Cardiac Glycosides in the Defensive Secretion of Chrysomelid Beetles: Evidence for Their Production by the Insects

Abstract. *The defensive secretions of some chrysomelid beetles belonging to the genera* Chrysolina, Chrysochloa, *and* Dlochrysa *contain complex mixtures of cardenolides. The spectral data for some of these compounds suggest that they are monohydroxylated digitoxigenin derivatives linked to a pentose (such as xylose or arabinose). Evidence indicates that the beetles do not sequester these steroid glycosides from their host plants.*

Chemical defense has been studied in several chrysomelid larvae (*1*), but somewhat neglected in adults (*2*). Many chrysomelid beetles, however, are brightly colored, and this suggests that they should be chemically protected. Reflex bleeding is frequent within the family and defensive glands are present in some species. Moreover, unpalatability or toxicity for various predators has been demonstrated in some instances (*3*). We report here the discovery of cardenolides in the defensive secretion of adults from several chrysomelid species and present evidence that these compounds are not sequestered from their food plants.

In many chrysomelid beetles, the defensive glands are located along the external edges of the elytra and at the anterior corners of the pronotum. In some species, a few defensive glands are also more centrally distributed in the elytra (*4*). When disturbed, the insect discharges copious amounts of secretion, which can be easily collected on bits of filter paper (Fig. 1). Most of our chemical analyses were performed on the defensive secretion stored in methanol; for some species, only methanolic extracts of whole beetles were available (Table 1).

The presence of cardenolides was assessed by silica gel thin-layer chromatography (TLC) (*5*), followed by spraying with Kedde reagent (*6*). In most cases, further identification was performed by using mass spectrometry on the total secretion or on purified compounds and, whenever possible, by infrared, ultraviolet, and nuclear magnetic resonance spectroscopy (Table 1) (*7*). All mass spectra exhibit the typical fragmentation pattern of cardenolides, with peaks at mass-to-charge ratio (*m/e*) 354, 336, and 321. The majority of the mass spectra also show intense peaks for fragments at *m/e* 244 and 201, suggesting that these compounds are monohydroxylated digitoxigenin derivatives (*8*). Mass spectra of acetylated compounds were obtained for four species (Table 1). They all show, besides the fragmentation of the acetylated aglycone, strong peaks at *m/e* 259, 199, 139, and 97, suggesting the presence of a triacetylated pentose (*9*). Indeed, xylose was identified as the only sugar of the two main glycosides of *Chrysolina coerulans* and arabinose as the main sugar of *Dlochrysa fastuosa* glycosides. This was established by acid hydrolysis followed by silylation and mixed injections in gas-liquid chromatography on a 3 percent OV-17 column at 120°C.

Inotropic activity was demonstrated for the main cardenolide of *C. coerulans*. The observed activity on electrically stimulated left atria of guinea pigs was of the same order of magnitude as that of ouabain (*10*).

So far, cardenolides were detected only in species belonging to the three related genera *Chrysolina*, *Dlochrysa*, and *Chrysochloa*. While each species is strongly specialized in its feeding habit (*11*), the total spectrum of food plants of the beetles in which cardenolides were found is quite large and includes plants from three different families, Labiatae, Compositae, and Scrofulariaceae (Table 1).

Thin-layer chromatographic analyses have shown that cardenolides are always found as complex mixtures, characteristic of the beetle species, whatever the beetles' diet. Beetles collected or bred on different host plants secrete the same spectrum of cardenolides—for example, *C. coerulans* feeding on *Mentha spicata* subsp. *glabrata*, *Mentha longifolia*, and *Mentha X villosa* (*12*), and *Chrysolina polita* feeding on *M. X villosa* or *Lycopus europaeus*. But different species collected on the same host plant show a different TLC pattern of cardiac glycosides—for example, *C. coerulans*, *C. herbacea*, and *C. polita* feeding on *M. X villosa*.

Cardiac glycosides are well-known constituents of the Scrofulariaceae, belonging to the genus *Digitalis*, but have

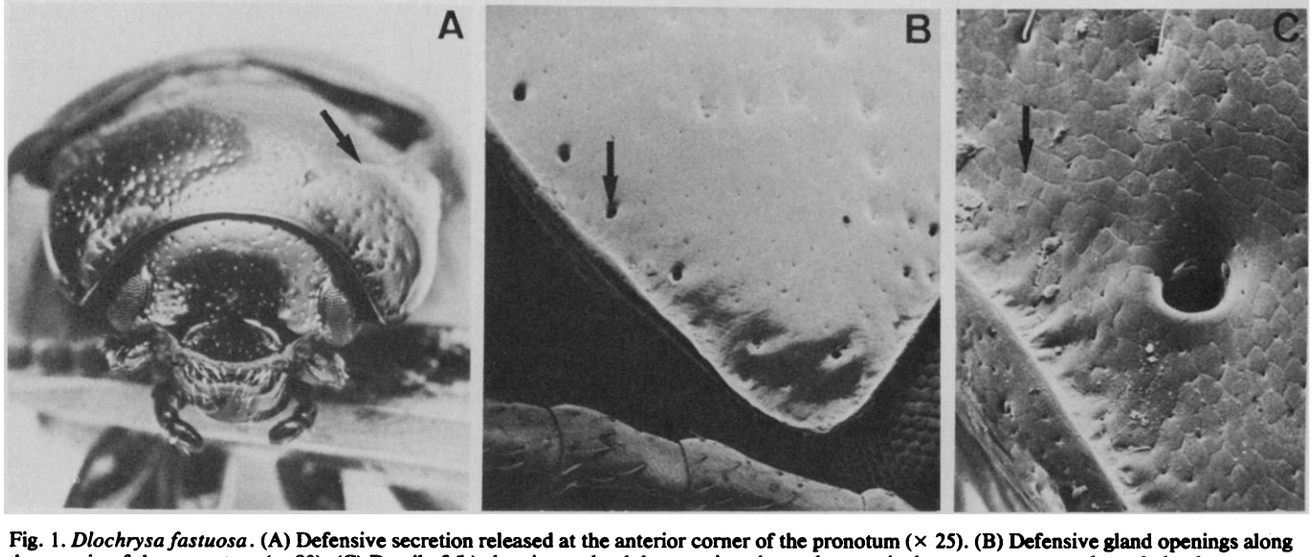

Fig. 1. *Dlochrysa fastuosa*. (A) Defensive secretion released at the anterior corner of the pronotum (× 25). (B) Defensive gland openings along the margin of the pronotum (× 80). (C) Detail of (b) showing a glandular opening above the marginal grove; numerous dermal gland pores are also apparent (arrow) (× 400). (B) and (C) are scanning electron micrographs; the dried specimens were coated with gold and examined with a Stereoscan scanning electron microscope.

not been reported from *Linaria* (*13*). They have also been described in a few Compositae (*13*) and tentatively in two Labiatae species (*14*), but in none of the food plants of the beetles found to secrete cardenolides. Some of these plants, such as mint or rosemary, are commonly used in cookery, and it is doubtful that they are very toxic. This suggests that the beetles might not take the cardenolides from their food plants. This was confirmed by the following experiment.

Chrysolina coerulans and *C. polita* were reared in the laboratory from the egg on *M. X villosa*. Both the secretion of the beetles and the leaves of the plants used as food were checked for cardenolides. The latter were easily detected in the secretion of a single beetle, but no Kedde-positive compound with infrared and mass spectra characteristic of cardenolides could be detected in an extract of 750 g (fresh weight) of leaves. Similarly, cardenolides could be detected neither in *Rosemarinus officinalis*, on which *C. americana* was found feeding, nor in *Galeopsis tetrahit*, the food plant of *D. fastuosa* (*15*).

Kedde-positive substances were detected in the eggs of several *Chrysolina* including *C. coerulans* and *C. polita*. The occurrence of cardenolides in the eggs has been reported for the grasshopper *Poekilocerus bufonius* (*16*) and the bug *Oncopeltus fasciatus* (*17*) feeding on milkweeds. In *Oncopeltus* the cardenolide content of the egg is not imparted to the newly hatched first-instar larva. But in *Poekilocerus* it has been suggested that such a maternal influence could at least partly explain the occurrence of cardenolides in hoppers fed on lettuce (*16*). Such a transfer from one generation to another cannot explain the content of cardiac glycosides in the *Chrysolina* feeding on plants devoid of them. First, as already stated, these oligophagous insects were never observed or reported on plants known to contain cardiac glycosides. They are, however, common and spectacular species, frequently collected by professional or amateur entomologists. Second, a colony of *C. herbacea* lives on the *M. X villosa* used in our feeding experiment. These beetles do secrete cardenolides absent in their food plant, on which they have probably fed for many generations. Finally, the quantity of Kedde-positive material present in one egg is insufficient to explain the amount found in adults bred on *M. X villosa*. More than one egg of *C. polita* is required to extract a quantity that can be detected by TLC. The extracts of two to five eggs sometimes give weakly positive and sometimes negative results, and only faint positive spots could be obtained from extracts of ten eggs. In contrast, the extracts of the secretion of single adults (15 days old) issued from eggs of the same parents and reared on *M. X villosa* always gave very distinct spots in TLC. The TLC patterns of eggs and adult extracts are different (*18*).

Cardiac glycosides have been detected in a number of insects feeding on Asclepiadaceae, Apocynaceae, or Scrofulariaceae (*Digitalis*), and it is considered that they are sequestered from the food plant (*2, 16, 17, 19*), and sometimes somewhat modified by the insects (*17*). Chrysomelid beetles seem to be the first example of insects that do not sequester their cardiac glycosides from their food and, thus, must synthesize them (*20*).

As far as we know, the steroid portions of these glycosides are related or perhaps even identical to those of some plant cardenolides (monohydroxylated digitoxigenins). The sugars, however, seem unusual. Arabinose has never been described as a constituent of plant cardenolides, and xylose has been so described on only one occasion (*21*).

The capability of insects to metabolize their dietary sterols into complex steroids is well documented. Two striking examples are the moulting hormones (*22*) and the defensive steroids (mainly pregnane derivatives) of the water beetles (*23*). It is thus most likely that chrysomelid beetles use dietary precursors such as sterols to synthesize the aglycone moiety of their cardiac glycosides.

The only three *Chrysolina* species found until now to be devoid of cardeno-

Table 1. Distribution of cardenolides in Chrysomelidae. Methods of identification are TLC, thin-layer chromatography; KR, Kedde reagent (*6*); IR, infrared spectroscopy; UV, ultraviolet spectroscopy; MS, mass spectrometry; MSAC, mass spectrometry of the acetylated compound; and NMR, nuclear magnetic resonance. The number detected is *N*.

Species	Material analyzed	Method	*N*	Food plant
		Criocerinae		
Lema cyanella	Total extract	TLC + KR	0	Gramineae sp.
Lema melanopus	Total extract	TLC + KR	0	Gramineae sp.
Lilioceris lilii	Total extract	TLC + KR	0	*Lilium sp.* (Liliaceae)
		Chrysomelinae		
Leptinotarsa decemlineata	Secretion	TLC + KR	0	*Solanum tuberosum* (Solanaceae)
Chrysolina coerulans	Secretion and total extract	TLC + KR, IR, UV, MS, MSAC, NMR	7*	Several *Mentha sp.* (Labiatae)
Chrysolina herbacea	Secretion	TLC + KR, MS, MSAC	8	*Mentha X villosa* (Labiatae)
Chrysolina polita	Secretion	TLC + KR, MS	8	*Mentha X villosa, Lycopus europaeus* (Labiatae)
Chrysolina grossa	Total extract	TLC + KR, MS	4	Labiatae sp.
Chrysolina americana	Total extract	TLC + KR, MS	2	*Rosemarinus officinalis* (Labiatae)
Chrysolina cerealis	Secretion	TLC + KR, MS, MSAC	5	Labiatae sp.
Chrysolina sanguinolenta	Secretion	TLC + KR	6	*Linaria vulgaris* (Scrofulariaceae)
Chrysolina hyperici	Secretion	TLC + KR	0	*Hypericum sp.* (Hypericaceae)
Chrysolina brunsvicensis	Secretion	TLC + KR	0	*Hypericum sp.* (Hypericaceae)
Chrysolina varians	Secretion	TLC + KR	0	*Hypericum sp.* (Hypericaceae)
Chrysochloa cacaliae	Secretion	TLC + KR	4	*Adenostyles sp.* (Compositae)
Dlochrysa fastuosa	Secretion and total extract	TLC + KR, MS, MSAC, IR, UV	6	*Galeopsis tetrahit* (Labiatae)
Gastroidea viridula	Secretion	TLC + KR	0	*Rumex sp.* (Polygonaceae)
Hydrothassa marginella	Secretion	TLC + KR	0	*Ranunculus sp.* (Ranunculaceae)
Phytodecta olivacea	Secretion	TLC + KR	0	*Sarothamnus scoparius* (Papilionaceae)
Phyllodecta laticollis	Secretion	TLC + KR	0	*Populus trichocarpa XPh. deltoides* (Salicaceae)

*The total was about 30 μg per beetle.

lides live on *Hypericum* (Table 1). The absence of cardenolides in these species could be explained if the host plant lacks the necessary precursors or if the beetles do not possess the enzymatic systems needed for such synthesis.

JACQUES M. PASTEELS
DÉSIRÉ DALOZE
Biologie Animale and Chimie Bioorganique, Université Libre de Bruxelles, Brussels 1050, Belgium

References and Notes

1. R. L. Wain, *Annu. Rep. Agric. Hortic. Res. Stn. Univ. Bristol.*, 108 (1943); M. Pavan, *Arch. Zool. Ital.* **38**, 157 (1953); B. P. Moore, *J. Aust. Entomol. Soc.* **6**, 36 (1967); T. H. Hsiao and G. Fraenkel, *Toxicon* **7**, 119 (1969); J. B. Wallace and M. S. Blum, *Ann. Entomol. Soc. Am.* **62**, 503 (1969); M. S. Blum, J. M. Brand, J. B. Wallace, H. M. Fales, *Life Sci.* **11**, 525 (1972).
2. M. Rothschild, *Symp. R. Entomol. Soc. London* **6**, 59 (1972); see table 1.
3. L. Cuénot, *Arch. Zool. Exp. Gen.* **4**, 655 (1896); W. L. Tower, *Carnegie Inst. Washington Publ.* **42**, 320 (1906); A. Ch. Hollande, *Arch. Anat. Microsc. Morphol. Exp.* **13**, 171 (1911); F. M. Jones, *Trans. R. Entomol. Soc. London* **80**, 345 (1932).
4. C. Deroe, unpublished results.
5. Thin-layer chromatography was performed on Macherey-Nagel precoated sil-G plates, using Ch_2Cl_2, methanol, and H_2O (85:14:1) as the eluent.
6. Cardenolides appear as purple spots when sprayed first with 2 percent methanolic *m*-dinitrobenzoic acid and then with 5 percent methanolic KOH [Kedde reagent: D. L. Kedde, *Pharm. Weekbl.* **82**, 741 (1947); I. E. Bush and D. A. H. Taylor, *Biochem. J.* **52**, 643 (1952); M. L. Lewbart, W. Wehrli, T. Reichstein, *Helv. Chim. Acta* **46**, 505 (1963)].
7. For infrared and ultraviolet spectra of cardenolides see K. Yamaguchi, [*Spectral Data of Natural Products* (Elsevier, Amsterdam, 1970)]; for nuclear magnetic resonance spectra of cardenolides see K. Tori and K. Aono [*Shionogi Kenkyusho Nempo* **15**, 130 (1965)].
8. P. Brown, F. Brüschweiler, T. Reichstein, *Helv. Chim. Acta* **55**, 531 (1972); M. B. E. Fayez and S. A. R. Negm, *J. Pharm. Sci.* **61**, 765 (1972).
9. N. K. Kochetkov and O. S. Chizhov, *Adv. Carbohydr. Chem.* **21**, 39 (1966).
10. We thank J. Roba (Continental Pharma) for performing these experiments; the details will be reported elsewhere (J. Roba, in preparation).
11. P. Jolivet and E. Petitpierre, *Ann. Soc. Entomol. Fr.* **12**, 123 (1976).
12. The systematics of the genus *Mentha* is confusing for a nonspecialist because of the existence of numerous hybrids. *Mentha X villosa* Huds is a hybrid of *M. spicata* L. and *M. suaveolens* Ehrh. (= *rotundifolia* auct.). The notomorph used is *alopecuroides* (Hull) [J.-E. De Langhe, L. Delvosalle, J. Duvigneaud, J. Lambinon, C. Vanden Berghen, *Nouvelle Flore de la Belgique, du Grand-Duché de Luxembourg, du Nord de la France et des Régions voisines* (Patrimoine Jardin Botanique National de Belgique, Brussels, 1973)].
13. R. Hegnauer, *Chemotaxonomie der Pflanzen* (Birkhäuser, Basel, 1964–1973).
14. T. V. Zinchenko, *Farm. Zh. Kiev* **14**, 47 (1959). Hegnauer (*13*) reported the occurrence of cardenolides in *Leonurus cardiaca*. In fact, in the original paper the compounds were described as bufadienolides [O. E. Schultz and H. J. Haack, *Arzneim. Forsch.* **11**, 830 (1961)].
15. *Mentha X villosa* (750 g, fresh weight), *R. officinalis* (450 g, dried), and *G. tetrahit* (300 g, dried) were screened for cardenolides by the classical procedure of Reichstein [for example, see M. Rothschild, J. von Euw, and T. Reichstein, *J. Insect Physiol.* **16**, 1141 (1970) → *Proc. R. Soc. London Ser. B* **183**, 227 (1973)].
16. J. von Euw, L. Fishelson, J. A. Parsons, T. Reichstein, M. Rothschild, *Nature (London)* **214**, 35 (1967).
17. S. S. Duffey and G. G. E. Scudder, *Can. J. Zool.* **52**, 283 (1974).
18. More details about the qualitative and quantitative variations in the content of cardenolides during the life cycle of these insects will be given elsewhere (J. M. Pasteels and D. Daloze, in preparation).
19. T. Reichstein, *Naturwiss. Rundsch.* **20**, 499 (1967); L. P. Brower and S. C. Glazier, *Science* **188**, 19 (1975).
20. In the animal kingdom, only some toads are known to synthesize true cardenolides, beside the well-known bufadienolides [N. Höriger, H. H. A. Linde, K. Meyer, *Helv. Chim. Acta* **53**, 1503 (1970); N. Höriger, D. Zivanov, H. H. A. Linde, K. Meyer, *ibid.*, p. 2051; Y. Fujii, K. Shimada, T. Nambara, *Chem. Ind. (London)* (1976), p. 614].
21. T. Reichstein and E. Weiss, *Adv. Carbohydr. Chem.* **17**, 65 (1962); I. F. Makarevich, M. Ya Tropp, D. G. Kolesnikov, *Dokl. Akad. Nauk SSSR* **136**, 617 (1961).
22. W. E. Robbins, J. N. Kaplanis, J. A. Svoboda, M. J. Thompson, *Annu. Rev. Entomol.* **16**, 53 (1971).
23. H. Schildknecht, *Angew. Chem. Int. Ed. Engl.* **9**, 1 (1970).
24. We thank T. Reichstein for much helpful advices and for generous gifts of reference compounds. M. Rothschild critically reviewed the manuscript. Thanks are due also to M. Colin and C. Moulard for technical assistance.

20 December 1976; revised 16 February 1977

Freeze-Fractured Purple Membrane Particles: Protein Content

Abstract. *Optical diffraction and image reconstruction can be used to correlate the electron microscope image of the biological membrane with its electron density projection. Such correlation shows that a single purple membrane particle contains 9 to 12 protein molecules—63 to 84 transmembrane alpha helices—a complexity two to ten times greater than that previously suggested for membrane particles.*

Current models of biomembranes show protein intercalated in the lipid bilayer (*1*). The interest in structural details of membrane proteins led to the extensive use of freeze-fracture as a tool to investigate the interior of the membrane bilayer (*2*). This use presupposes that membranes split upon freeze-fracture (*3*) and that the particles seen represent protein (*2*). But the question of what constitutes a "particle" in situ remains unknown, in part because of lack of sufficient structural information about individual membrane-associated proteins.

Recently a membrane protein, bacteriorhodopsin, was described in three dimensions, at 7-Å resolution, by computer analysis of electron diffraction patterns and corresponding micrographs (*4*). Bacteriorhodopsin is a 26,000-dalton polypeptide with covalently bound retinal (*5*) found in *Halobacterium halobium* (*6*). It has an absorption maximum at 570 nm and functions as a light-driven proton pump (*7*). Multiple copies of this protein are associated in vivo in the plasma membrane, in regular arrays that can be visualized by freeze-fracture electron microscopy (*8*).

By combining freeze-fracture with diffraction techniques, we can define the polypeptide content of a particle. We measure particle dimensions and examine packing geometry in freeze-fracture electron micrographs, and obtain the particle lattice by optical filtration. We then verify that the primary structure of the membrane polypeptide after freeze-fracture is the same as in native membrane (that is, no major covalent bonds are broken) and compare the particle image to the filtered lattice. If those lattice points can be superimposed on the previously derived planar electron density projection (*9*), one can relate the particles seen in freeze-fracture to the 7-Å map.

Conventional freeze-fractured preparations (*10*) of light-grown and oxygen-starved cells of *H. halobium* (*11*) reveal quasi-crystalline aggregates of particles in the plane of the membrane half (P face) closest to the cytoplasm (Fig. 1, a and b). Particles can be identified (Fig. 1a) as irregular accumulations of electron-scattering material bordered on one side by a crescent-shaped electron-transparent region (shadow). The complementary half (E face) is devoid of particles but shows a fine lattice at favorable shadow angles.

Particles were selected at random from electron micrographs of fractured *H. halobium* cells at calibrated magnifications of 600,000 ×. Their dimensions were measured with an optical comparator, using lines drawn normal to the shadowing direction. Purple membrane particles were 11.9 ± 2.6 nm wide (mean ± standard deviation, $N = 50$) and adjacent nonaggregated red membrane particles 10.4 ± 1.3 nm wide ($N = 50$; uncorrected for shadowing material). The purple particles were arranged in rows offset from each other by one-half particle width (Fig. 1a). No distinct hexagonal patterns were observed.

Because fractures pass through the cells at many angles, both low-angle and high-angle shadows can be found in a single replica. The low-angle regions, identified by long shadows or minimal Pt-C deposition, are especially useful for detection of particle substructure (Fig. 1b). Scrutiny of such areas reveals subparticle grains of Pt-C occasionally arranged hexagonally (Fig. 1b, circles). Whether this preferential accumulation of Pt-C is primarily due to "decoration" (*12*) or to true shadowing is not known. Our interest, however, was not to exam-