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ALDEHYDIC CONTACT POISONS AND ALARM PHEROMONE OF THE ANT *Crematogaster scutellaris* (Hymenoptera: Myrmicinae) Enzyme-Mediated Production from Acetate Precursors

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Abstract—The Dufour gland of *Crematogaster scutellaris* stores a mixture of long-chain primary acetates bearing a cross-conjugated dienone (Scheme 1, 1a–c). The poison gland contains two highly active enzymes: an acetate esterase and an alcohol oxidase. During venom emission, the constituents of both glands mix and accumulate on the sting, where the formation of the highly electrophilic aldehydes (Scheme 1, 2a–c) from their acetate precursors is initiated. Acetic acid, produced during the reaction, acts as alarm pheromone. The toxicity of the acetates (Scheme 1, 1a–c) and of the crude secretion has been assessed by topical application on *Myrmica rubra*. The acetate-containing secretion from the Dufour gland was less toxic than the enzymatically altered secretion that was rich in aldehydes. The production of acids (Scheme 1, 3a–c) was an artifact resulting from the nonenzymatic oxidation of the unstable aldehydes.

Key Words—*Crematogaster scutellaris*, ants, Hymenoptera, Formicidae, Dufour gland, contact poisons, esterase, alcohol oxidase, alarm pheromone, aldehydes.

INTRODUCTION

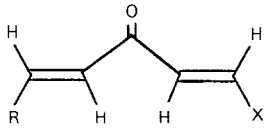
Ants of the genus *Crematogaster* are characterized by a spatulate sting, which is not a suitable injection device (Buren, 1958). In many species, the venom is emitted as a froth that accumulates on the spatulate portion and at the base of the sting, from whence it can be applied easily to the integument of attacking insects. Application is further facilitated by the fact that the abdominal tip can be pointed in nearly all directions. Indeed, *Crematogaster* species are remarkable among the ants by their ability to raise the abdomen forward and over the thorax and head. The hypertrophied Dufour gland of *Crematogaster,* which is connected to the sting, is considered to be the origin of the venom (Maschwitz, 1975; Buschinger and Maschwitz, 1984). During venom emission, an alarm pheromone is also emitted, which mediates a general excitement (i.e., frenzied alarm) in the aggregations of workers inside and outside the nest (Leuthold and Schlunegger, 1973).

In a preceding paper, we reported that the venom of *C. scutellaris* contained a series of C_{23} long-chain derivatives, characterized by the presence of a cross-conjugated dienone linked to either a primary acetate (Scheme 1, 1a–c), an aldehyde (Scheme 1, 2a–c), or a carboxylic acid function (Scheme 1, 3a–c). The aldehydes 2a–c are powerful electrophiles and could be responsible for the toxicity of the venom (Daloze et al., 1987). However, the aldehydes are highly unstable and cannot be stored as such in the Dufour gland. We report here that the Dufour gland of *C. scutellaris* only stores the acetates 1a–c and that aldehyde production is triggered at the moment of discharge of the esters to the exterior by mixing with enzymes located in the simultaneously discharged fluid of the poison gland. This enzymatic reaction also liberates acetic acid, which is responsible for the alarm reaction observed in grouped ants. The production of homologous acids 3a–c is an artifact resulting from nonenzymatic oxidation of the aldehydes 2a–c.

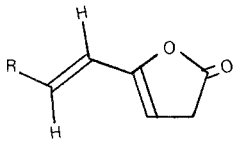
METHODS AND MATERIALS

Biological Material. Portions of dead pine logs containing ant colonies were collected in south of France near Salernes and Banyuls. These colonies could be maintained in these pieces of log for several years in the laboratory, merely by feeding them continuously with a solution of brown sugar and freshly killed cockroaches.

Chemical Analyses. The venom of workers was collected as described previously (Daloze et al., 1987) on bits of filter paper that were immediately dropped into *n*-hexane or CH_2Cl_2. For the kinetic studies, the venom was collected in the same manner, but the filter papers were maintained at 19 $\pm$ 1°C



	R	X		R	X
1a	R^1	CH_2OAc	3a	R^1	COOH
1b	R^2	CH_2OAc	3b	R^2	COOH
1c	R^3	CH_2OAc	3c	R^3	COOH
2a	R^1	CHO	4a	R^1	CH_2OH
2b	R^2	CHO	4b	R^2	CH_2OH
2c	R^3	CHO	4c	R^3	CH_2OH



	R
5a	R^1
5b	R^2
5c	R^3

$R^1 = CH_3-(CH_2)_5-CH=^{Z}CH-(CH_2)_9-$

$R^2 = CH_3-(CH_2)_7-CH=^{Z}CH-(CH_2)_7-$

$R^3 = CH_3-(CH_2)_5-CH=^{Z}CH-CH=^{Z}CH-(CH_2)_7-$

SCHEME 1.

for 0, 5, 10, or 15 min before being dipped into CH_2Cl_2. The resulting solutions were analyzed by photodensitometry using TLC plates (Merck Silicagel G F 254); eluent 1: hexane–acetone 8:2; eluent 2: CH_2Cl_2. The compounds were made visible by quenching of the UV fluorescence of silica. Absorbances were measured at 250 nm on a Shimadzu CS 930 dual-wavelength TLC scanner. Percentages given in Table 1 are mean values, obtained by averaging the values of three replications. Similar assays were performed under a nitrogen atmosphere. Enzyme specificity assays were conducted in 0.05 M phosphate buffer (pH 7.0), containing 0.1% of Tween 60 as detergent. One or 2 μg of the acetate substrate was suspended into 20 μl of the buffer solution, and two poison glands were added. After sonication for 2 min, the solution was incubated for 20 min at 20°C, then extracted twice with 5 μl of CH_2Cl_2. The organic extract was spotted on a TLC plate and analyzed for the presence of alcohol or aldehydes.

As previously reported (Daloze et al., 1987), neither the major constituents

TABLE 1. KINETICS OF PRODUCTION OF ALDEHYDES WHEN DUFOUR GLAND AND POISON GLAND WERE CRUSHED TOGETHER

Time (min)	Acetates (%)[a] (1a–c)	Aldehydes (%)[a] (2a–c)
0	100	0
5	52.8 ± 6.5	47.2 ± 6.5
10	41.0 ± 8.5	59.0 ± 8.5
15	22.2 ± 4.4	77.8 ± 4.6

[a] Mean and standard deviation of three replications.

(a–c) of each fraction (1–3) of the secretion nor the corresponding alcohols (4a–c) produced by the enzyme under nitrogen can be separated from each other by TLC. The alcohols 4a–c were isolated by preparative TLC (eluent 1), and identified by mass spectrometry (Micromass VG 7070). Acetic acid was identified by GLC (25-m OV-1 capillary column, Varian 3700) after derivatization with *N*-methyl-*N*-*t*-butyldimethylsilyl trifluoroacetamide (Schooley et al., 1985) and coinjection with a similarly derivatized authentic sample.

RESULTS

Variation in Proportions of Constituents in Stored Venom. Variations in the proportions of the chemical constituents in the venom depend on how the venom was handled after its collection on paper. Two factors are particularly critical: (1) the nature of the storage solvent, and (2) the time elapsed between emission of the venom and its immersion in solvent. Mixtures of 1a–c, 2a–c, and 3a–c were always found when the storage solvent was *n*-hexane or when the venom was immersed in the solvent 5 or 10 min after its emission. In contrast, only acetates 1a–c were detected by TLC when the venom was placed in CH_2Cl_2 at the very instant of discharge (Figure 1). These observations strongly suggest that *C. scutellaris* uses a defensive mechanism involving the storage of relatively stable acetates and the emission of highly reactive electrophilic aldehydes via enzymatic conversion.

Storage of Precursor in the Dufour Gland. Dufour glands of workers were dissected in H_2O and the glandular fluid briefly extracted with CH_2Cl_2. TLC analyses demonstrated the presence of acetates 1a–c only. The same results were obtained when the dissected glands were crushed on bits of filter paper and kept in contact with air for several hours before extraction. Thus, the acetates are the only precursors stored in the Dufour gland. Their transformation into aldehydes must be mediated by catalysts from another origin.

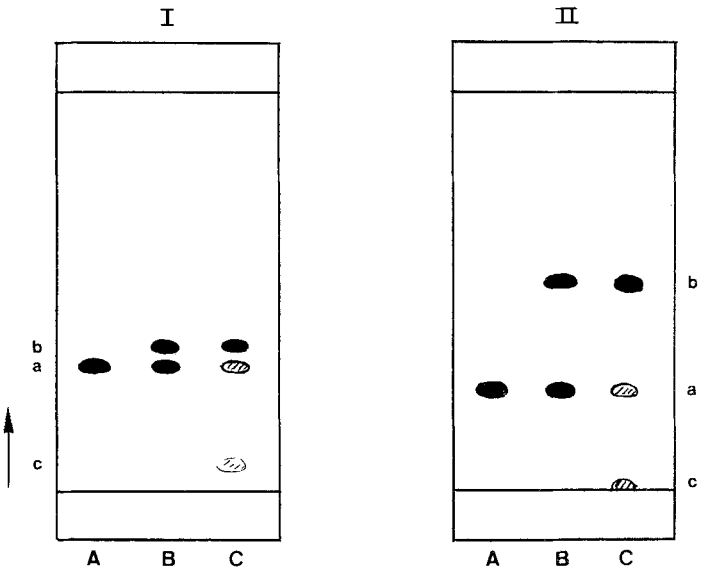


FIG. 1. Redrawn thin-layer chromatographic plates showing the kinetics of ageing of the venom in open air: (A) venom immediately immersed in CH_2Cl_2; (B) venom immersed in CH_2Cl_2 after 5 min; (C) venom immersed in CH_2Cl_2 after 15 min; (a) acetates (1a–c); (b) aldehydes (2a–c); (c) acids (3a–c); I, eluent: hexane–acetone, 8:2; II, eluent: CH_2Cl_2.

Poison Gland as Source of Enzymes Responsible for Aldehyde Production. In contrast to the above-mentioned results, when dissected Dufour and poison glands were crushed together on a piece of filter paper, left to sit for 15 min, and subsequently extracted with CH_2Cl_2, TLC analyses showed the presence of mixtures of acetates (1a–c) and aldehydes (2a–c) accompanied by traces of acids (3a–c) (Figure 2). Crushing Dufour glands with pieces of fat body only led to the recovery of the acetates. Thus, the poison gland appears to contain the requisite enzymes to transform the Dufour gland acetates into the corresponding aldehydes. This type of transformation requires two enzymes, an acetate esterase and an alcohol oxidase. The evidence presented below shows that both kinds of enzymes are operative in the defensive secretion of *C. scutellaris*. The fact that only traces of acids were observed suggest that these are not formed by an enzyme-mediated process, but rather by air oxidation of the highly reactive aldehydes. This reactivity is further demonstrated by a spontaneous rearrangement that will be described at the end of this paper.

Identification of Acetic Acid in Secretion. The hydrolysis of acetates 1a–c, triggered by the acetate esterase should produce acetic acid and the alco-

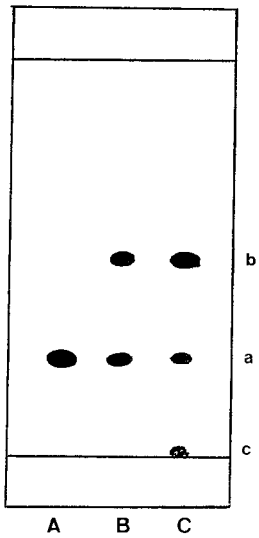


FIG. 2. Redrawn TLC (eluent: CH_2Cl_2) showing the pattern obtained by: (A) extraction of dissected Dufour gland after 15 min; (B) extraction of dissected Dufour and poison gland crushed together for 5 min; (C) as in B for 15 min; (a) acetates (1a–c); (b) aldehydes (2a–c); (c) acids (3a–c).

hols 4a–c. Venom emission by workers is always accompanied by a characteristic odor of acetic acid. The presence of acetic acid in venom samples was indeed demonstrated by GLC of its *t*-butyldimethylsilyl derivative and was always correlated with the presence of the aldehydes (2a–c). The other products of acetate hydrolysis, namely the alcohols 4a–c could never be isolated from crude venom extracts, presumably because their oxidation into the aldehydes is too fast. The alcohols, however, could be trapped under nitrogen.

Accumulation of Alcohols (4a–c) under Nitrogen. The venom, collected on pieces of filter paper, was immediately placed under a nitrogen atmosphere. Under these conditions, TLC analyses of the venom extracts showed that after 1 hr, about 30% of the acetates was transformed into a more polar fraction (Figure 3). The R_f of the latter suggested that it could be the corresponding alcohols (4a–c). Preparative TLC (eluent 1) afforded 0.5 mg of this fraction whose EI-MS ($M^{+\cdot}$ at *m/z* 348 and 346; fragments at *m/z* 330, 328, 320, 318, 302, 300, . . .) confirmed it contained the alcohols 4a–c.

This result demonstrated that the aldehydes arise from the alcohols under the action of an oxygen-dependent alcohol oxidase. The marked slow-down of acetate hydrolysis under nitrogen (see below and Table 1) may be ascribed to the well-known phenomenon of product inhibition (Mahler and Cordes, 1969),

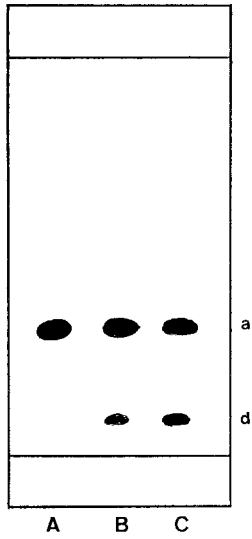


FIG. 3. Redrawn TLC (eluent: hexane–acetone, 8:2) showing the aging of the venom under N_2: (A) venom immediately immersed in CH_2Cl_2; (B) venom after 20 min; (C) venom after 1 hr; (a) acetates (1a–c); (d) alcohols (4a–c).

because the alcohols were not removed from the reaction medium by the oxidase, which is inhibited under N_2.

Semiquantitative Analysis of Kinetics of Aldehyde Production. The venom, collected on bits of filter paper, was kept in air and dipped in CH_2Cl_2 at different time intervals. TLC analyses of the resulting solutions were performed by photodensitometry at 260 nm. Typical results are shown in Figure 1, and the variations of the percentages of acetates and aldehydes with time are given in Table 1. Furthermore, if the same experiments were run with *n*-hexane as storage solvent, mixtures always resulted, even if attempts were made to stop the transformation at time zero. These results show that the enzyme is still active in hexane. Under our experimental conditions, 47.2% ± 6.5 of the acetates were metabolized into the aldehydes in 5 min (Table 1).

Substrate Specificity of Acetate Esterase. Enzyme activity was first determined *in vitro* on the natural substrates 1a–c in 0.05 M phosphate buffer, in the presence of poison gland extracts. Under these conditions, about 50% of 1a–c were metabolized into the alcohols 4a–c in 20 min. The same experiment performed on *n*-octadecylacetate afforded small amounts of octadecanol, thus showing that the esterase of *C. scutellaris* is not strictly substrate-specific. No aldehydes were produced under these conditions.

Spontaneous Rearrangement of Aldehydes (2a–c). We would like to dis-

cuss here the structure of an artifact that is repeatedly obtained during storage and handling of the secretion of *C. scutellaris*. This artifact, which is less polar than the other derivatives, is obtained in good yields by chromatography of the secretion under daylight on Sephadex LH 20 as described previously (Daloze et al., 1987). It must be derived from the aldehydic fraction (2a–c), since the latter disappears during the chromatographic process.

The spectroscopic properties of the artifact [MS: $M^{+\cdot}$ at *m/z* 346 and 344; UV: λ max 235 nm; IR: ν C=0 1800 cm^{-1}; [^{1}H] NMR: δ 6.20, 1H, δt, 15.8 and 7 Hz; δ 5.89, 1H, d, 15.8 Hz; δ 5.35, 2H, t, J = 4.7 Hz; δ 5.20, 1H, t, J = 2.6 Hz; δ 3.27, 2H, m; characteristic signals of a (Z,Z)-diene are also present at δ 6.24, bd, and 5.43, m] strongly suggest its components should have structures 5a–c. This hypothesis was confirmed by decoupling experiments in [^{1}H]NMR ($J_{2,3}$ = 2.5 Hz; $J_{5,6}$ = 15.8 Hz; $J_{6,7}$ = 6.5 Hz; $J_{2,5}$ = 2.0 Hz; $J_{3,5}$ = 1.0 Hz) and by comparison of the spectral properties of 5a–c with those of model compounds [e.g., α-angelica lactone (Gagnaire and Payo-Subiza, 1963; Hirsch and Szur, 1972)]. The formation of this type of lactone from 4-oxo-2-enals has been well documented (Hirsch and Szur, 1972; Macleod et al., 1977).

Biological Activities of Secretion. Toxicity was measured by counting dead ants (*Myrmica rubra*) observed 48 hr after topical application of 0.1 μl of hexane solution of the compounds. It was not possible to measure the toxicity of pure aldehydes because of their instability. Thus, the toxicity of pure acetates dissolved in hexane was compared to that of the crude secretion kept in dry air during 20 min before its extraction with hexane. The aldehydic content of this secretion was not precisely determined, but substantial amounts of aldehydes together with acetates and acids were detected by TLC (see also Table 1). Only five ants of 20 were found dead 48 hr after 30 μg of the acetates per ant was applied, whereas 19 ants of 20 were dead or paralyzed when the same amount of crude secretion was applied ($P < 0.00001$, Fisher exact probability test). No mortality was observed with pure hexane. It is important to note that 30 μg is about half of what can be collected as a mean in one *Crematogaster* ant by gently squeezing it between fingers (Daloze et al., 1987). Thus, the secretion was more toxic after the enzymatic transformation than before transformation when the acetates alone were present.

C. scutellaris feeding in groups are excited and dispersed in the presence of acetic acid vapors, as demonstrated by placing a bit of filter paper impregnated with acetic acid on a platform 1 cm above ants feeding at its basis.

DISCUSSION

The cooperation between the Dufour and the poison glands in the production of topical poison and alarm pheromone is unique in ants. However, it is worth mentioning that the content of the poison gland of many hymenopterans, includ-

ing ants, is rich in enzymes, among others, esterases (Schmidt, 1982), and the esterase needed to hydrolyze the acetates stored in the Dufour gland is not strongly specific. The uniqueness of the *Crematogaster* system arises more from the particular chemistry of the Dufour gland secretion than from the enzymatic participation of the poison gland. In most ants, the Dufour gland secretion is lipophilic but usually contains much simpler and lighter aliphatic derivatives or sesquiterpenes (Blum and Hermann, 1978; Attygalle and Morgan, 1984).

Similar defensive strategies based on the storage of stable precursors in specialized reservoirs and their transformation by enzymes into highly reactive compounds have already been reported in other arthropods (e.g., Eisner et al., 1963; Schildknecht et al., 1968; Aldrich et al., 1978). The enzymatic transformation of aliphatic acetates into aldehydes by an acetate esterase and an oxygen-dependent alcohol oxidase has been well documented recently in the biosynthesis of aldehydic moth pheromones (Morse and Meighen, 1984, 1986). The enzymes of *Crematogaster* are still active when dissolved in hexane, a well-known property of esterases and related enzymes (Tanaka et al., 1981, Fukui and Tanaka, 1985). However, catalytic activity was totally lost in CH_2Cl_2, thus indicating that catalysis is due to an enzyme rather than an organic catalyst.

The alarm reaction induced by the vapor of acetic acid is certainly not specific and can be induced by many "nonspecific irritants" (sensu Eisner, 1972), such as volatile acids, aldehydes, or terpenes (Boevé, 1988). However, since acetic acid is produced during poison emission and since it is the only volatile compound detected in the secretion, it is most probably responsible of the alarm pheromonal function of the secretion described by Leuthold and Schlunegger (1973). A more refined analysis of this alarm communication would require a quantification of the emission rate of acetic acid during the enzymatic reaction, which is beyond the scope of this paper.

As expected, the precursor acetates were found to be less toxic than the secretion enriched with the strongly electrophilic aldehydes. The enzymatic transformation of acetates into aldehydes is far from being instantaneous. In our experiments, only half of the acetates was transformed 5 min after their secretion, and significant amounts of acetates still persisted after 15 min. This slow reaction could be in part an artifact of our collecting process, during which we tried to collect as much material as possible by gently squeezing the ants between our fingers. During normal emission, less acetates could be secreted at once and more quickly transformed. However, a "delayed" defense offers obvious advantages, especially since the toxin is unstable. The total conversion of esters to aldehydes in an instant would be an all-or-nothing strategy, because the aldehydes would quickly loose their activity by spontaneous oxidation to acids and rearrangement. On the other hand, a foe contaminated by a mixture that progressively releases its toxins, while penetrating the organism, will be handicapped for a longer time.

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