 - NO_2

(10) R = R_1 = Ac

(11) R = Ac ; R_1 = C(=O)H - CH_2 - CH_2 - NO_2

In contrast with the Chrysolinini, the defensive secretion of the Phaedonini is characterized by the presence of isoxazolin-5-one glycosides. The structure determination of 2 of these compounds (**8** and **9**) first found in *Chrysomela tremulae*, is described hereunder.

The more polar compound **8** from the defensive secretion of *C. tremulae* (Experimental) was identified as 2 - (β - D - glucopyranosyl) - 3 - isoxazolin - 5 - one,[23,24] on the basis of the following data. Its IR spectrum displays bands at 3400 cm^{-1}(ν_{OH}), 1740 cm^{-1} ($\nu_{C=O}$) and 1550 cm^{-1} (heterosubstituted double bond), whereas its UV spectrum (λ_{max} 260 nm, ϵ = 6500) is characteristic of an N - substituted - 3 - isoxazolin - 5 - one system.[25]

The ^{1}H NMR spectrum of **8** (Table 4) was particularly informative, showing besides the signals of a β-glucopyranosyl moiety, two doublets at 8.42 and 5.31 ppm, with a small coupling constant (3.5 Hz), which are diagnostic peaks for the 3-isoxazolin-5-one heterocycle.[25,26] These conclusions are corroborated by the ^{13}C NMR spectrum of **8** (Table 5). The EI-MS of **8** shows no peak attributable to a molecular ion, but a strong peak is present at *m/z* 84 (isoxazolin-5-one).

Treatment of **8** with Ac_2O-pyridine afforded tetraacetate **10**, resulting from acylation of the C-2', C-3', C-4' and C-6' OH groups of the sugar, as demonstrated by comparison of the ^{1}H NMR spectra of **8** and **10**. It follows that the aglycone must be attached at C-1' of the sugar. In the CI-MS of **10** (isobutane), the peak of highest mass number appears at m/z 416 and was attributed to $(M+H)^+$. This was confirmed by D/CI-MS analysis,[27] using NH_3 as the reactant gas, which afforded peaks at m/z 433 $(M+NH_4)^+$ and 416 $(M+H)^+$. These results point to a molecular weight of 415 dalton for **10**, corresponding to an empirical formula of $C_{17}H_{21}O_{11}N$.

Hence compound **8** has an empirical formula of $C_9H_{13}O_7N$. The presence of a glucose moiety in **8** was further indicated[28] by peaks at *m/z* 331 [M-84(isoxazolin-5-one)], 271, 211, 169 and 109 in the CI-MS spectrum of **10**, and unambiguously demonstrated by acid hydrolysis of **8**. The resulting sugar was identified as glucose by silylation (GC analysis) and by acetylation (GC and MS analyses). Since **8** contains both a glucose and a 3-isoxazolin-5-one heterocycle, it must be identical with 2 - (β-D-glucopyranosyl)-3-isoxazolin-5-one, previously isolated from *Lathyrus odoratus* seedlings.[23,24] Indeed, comparison of the spectral properties of both compounds shows that they are identical.

The major, less polar compound **9** from the defensive secretion of *C. tremulae*, was shown to be 2 - [6' - (3'' - nitropropanoyl) - β - D - glucopyranosyl] - 3 - isoxazolin - 5 - one, mainly on the basis of spectral data.

Indeed, the presence in **9** of the N-substituted 3-

Table 4. ^{1}H NMR spectra of **8**, **9**, **10**, **11** and reference compounds

	HC-3	HC-4	HC-1'	HC-2'	HC-3'	HC-4'	HC-5'	H_aC-6'	H_bC-6'	H_2C-2''	H_2C-3''
8 (CD_3OD)	8.42, d	5.31, d	4.90, d	3.74, t	3.44, t	3.32, t	3.40, m	3.64, dd	3.84, dd	-	-
	(3.5)	(3.5)	(9)	(9,9)	(9,9)	(9,9)	(9,5,2)	(12,5)	(12,2)		
9 (CD_3OD)	8.40, d	5.35, d	4.9, d	3.75, t	3.45, t	3.30, t	3.58, ddd	4.20, dd	4.45, dd	3.01, t	4.70, t
	(3.5)	(3.5)	(9)	(9,9)	(9,9)	(9,9)	(9,6,2)	(12.5,6)	(12.5,2)	(6)	(6)
10 ($CDCl_3$)	7.98, d	5.48, d	~5.30 *		~5.05 *		3.77, ddd	4.24, dd	4.06, dd	-	-
	(3.7)	(3.7)						(12,5)	(12,2)		
11 ($CDCl_3$)	8.01, d	5.49, d	~5.35 *	~5.35 *	~5.00 * and ~5.10 *		3.77, m	~4.23, m **		3.02, t	4.66, t
	(4.1)	(4.1)	(9)							(6)	(6)
β-methylgluco-pyranoside (32) (D_2O)	-	-	4.38, d	3.26, dd	3.51,dd	3.38,dd	3.47, ddd	3.73	3.93	-	-
			(7.9)	(7.9,9.1)	(9.1,9)	(9,9.8)	(9.8,5.8,2)	(12,5.8)	(12,2)		
3-nitropropanoic acid ($CDCl_3$)	-	-	-	-	-	-	-	-	-	3.05, t	4.65, t
										(6)	(6)

* These 4 protons appear as an ABCD system.

** These 2 protons appear as the AB part of an ABX system.

Table 5. ^{13}C NMR spectra of **8**, **9** and reference compounds in D_2O

	C-3	C-4	C-5	C'-1'	C-2'	C-3'	C-4'	C-5'	C-6'	C-1''	C-2''	C-3''
8	157.0	93.3	177.0	91.0	72.3	78.9	71.8	81.0	63.3	-	-	-
9	157.3	94.0	177.0	91.8	72.1	78.8	71.9	78.4	66.3	174.5	33.7	73.0
β-methylgluco-pyranoside [33]	-	-	-	104.5	74.6	77.3	71.2	77.3	62.4	-	-	-
3-nitropropanoic acid ($CDCl_3$)	-	-	-	-	-	-	-	-	-	175.1	30.8	69.3

isoxazolin-5-one and the glucose unit of **8** was immediately apparent from its IR, UV, ^1H and ^{13}C NMR spectra (Experimental and Tables 4 and 5).

As in the case of **8**, no molecular ion was obtained by EI-MS of **9**. In the D/CI-MS spectrum[27] of **9**, the highest peaks were observed at 322, 319, 305 and 302 corresponding to $(304+NH_4)^+$, $(301+NH_4)^+$, $(304+H)^+$ and $(301+H)^+$ respectively. This observation suggests that even this soft ionization technique[27] did not afford the molecular ion. Brief treatment of **9** with Ac_2O/pyridine afforded a triacetate (**11**),† arising from acylation of C-2', C-3' and C-4' of the sugar (see ^1H NMR spectra of **9** and **11**, Table 4). D/CI-MS of **11** (NH_3) afforded ions at 492 $(M+NH_4)^+$ and 475 $(M+H)^+$, i.e. a molecular weight of 474 dalton and an empirical formula of $C_{18}H_{22}O_{13}N_2$ which fits all the other spectral data. It follows that **9** has a molecular weight of 348 dalton, corresponding to $C_{12}H_{16}O_{10}N_2$, and that it differs from **8** by $C_3H_3O_3N$. This fragment was identified as a 3-nitropropanoyl group on the following evidence. The peak at *m/z* 302 in the D/CI-MS spectrum of **9** corresponds to the loss of 47 mass units (HNO_2) from the undetected $(M+H)^+$ peak at *m/z* 349. A loss of 47 dalton has also been observed in the CI-MS of 3-nitropropanoylglucosides from plant origin.[29] The presence of a nitro group in **9** was further substantiated by a positive Gries-Ilosvay test[30] and strong IR bands at 1550 and 1380 cm^{-1}.[31] The ^1H and ^{13}C NMR spectra of **9** clearly indicate the existence of a 3-nitropropanoyl moiety which must be attached at C-6' of the sugar. Indeed, two characteristic CH_2 signals coupled to each other appear at 4.70 ppm (CH_2–NO_2, t, J = 6 Hz) and 3.01 ppm (CO–CH_2, t, J = 6 Hz) in the ^1H NMR spectrum of **9**, whereas its ^{13}C NMR spectrum shows signals at 174.50 (C=O), 73.00 (CH_2–NO_2) and 33.70 ppm CH_2–CO. These values are in complete agreement with those expected for a 3-nitropropanoyl group.[29] Moreover, the CH_2 group at 4.70 ppm exhibits a marked acidity since its two hydrogen atoms are readily exchanged by deuterium when the NMR spectrum is run in D_2O in the presence of the mildly basic DSS as internal reference.

Finally, a comparison between the ^1H NMR spectra of **8** and **9** and their acetylated derivatives **10** and **11** clearly demonstrates that the primary hydroxyl group at C-6' of the glucose is acylated by the 3-nitropropanoyl group. Consequently, **9** is thus 2 - [6' - (3'' - nitropropanoyl) - β - D - glucopyranosyl] - 3 - isoxazolin - 5 - one.

The major compound **9** was further detected in the secretions of all Phaedonini examined so far, as well as in all *Phratora* sp. from the Phratorini, but not in the secretion of *Gonioctena rufipes* belonging to this last tribe (Table 3). The presence of **8** was only detected with certainty in the secretion of *Gastrophysa viridula*, *Chrysomela tremulae* and *C. populi*. No traces of compounds **8** and **9** were detected in the leaves of *Rumex obtusifolius* and *Populus* sp., food plants of *Gastrophysa viridula* and *C. populi* and *tremulae* respectively. To our knowledge, isoxazolin-5-one derivatives have been found only in some Leguminosae, such as *Pisum sativum* and *Lathyrus odoratus*,[25] whereas 3-nitropropanoic acid glycosides are widely distributed in other Leguminosae genera (e.g. *Astragalus*, *Indigofera*).[29,30,34,35]

Table 3 also shows that other differences exist between the Chrysolinini and the Phaedonini in the composition of their defensive secretion. Indeed, both groups secrete mixtures of unidentified saturated hydrocarbons. However, the amounts found in the secretions of both *C. tremulae* and *C. populi* largely exceed those found in *C. coerulans* and other Chrysolinini. Furthermore, amino-acid derivatives have been found in the Chrysolinini (*C. coerulans*, *C. herbacea*, *C. hyperici*, *L. decemlineata*) and never in the Phaedonini. These compounds behave as amino-acids or small peptides in tlc and show a positive reaction with ninhydrin. Two of them (from *C. coerulans* and *L. decemlineata*) afforded essentially glutamic acid after 6N HCl hydrolysis. The structure of these derivatives is currently under investigation.

DISCUSSION

From Tables 1 and 3, it is clear that major differences are observed in the chemistry of defensive secretions between different tribes. The Phaedonini appear as an homogeneous taxon, the defensive secretions of the adults being characterized by isoxazolin-5-one glycosides and those of the larvae dominated by methylcyclopentanoid monoterpenes. Exceptions to this rule could be interpreted as a secondary adaptation to particular host-plants. For example, salycilaldehyde found in the secretion of *Salix* or *Populus*-feeders could be derived from salicin, whereas juglone produced by *Gastrolina depressa*[17] could easily be sequestered from the walnut on which it feeds. We have identified glucose in the larvae secretion of *Chrysomela tremulae* and *C. populi*, thus reinforcing this hypothesis. Incorporation experiments are underway to demonstrate the origin of salicylaldehyde unambiguously.

†With longer reaction times, the initially formed triacetate is transformed into more polar compounds. The same behaviour was observed for other nitroderivatives (e.g. miserotoxin[34]).

The Chrysolinini look less homogeneous, but too few genera have been studied so far to draw any conclusions.

A major taxonomic consequence of this study is the fact that the tribe Phratorini appears to be an unnatural entity. The genus *Phratora* (= *Phyllodecta*) is undoubtedly closely related to the Phaedonini, as demonstrated by their larval defence glands and the chemistry of the adult secretion, which is not the case for the genus *Gonioctena* (= *Phytodecta*).

Some results remain poorly understood. What is the biological significance of the differences observed between larval and adult secretions within the same species, both stages being found close to each other on the same plant? We would like to suggest that the secretions are principally aimed at different kinds of predators, vertebrates like birds for the adults and arthropods like ants for the larvae. Indeed, the aposematic coloration of the adults favours the idea that they are protected against birds. On the other hand, larval secretions were often reported to be potent repellents for ants.[8,10,14,16,17] Another intriguing question concerns the differences in the composition of defensive mixtures from closely related species living in the same biotope, or from different populations of the same species. Have they any ecological sense or are they the mere result of non-consequent genetic drift? Only a detailed analysis of the functioning of the communities in which these species live could bring some answer to these questions.

EXPERIMENTAL

The following instruments were used for measuring the physical data: IR: Pye-Unicam SP 1000; UV: Perkin–Elmer 137; ^{1}H NMR: Bruker HFX 270; ^{13}C NMR: Bruker WP60; GC/MS: Finnigan 3000 D; MS: Micromass 7070 F; DCI/MS: Varian MAT 311A and Varian MAT 44S; GC: Hewlett–Packard 402.

(A) *Larvae*

The larvae secretions were collected with bits of filter paper, dropped in hexane and stored at −20°. After filtration the solution was analyzed by GC [Column I: 10% Carbowax 20M (1.10 m) at 170°; Column II: 5% DEGS (1.80 m) at 150°] and tlc (silica gel; eluent: hexane/acetone 7:3; spray reagent: phosphomolybdic acid). Rt = Rt plagiolactone/Rtx; R_f = distance of spot centre from start point/distance of solvent front from start point.

Chromatographic behaviour of the methylcyclopentanoid monoterpenes

	Rt (GC I)	Rt (GC II)	R_f (tlc)
Chrysomelidial (**1**)	0.83	0.74	0.34
Plagiolactone (**4**)	1	1	0.42
Plagiodial (**3**)	1.68	1.60	0.37

Gastrophysa viridula. GC column I: 1 peak at Rt 0.83; GC column II: 1 peak at Rt 0.74; tlc: 1 spot at R_f 0.34. The mass spectrum obtained by GC/MS is identical to the one described for **1**.[6,7] Pure chrysomelidial was obtained by column chromatography on silica gel (eluent: pentane/acetone 85:15). ^{1}H NMR (270 MHz, $CDCl_3$, TMS): 3H d (J = 7.5 Hz) at 0.89 ppm, 3H bs at 2.19 ppm, 1H ddq at 3.10 ppm, 1H s at 9.73 ppm and 1H d (J ≈ 1 Hz) at 10.02 ppm. A weak doublet attributable to small amounts (~10%) of epichrysomelidial is observed at 1.09 ppm. On standing, chrysomelidial is transformed into a more polar derivative (R_f = 0.12): IR: broad ν_{OH} from 3300 to 2500 cm^{-1}, $\nu_{C=O}$ at 1725, 1710 and 1664 cm^{-1}; ^{1}H NMR: 3H d (J = 7 Hz) at 0.93 ppm and 1H ddq at 3.28 ppm.

Plagiodera versicolora. GC column I: 2 peaks at Rt 1 and 1.68; GC column II: 2 peaks at Rt 1 and 1.60; tlc: 2 spots at R_f 0.37 and 0.42. The mass spectrum obtained by GC/MS for the more polar peak (Rt = 1) is identical to that of **4**.[6] The one obtained for the less polar peak (Rt = 1.68) is identical to that of **3**.[11] Pure **3** and **4** were obtained by column chromatography on silica gel (eluent: pentane/acetone 85:15).

Compound **3**. ^{1}H NMR (270 MHz, $CDCl_3$, TMS): 3H d (J = 7.5 Hz) at 1.13 ppm, 3H s at 1.72 ppm, 1H ddq at 2.48 ppm, 1H bs at 5.54 ppm, 1H d (J = 2.5 Hz) at 9.88 ppm and 1H s at 9.94 ppm.

Compound **4**. ^{1}H NMR (idem): 3H d (J = 7.5 Hz) at 1.31 ppm, 3H s at 1.79 ppm, 1H ddq at 2.46 ppm, 1H bs at 5.76 and 6.53 ppm.

Hydrothassa marginella. GC column II: 2 peaks at Rt 1 and 0.74; tlc: 2 spots at R_f 0.42 and 0.34.

Phaedon cochleariae. GC column I: 1 peak at Rt 0.83; tlc: 1 spot at R_f 0.34.

Prasocuris phellandrii. GC column I: 1 peak at Rt 1.68; tlc: 1 spot at R_f 0.37.

Chrysomela aenea. GC column II: 2 peaks at Rt 1.6 and 1; tlc: 2 spots at R_f 0.37 and 0.42.

Individual variations of the methylcyclopentanoid monoterpene content of *Plagiodera versicolora* was determined by GC of the crude secretion of 10 specimens on DEGS (5%) at 150°. The plagiodial/plagiolactone ration was estimated after the heigth of the peaks.

(B) *Adults*

Adult *C. tremulae* were "milked" with bits of filter papers and the secretion was stored in MeOH. After filtration, a partition between MeOH–H_2O–hexane (40:10:50) afforded a separation between polar and lipid material. IR, MS and NMR of the latter show that it is a complex mixture of saturated hydrocarbons. Similar mixtures were found in the defensive secretions of other chrysomelids (Table 3). Tlc of the polar fraction (CH_2Cl_2–MeOH–H_2O 80:19:1) shows the presence of four spots (detected by UV at 254 nm) with R_f = 0.11, 0.39, 0.57, 0.62 respectively. Compound **9** (R_f = 0.39) is the major one, accompanied by variable amounts of compound **8** (R_f = 0.11). The presence of **8** and **9** in the defensive secretion of other chrysomelids (Table 3) was checked by tlc, using the same conditions as above. The identification of **9** in the secretion of *C. populi* was further confirmed by IR and NMR. Compounds **8** and **9** from *C. tremulae* were isolated by flash chromatography on SiO_2. The two less polar compounds could not be obtained in sufficient amounts for chemical study.

Compound **8**. $C_9H_{13}O_7N$, amorphous solid; EI-MS: highest peak at *m/z* 172, 156, 125, 115, 84, 60, 44; IR: ν_{OH} 3400 cm^{-1}, $\nu_{C=O}$ 1740 cm^{-1}; UV: λ_{max} 260 nm (ϵ = 6500); ^{1}H NMR: see Table 4; ^{13}C NMR: see Table 5.

Compound **9**. $C_{12}H_{16}O_{10}N_2$, amorphous solid; D/CI-MS (NH_3): 322 $(M-CO_2+NH_4)^+$, 319 $(M-HNO_2+NH_4)^+$, 305 $(M-CO_2+H)^+$, 302 $(M-HNO_2+H)^+$, 258, 204; IR: ν_{OH} 3400 cm^{-1}, $\nu_{C=O}$ 1740 cm^{-1}, $\nu_{C=C}$ and νas_{NO_2} 1550 cm^{-1}, νs_{NO_2} 1380 cm^{-1}; UV: λ_{max} 260 nm (ϵ = 6500); ^{1}H NMR: see Table 4; ^{13}C NMR: see Table 5.

Acetylation of **8**

Compound **8** (10 mg) was treated at room temp with a mixture of pyridine (2 ml) and Ac_2O (1 ml) for 24 hr. Usual work up and purification by silica gel column chromatography afforded tetraacetate **10** (15 mg).

Compound **10**: $C_{17}H_{21}O_{11}N$ amorphous solid; CI-MS (isobutane): 416 $(M+H)^+$, 390, 374 (M^+-CH_2CO), 331 (M^+-isoxazolin-5-one), 314, 271 (331-AcOH), 254, 227, 213, 211 (331-2xAcOH), 194, 169, 109, 85; IR: no ν_{OH}, $\nu_{C=O}$ 1740 cm^{-1}, $\nu_{C=C}$ 1550 cm^{-1}, ν_{C-O} 1215 cm^{-1}; ^{1}H NMR: see Table 4.

Acetylation of **9**

Compound **9** (10 mg) was acetylated as **8** but only for 2 hr, yielding triacetate **11** (6 mg). With longer reaction times, the initially formed **11** is transformed into more polar compounds, which were not investigated.

Compound **11**: $C_{18}H_{22}O_{13}N_2$ amorphous solid; D/CI-MS

(NH_3): 492 $(M+NH_4)^+$, 475 $(M+H)^+$, 433, 390 (M^+-isoxazolin-5-one), 330, 310, 289, 272, 254, 228, 194; IR: no ν_{OH}, $\nu_{C=O}$ 1750 cm^{-1}, $\nu_{C=C}$ and νas_{NO_2} 1550 cm^{-1}, νs_{NO_2} 1380 cm^{-1}, ν_{C-O} 1220 cm^{-1}; ^1H NMR: see Table 4.

Acid hydrolysis of **8**

Compound **8** was refluxed in a soln of 1N H_2SO_4 and MeOH (1:1) for 4 hr. After cooling, neutralization with Na_2CO_3 and evaporation of MeOH, the aqueous phase was extracted three times with $CHCl_3$. Desalting of the aqueous soln with Dowex 50 (H^+) and Dowex 1 (OH^-) resins and evaporation of the water yielded the sugar moiety which was dissolved in pyridine (1 ml). One half of this soln was treated overnight with Ac_2O (0.5 ml), the other half was silylated with HMDS (0.1 ml) and TMCS (0.05 ml). The resulting compounds were analyzed by GC (Silicone Dow 710 3 m, 180° for the acetate and UCON Polar 3 m, 170° for the TMS derivative) and by MS in the case of the acetate. They were shown to be identical with pentaacetyl- and pentaTMS-glucose respectively.

Amino-acid derivatives

The presence of amino-acid derivatives was checked in several Chrysolinini and Phaedonini by tlc (eluent: n-BuOH-AcOH-H_2O, 8:2:2 v/v) and visualization with ninhydrin (Table 3). In the cases of *L. decemlineata* and *C. coerulans* the ninhydrin positive compound was hydrolyzed with 6N HCl for 24 hr and the resulting mixture analyzed on a Biotronic LC2000. In both cases, glutamic acid was by far the major amino-acid detected.

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REFERENCES

[1]Maître de Recherche du Fonds National de la Recherche Scientifique Belge.
[2]C. Deroe and J. M. Pasteels, *J. Chem. Ecol.* **8**, 67 (1982).
[3]G. Garb, *J. Entom. Zool.* **8**, 88 (1915).
[4]A. C. Hollande, *Ann. Univ. Grenoble* **21**, 459 (1909).
[5]B. P. Moore, *J. Austr. Ent. Soc.* **6**, 36 (1967).
[6]J. Meinwald, T. H. Jones, T. Eisner and K. Hicks, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2189 (1977).
[7]J. Meinwald and T. H. Jones, *J. Am. Chem. Soc.* **100**, 1883 (1978).
[8]M. S. Blum, J. B. Wallace, R. M. Duffield, J. M. Brand, H. M. Fales and E. A. Sokoloski, *J. Chem. Ecol.* **4**, 47 (1978).
[9]T. H. Jones, M. S. Blum and H. M. Fales, *Tetrahedron Letters* 1701 (1980).
[10]F. Sugawara, K. Matsuda, A. Kobayashi and K. Yamashita, *J. Chem. Ecol.* **5**, 635 (1979).
[11]F. Sugawara, K. Matsuda, A. Kobayashi and K. Yamashita, *Ibid.* **5**, 929 (1979).
[12]F. Sugawara, A. Kobayashi, K. Yamashita and K. Matsuda, *Agr. Biol. Chem.* **42**, 687 (1978).
[13]M. Pavan, *Arch. Zool. It.* **38**, 157 (1953).
[14]J. B. Wallace and M. S. Blum, *Ann. Ent. Soc. Amer.* **62**, 503 (1969).
[15]R. L. Wain, *Ann. Rep. Agr. Nat. Res. Sta.* 108 (1943).
[16]M. S. Blum, J. M. Brand, J. B. Wallace and H. M. Fales, *Life Sci.* **11**, 525 (1972).
[17]K. Matsuda and F. Sugawara, *Appl. Ent. Zool.* **15**, 316 (1980).
[18]C. Deroe and J. M. Pasteels, *Arch. Biol. Bruxelles* **88**, 289 (1977).
[19]P. Jolivet, *Act. Zool. Path. Antverpiensia* **70**, 167 (1978).
[20]J. M. Pasteels and D. Daloze, *Science* **197**, 70 (1977).
[21]D. Daloze and J. M. Pasteels, *J. Chem. Ecol.* **5**, 63 (1979).
[22]J. M. Pasteels, D. Daloze, W. van Dorsser and J. Roba, *Comp. Biochem. Physiol.* **63C**, 117 (1979).
[23]L. van Rompuy, F. Lambein, R. de Gussem and R. van Parys, *Biochem. Biophys. Res. Comm.* **56**, 199 (1974).
[24]F. Lambein, L. van Rompuy, A. de Bruyn and R. van Parys, *Arch. Int. de Physiol. et Biochim.* **82**, 187 (1974).
[25]F. Lambein, Y. H. Kuo and R. van Parys, *Heterocycles* **4**, 567 (1976).
[26]L. van Rompuy, N. Schamp, N. de Kimpe and R. van Parys, *J. Chem. Soc.* P.I., 2503 (1973).
[27]K. Hostettman, J. Doumas and M. Hardy, *Helv. Chim. Acta* **64**, 297 (1981).
[28]K. Biemann, D. C. de Jongh and H. K. Schnoes, *J. Am. Chem. Soc.* **85**, 1763 (1963).
[29]F. R. Stermitz, W. T. Lowry, E. Ubben and I. Sharifi, *Phytochem.* **11**, 3525 (1972).
[30]M. C. Harlow, F. R. Stermitz and R. D. Thomas, *Ibid.* **14**, 1421 (1975).
[31]K. Nakanishi, *Infrared Absorption Spectroscopy*, p. 50. Holden-Day, San Francisco, (1962).
[32]A. de Bruyn, M. Anteunis and P. Kovac, *Coll. Czech. Chem. Comm.* **42**, 3057 (1977).
[33]T. Usui, N. Yamaoka, K. Matsuda, K. Tuzimura, H. Sugiyama and S. Seto, *J. Chem. Soc.* P.I., 2425 (1973).
[34]F. R. Stermitz, W. T. Lowry, N. A. Norris, F. A. Buckeridge and M. C. Williams, *Phytochem.* **11**, 2117 (1972).
[35]R. A. Finnegan, W. H. Mueller and M. P. Morris, *Proc. Chem. Soc.* 182 (1963).