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Chemical defense in the three European species of *Crematogaster* ants

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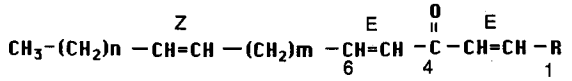
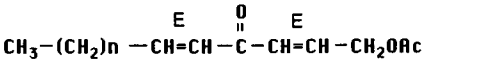
Abstract. The composition of the Dufour gland of the ant *C. scutellaris* has been reinvestigated by gas chromatography/mass spectrometry. The major components of the gland are (2E,5E,12Z)-4-oxoheneicosa-2,5,12-trien-1-ol acetate (**1a**) its Δ^{14} and Δ^{16} double bond isomers (**1b** and **1c**), and the corresponding (Z,Z)-dienes **5a** and **5b**, all containing an acetylated C_{21} chain. The previously proposed structures **1d**, **1e**, and **5c**, which are based on an homologous acetylated C_{23} chain, correspond to minor derivatives present in the gland. Traces of acetylated C_{19} homologs, tentatively identified as **1g–1i**, have also been found. The Dufour gland contents of the two other European *Crematogaster* species have also been studied. *C. auberti* is very similar to *C. scutellaris* in producing mainly **1a**, **1b** and **1c**, together with the same higher and lower homologs, but it lacks the dienic derivatives **5**, whereas *C. sordidula* contains essentially the acetylated C_{19} compounds **1g**, **1h**, and **1i**, accompanied by acetylated C_{17} homologs.
Key words. Ants; *Crematogaster*; Dufour gland; chemical defense; electrophilic contact poisons.

Crematogaster ants are characterized by a peculiar defense strategy. Instead of injecting their venom as most primitive ant species do, they use their spatulate sting to apply their venom topically to the integument of attacking insects. In a previous paper[1], we reported that the venom of *C. scutellaris*, collected on bits of filter paper and stored in hexane, was an exceedingly complex mixture of unstable compounds. The latter were only partially separated by Sephadex LH-20 chromatography, affording five major fractions named CS1 to CS5 in order of increasing polarity. The compounds of one fraction differed from those of the other fractions by the nature of the functional group at the end of the chain: aldehyde (CS1), acetate (CS2), and carboxylic acid (CS5). In all compounds, a cross-conjugated dienone was linked to the terminal functional group. The minor fraction CS3 polymerised immediately after its isolation, whereas CS4 was an artifact[1]. Each fraction was still a mixture of compounds differing by the number or position of the central double bond(s) in the carbon chain. The most stable acetate fraction, CS2, was shown by HPLC[1] to consist of at least six components. Two of the major compounds of this fraction were assigned the molecular formula $C_{25}H_{42}O_3$ and structures **1d** and **1e** (fig. 1), differing only by the position of the central double bond in the chain. A third major derivative was shown by ^{1}H NMR to contain a Z,Z-diene and was assigned the molecular formula $C_{25}H_{40}O_3$ and, tentatively, structure **5c**[1] (fig. 1). Further work[2] led us to discover that only the acetates (fraction CS2) were stored in the Dufour gland of *C. scutellaris*. During venom emission, the acetates were transformed into the corresponding aldehydes by two enzymes, an esterase and an oxygen-dependent alcohol oxidase, which are present in the poison gland. The aldehydes, for which structures **3d**, **3e** and **7c** (fig. 1) were proposed, are powerful electrophiles and thus responsible, at least in part, for the toxicity of the venom[2].

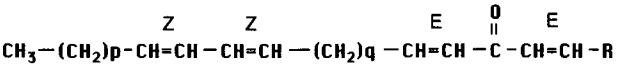
We now report on a reinvestigation of the venom constituents of *C. scutellaris*, demonstrating that the Dufour gland content of this ant is more complex than was originally found[1]. Indeed, gas chromatography-mass spectrometry analyses (GC-MS) and chemical degradations have shown that the major constituents are acetates **1a–1c** and **5a**, **5b**, containing an acetylated C_{21} chain. These compounds are accompanied by small amounts (less than 10%) of acetylated C_{23} homologs for which we propose structures **1d**, **1e**, **1f**, **5c** and **5d** and by traces of



n	m	R = CH_2OAc	R = CH_2OH	R = CHO
7	5	1a	2a	3a
5	7	1b	2b	3b
3	9	1c	2c	3c
7	7	1d	2d	3d
5	9	1e	2e	3e
3	11	1f	2f	3f
7	3	1g	2g	3g
5	5	1h	2h	3h
3	7	1i	2i	3i



n= 12; 4a
n= 14; 4b
n= 16; 4c



p	q	R = CH_2OAc	R = CH_2OH	R = CHO
5	5	5a	6a	7a
3	7	5b	6b	7b
5	7	5c	6c	7c
3	9	5d	6d	7d

Figure 1. Proposed structures for the major venom constituents of *C. scutellaris*, *C. auberti* and *C. sordidula*.

acetylated C_{19} homologs, tentatively identified as **1g–1i**. We also report the results of an analysis of the venoms of the two other European species belonging to the genus *Crematogaster*, *C. auberti* and *C. sordidula*.

Materials and methods

Biological material. Arboreal nests of *C. scutellaris* were collected by removing inhabited dead logs from pine forests in the South of France (Mont Ventoux). Ground-excavated nests of *C. auberti* were found at the same site and those of *C. sordidula* in the 'Corbières' ridge (Pyrénées Orientales). These three species were identified by one of us (J. M. P.). *C. scutellaris* ants were kept in the laboratory in pieces of dead logs. *C. auberti* and *C. sordidula* ants were kept in test tubes moistened by a water reservoir separated from the nest by a cotton plug. All these nests were maintained at a constant temperature (22 ± 1 °C) and the ants were fed with freshly killed cockroaches and a solution of brown sugar.

Venom collection. Ant workers were seized by tweezers and the venom that appeared at the tip of the sting was collected on small bits of filter paper. The latter were *immediately* dipped in dichloromethane to stop or slow down the enzymatic reactions, and the resulting solution was stored at − 18 °C.

Chemical analyses. Thin layer chromatographic analyses (TLC) were performed on precoated silica gel G plates (Macherey-Nagel, UV 254) using hexane-acetone 8:2 or hexane-diethyl ether 7:3 as eluent, and visualized under UV 254 light. The preparative TLC was run on precoated glass silica gel plates (Merck, UV 254, 5 × 10 cm, 0.5 mm thick) using the same eluents as above. The ozonolysis, epoxidation and epoxide cleavage reactions were performed as described in Daloze et al. [1], on venom samples obtained from 10–30 workers.

Capillary gas-liquid chromatographic analyses (GLC) of freshly collected venom samples were performed with nitrogen as carrier gas on a Varian 3700 apparatus equipped with:

a) a 25-m OV 1 column (Alltech), at 240 °C (5 min), programmed at 5 °C/min to 260 °C (hold).
b) a 25-m OV 1701 column (Alltech) at 200 °C (2 min), programmed at 4 °C/min to 240 °C (hold).
c) a 12-m PEG column (Alltech) at 180 °C (5 min), programmed at 5 °C/min to 225 °C (hold).

The GC-MS analyses of the venom samples and of the epoxide fractions derived therefrom were conducted on a Finnigan ion trap detector (ITD 800), coupled to a Tracor 540 gas chromatograph equipped with a 10-m SE-54 capillary column (Rescom) at 50 °C (5 min), programmed at 30 °C/min to 160 °C (3 min), programmed at 5 °C/min to 260 °C, using helium as carrier gas (15 psi). Ion intensities were recorded using chemical ionization (CI) with isobutane as the reactant gas. Data are reported in tables 1–3.

Identification of the aldehydes arising from the ozonolysis and epoxide cleavage reactions was realized by GLC:

a) on a Varian 3700 gas chromatograph equipped with:
   – a 25-m OV 1 capillary column, at 60 °C (2 min), programmed at 6 °C/min to 120 °C.
   – a 50-m capillary CPwax 52B column (Chrompack) at 120 °C (15 min), programmed at 6 °C/min to 220 °C.
b) on a Delsi D200 gas chromatograph equipped with a 2-m packed column of 10% Carbowax 20M on acid-washed Chromosorb W at 50 °C (2 min), programmed at 8 °C/min to 180 °C.

Heptanal and nonanal were identified by mixed injections with authentic samples on the three columns and by GC-CIMS (isobutane) on the SE 54 column {50 °C (3 min), programmed at 6 °C/min to 140 °C (20 min)} coupled to the ITD 800. Pentanal was identified on the Carbowax 20M packed column and on the CP wax 52B capillary column by mixed injections with an authentic sample. The aldehyde ratios given in the text are mean

Table 1. CIMS (isobutane) data for a venom sample of *C. scutellaris*

Scan No.	R_T (min)	Peak[a]	Diagnostic ions in CIMS (isobutane)	Formula	Structure
1128	21.30	A	275	$C_{21}H_{34}O_3$	**1g–1i**
1128	21.30	A	277	$C_{21}H_{36}O_3$	**4a**
1227	23.26	B	303	$C_{21}H_{36}O_2$	**2a–2c**
1270	24.16	C	301	$C_{21}H_{34}O_2$	**6a–6b**
1278	24.26	D	303	$C_{23}H_{38}O_3$	**1a–1c**
1290	24.38	D	303	$C_{23}H_{38}O_3$	**1a–1c**
1312	25.03	E	305	$C_{23}H_{40}O_3$	**4b**
1327	25.21	F	301	$C_{23}H_{36}O_3$	**5a, 5b**
1425	27.15	G	331	$C_{25}H_{42}O_3$	**1d–1f**
1478	28.17	H	329	$C_{25}H_{40}O_3$	**5c, 5d**

[a] Letters refer to figure 4.

Table 2. CIMS (isobutane) data for a venom sample of *C. auberti*

Scan No.	R_T (min)	Peak[a]	Diagnostic ions (CIMS isobutane)	Formula	Structure
1110	21.06	A	275	$C_{21}H_{34}O_3$	**1g–1i**
1130	21.22	A	277	$C_{21}H_{36}O_3$	**4a**
1219	23.02	B	321, 303	$C_{21}H_{36}O_2$	**2a–2c**
1275	24.01	C	363, 303	$C_{23}H_{38}O_3$	**1a–1c**
1291	24.13	C	363, 303	$C_{23}H_{38}O_3$	**1a–1c**
1308	24.26	C	303	$C_{23}H_{38}O_3$	**1a–1c**
1308	24.26	C	305	$C_{23}H_{40}O_3$	**4b**
1437	26.52	D	331	$C_{25}H_{42}O_3$	**1d–1f**
1437	26.52	D	333	$C_{25}H_{44}O_3$	**4c**

[a] Letters refer to figure 6.

Table 3. CIMS (isobutane) data for a venom sample of *C. sordidula*

Scan No.	R_T (min)	Peak[a]	Diagnostic ions CIMS (isobutane)	Formula	Structure
950	18.04	A	249	$C_{19}H_{32}O_3$	
			247	$C_{19}H_{30}O_3$	
1048	19.57	B	275	$C_{19}H_{32}O_2$	**2g–2i**
1086	20.42	C	275	$C_{21}H_{34}O_3$	**1g–1i**
1108	21.07	D	275	$C_{21}H_{34}O_3$	**1g–1i**
1125	21.27	D	275	$C_{21}H_{34}O_3$	**1g–1i**
			277	$C_{21}H_{36}O_3$	**4a**

[a] Letters refer to figure 7.

values obtained by combining the ratios measured on the different columns.

The 600 MHz ^{1}H NMR spectrum (table 4) was recorded on a Varian VXR 600 spectrometer on a venom sample of *C. sordidula* obtained from 80 workers as described above, but collected in $CDCl_3$ instead of CH_2Cl_2. Chemical shifts are reported in δ from tetramethylsilane as internal standard. High resolution EI mass measurements were performed on a VG Micromass 7070 F mass spectrometer.

Results and discussion

All GC-MS data reported in this paper were obtained, within 10 min of their collection, on venom samples of *C. scutellaris*, *C. auberti* and *C. sordidula*, freshly collected in dichloromethane. This method allowed us to get a better picture of the true composition of the Dufour gland secretions. Indeed, when performed within a few minutes after collection, TLC analyses consistently showed the presence of only one major spot which, on the basis of previous results[1, 2] corresponded to the acetate fraction. A minor, more polar spot, originating from the corresponding alcohol fraction (see below), was also detected in some samples. It was in fact observed that, when the dichloromethane solutions were kept in the refrigerator, the acetates were progressively hydrolyzed by the esterase of the poison gland[2] into the corresponding alcohols. This hydrolysis reaction was always faster for *C. auberti* than for the two other species. Thus, after about 48 h, *C. auberti* venom samples contained almost exclusively the alcohols. It is also worth mentioning that when the venom samples were immediately stored in dichloromethane, the aldehyde fraction was usually barely detectable by both TLC and GLC. This showed that the activity of the alcohol oxidase of the poison gland is very low in dichloromethane. In contrast, in hexane solutions[1] the oxidation of the alcohols into the aldehydes was usually so quick that the former were never detected in crude venom samples, except when the venom was allowed to age under a nitrogen atmosphere[2]. Thus, it

should be emphasized that the venom compositions are time-dependent and that the results reported here are only valid for the particular sample analyzed.

Capillary GLC trials on three different stationary phases (see Materials and methods) disclosed that the constituents of the Dufour gland secretion of *C. scutellaris* could be only partially resolved on the OV 1 and OV 1701 columns, whilst none of the sought-after compounds eluted from the PEG column. Preliminary GC-MS analyses on 25-m OV 1 and 10-m SE 54 columns led us to select the latter as offering the best overall results. However, it should be pointed out that we have not been able to find GLC conditions allowing all the constituents of these complex mixtures to be separated. Two or more compounds generally coeluted as one GLC peak as shown by the CI mass spectra (tables 1–3) or by the results of ozonolysis reactions (see below). Comparison of EI, Cl(NH_3) and Cl(isobutane) mass spectra showed that the latter consistently gave diagnostic peaks of higher intensity (compare figs 2 and 3). The latter ionisation mode was thus used in all our GC-MS analyses.

C. scutellaris. The total ion chromatogram (CIMS, isobutane) of a freshly collected venom sample of *C. scutellaris* is shown in figure 4. The CI mass spectra of the acetates (peaks D, E, F, G and H in fig. 4) were always characterized by a base peak arising from the loss of acetic acid from the $(M + H)^+$ ions (see table 1). This information, together with our previous MS and HRMS studies [1], allowed us to propose the molecular formulae reported in table 1. It turns out that all the compounds

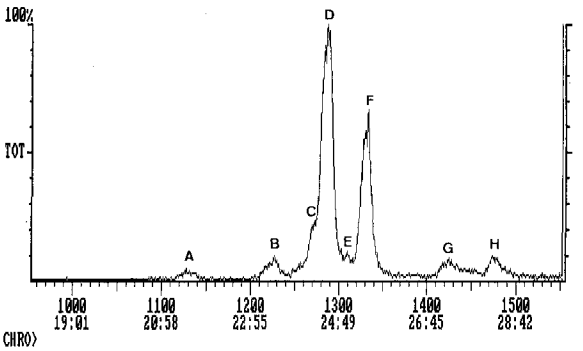


Figure 4. Total ion chromatogram of a fresh sample of *C. scutellaris* venom. Letters identify peaks whose compositions are listed in table 1.

present under peak D are isomers possessing the molecular formula $C_{23}H_{38}O_3$, whereas those present under peak F are their corresponding dehydroderivatives, which have the formula $C_{23}H_{36}O_3$. These data unambiguously demonstrate that the major components of *C. scutellaris* venom possess an acetylated C_{21} chain, whilst the minor components (peaks G and H, base peak at 331 and 329, resp. in CIMS, amounting to less than 10% of the major ones) possess the homologous acetylated C_{23} chain that was previously proposed [1]. This error is ascribable to earlier HPLC results. Indeed, by submitting the acetate fraction of the venom to HPLC, we isolated a fraction which eluted as a single peak [1]. Its EI mass spectrum showed ions at m/z 390 ($C_{25}H_{42}O_3$, by exact mass measurements), 362 ($C_{23}H_{38}O_3$, by exact mass measure-

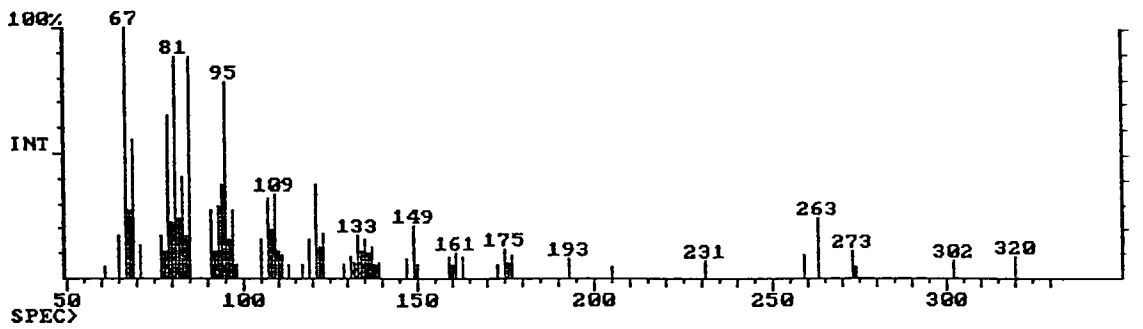


Figure 2. EI mass spectrum of compounds **1a–1c**.

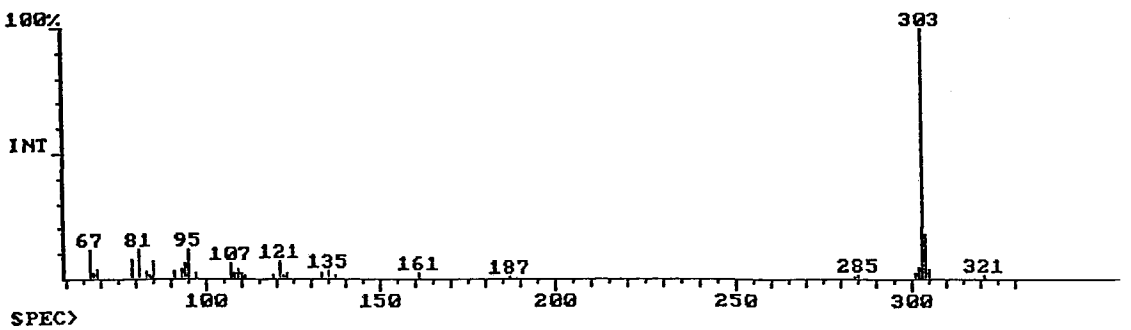


Figure 3. CI mass spectrum (isobutane) of **1a–1c** {peak D (scan n° 1290) in fig. 4}.

ments), 348, 330, 320, 302... The 28 dalton difference between m/z 390 and 362, which was attibuted to a then-unexplained loss of C_2H_4 from the m/z 390 ion [1], is now simply seen as arising from the simultaneous presence of acetylated C_{21} and C_{23} homologs in this sample. The present GC-MS data also permitted us to detect very small amounts of acetylated C_{19} homologs (peak A in fig. 4, base peaks at 275 and 277) which have the same retention time as the constituents of *C. sordidula* venom, and are thus tentatively formulated as **1g–1i**, and **4a** (fig. 1) (see later).

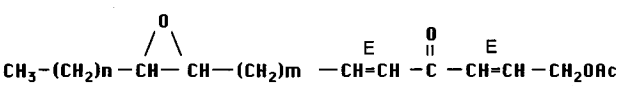
With the lengths of these chains now firmly established, and their structural features already deduced from spectroscopic data [1], only the position of the isolated and Z,Z-dienic double bonds remained to be established. In our previous paper [1], we reported the formation of heptanal and nonanal through ozonolysis of both the whole secretion and the acetate fraction. However, the discovery that minor homologs are present in the venom prompted us to reinvestigate this problem and, specifically, to search carefully for the presence of traces of other aldehydes in the ozonolysis mixture. Thus, microozonolysis [3] of a freshly collected venom sample, and analysis of the reaction mixture by GLC on three different columns (see Materials and methods), led to the identification of pentanal, heptanal and nonanal, in the ratio 6:3:1, whereas only heptanal and nonanal had been previously detected [1]. This discrepancy stems from the fact that pentanal is too volatile to be detected on either the OV 1 or the OV 1701 capillary column, under the conditions used. This, of course, raises the question of the possible presence of propanal, which would not be detected even on the CPwax 52B column, under our conditions. However, this possibility may be excluded since, in the high field ^{1}H NMR spectrum of the venom, such double bond isomers would give signals characteristic of an ethyl group on an sp^2 carbon.

Epoxidation of the Dufour gland secretion by treatment with *m*-chloroperbenzoic acid, followed by preparative TLC, afforded a monoepoxide fraction, {EIMS: $M^{+\cdot}$ at m/z 378 (major components) and 406 (minor components)}, originating only from the compounds possessing one isolated double bond in the chain. The absence in this fraction of any epoxide coming from the Z,Z-dienes was clearly shown by ^{1}H NMR [1]. Cleavage [4] of this monoepoxide fraction with HIO_4 again afforded pentanal, heptanal and nonanal, but in a 3.8:1.5:1 ratio, differing from the ratio found in the ozonolysis experiments. These results established that in the major $C_{23}H_{38}O_3$ derivatives (peak D in fig. 4) the central double bond is located in the Δ^{12}, Δ^{14} or Δ^{16} position. Thus, since we already know [1] by ^{1}H NMR that this double bond is Z whereas those of the cross-conjugated dienone moiety are E, we may assign structures **1a**, **1b** and **1c** to the peak D components. Since no traces of other aldehydes could be detected in the ozonolysis mixtures, the minor $C_{25}H_{42}O_3$ derivatives (peak G in fig. 4) are tentatively assigned structures **1d**, **1e** and **1f** although we have no definite proof that all three compounds are present.

The relative amounts of pentanal and heptanal versus nonanal, obtained by epoxide cleavage, are significantly lower than those found by ozonolysis of the total secretion. Consequently, we propose that the major Z,Z-dienic derivatives which eluted under peak F (CIMS, base peak at m/z 301, $C_{23}H_{36}O_3$) have $\Delta^{12,14}$ or $\Delta^{14,16}$ double bonds. Since the stereochemistry of these dienes is Z,Z [1], they must have structure **5a** and **5b**. Clearly, other dienic double bond isomers may also be present under peak F, but our data do not allow us to prove this point at the present time. We also tentatively assign $\Delta^{14,16}$ or $\Delta^{16,18}$ double bonds to the homologous $C_{25}H_{40}O_3$ dienic derivatives (peak H in fig. 4), which accordingly are formulated as **5c** and **5d**.

It follows from these results that we may assign structures **8a–8c** (fig. 5) to the major components of the above-mentioned epoxide fraction. The fragmentation pattern of the latter in EIMS was also used to study the position of the isolated double bonds in the carbon chains. Indeed, prominent peaks arising from cleavage α to the epoxide function were found at m/z 279.19672 ($C_{17}H_{27}O_3$), 251.16393 ($C_{15}H_{23}O_3$) and 223.13343 ($C_{13}H_{19}O_3$). Subsequent loss of AcOH from these ions afforded peaks at 219.17482 ($C_{15}H_{23}O$, metastable peak at m/z 172), 191.14428 ($C_{13}H_{19}O$, metastable peak at m/z 145) and 163.11236 ($C_{11}H_{15}O$, metastable peak at m/z 119), respectively. Thus, these fragment ions do not contain the epoxide moiety, in contrast to the usual fragmentation of long chain epoxide acetates [4]. We plan to address this problem in the near future.

GC-MS experiments on the monoepoxide mixture proved to be disappointing, since its constituents could not be better resolved on either the SE 54 or OV 1 column than those of the starting olefin mixture. The data thus obtained confirmed the presence of several monoepoxide acetates based on an acetylated C_{21} long chain {$(M + H)^+$ at m/z 379, base peak at m/z 319}, accompanied by minor homologs based on an acetylated C_{23} chain {$(M + H)^+$ at m/z 407; base peak at m/z 347}, and



n	m	Compound
7	5	8a
5	7	8b
3	9	8c
7	7	8d
5	9	8e
3	11	8f
7	3	8g
5	5	8h
3	7	8i

Figure 5. Structures of the components of the epoxide fractions derived from the venoms of the three *Crematogaster* species.

formulated as **8d–8f**. The acetylated C_{19} epoxides deriving from **1h–1i** could not be found, however.

The GC-MS analysis of the venom also made it possible to find traces of dihydroderivatives[1] {peaks A and E (fig. 4), $(M + H)^+$-AcOH at m/z 277 and 305, respectively (table 1)}. On the basis of ^1HNMR data we propose structures **4a** and **4b**, respectively for these dihydroderivatives, without the isolated double bond in the carbon chain. Indeed, any structural change at the level of the cross-conjugated dienone would change the chemical shift of the different protons of that system, thus allowing small amounts of such compounds to be detected in complex mixtures. The corresponding acetylated C_{23} dihydro homolog **4c**, which gave a very small $M^{+\cdot}$ at m/z 392 in the EI mass spectrum of whole venom samples, could not be detected in this particular analysis, but was found in other runs.

Finally, a small amount of the alcohols was also present {peaks B and C, fig. 4, base peaks at m/z 303 and 301 $(M + H)^+$-H_2O in CIMS (table 1)}. Since these components have the same base peak in CIMS as the corresponding acetates, their identification needed confirmation. This was done by comparison with GC-CIMS data (not shown) obtained on an older venom sample containing much higher proportions of the alcohol components, and by subjecting the venom to preparative TLC, which afforded a small sample of the alcohol fraction {EIMS: $M^{+\cdot}$ at m/z 348 and 346 (minors) and 320 and 318 (majors); fragment ions at m/z 330,328 (minors-H_2O), 302,300 (majors-H_2O); IR: ν_{OH} 3,400 cm^{-1}; cross-conjugated dienone: 1,666, 1,640 and 1,620 cm^{-1}}. This fraction coeluted with peaks B and C when it was co-injected with a freshly collected venom sample in capillary GLC. Since we have shown[2] that the alcohol and aldehyde components of the venom arise directly from the acetates, the structures of the alcohols with an isolated double bond in the chain may be represented by **2a–2i**, whereas those of the corresponding dienic compounds are **6a–6d**. Similarly, the aldehyde components are formulated as **3a–3i** and **7a–7d** (fig. 1).

C. auberti. The Dufour gland secretion of *C. auberti*, collected as described for *C. scutellaris*, had the same Rf as the latter in TLC. Its UV spectrum (λ_{max} 234 nm, hexane) and EI mass spectrum ($M^{+\cdot}$ at m/z 390 and 362; fragment ions at m/z 348, 330, 320, 302...) were similar to those of *C. scutellaris* except for the lack in EIMS of ions corresponding to the dienic derivatives **5a–5d**. The total ion chromatogram of the Dufour gland secretion is shown in fig. 6 and the corresponding CIMS (isobutane) data are reported in table 2. These data, whilst confirming the lack of dehydro derivatives (peaks F and H in fig. 4), demonstrated the simultaneous presence of the same major acetate homologs as in *C. scutellaris* {peak C in fig. 6 $(M + H)^+$-AcOH at m/z 303}, accompanied by the same higher {peak D, $(M + H)^+$-AcOH at m/z 331} and lower homologs {peak A, $(M + H)^+$-AcOH at m/z 275}, although the relative

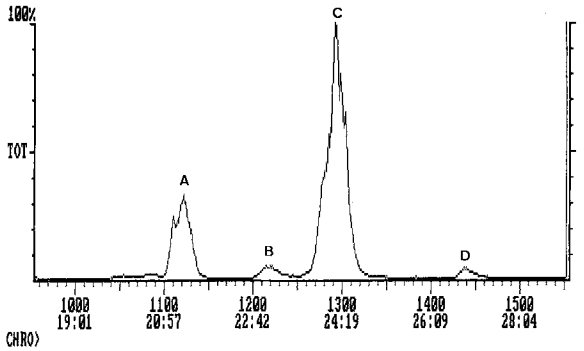


Figure 6. Total ion chromatogram of a fresh sample of *C. auberti* venom. Letters identify peaks whose compositions are listed in table 2.

amount of the latter is larger than in *C. scutellaris*. Again, small quantities of alcohols (peak B in fig. 6) arising from peak C acetates were also found. Their identity was proved as above by GC-MS comparison with an 'aged' venom sample (1 week old) containing almost exclusively the alcohol components.

Double bond location was also performed by ozonolysis experiments[3] leading in this case to the identification of pentanal, heptanal and nonanal in the ratio 1:4:2. Since the acetate components of *C. auberti* have the same Rf in TLC and the same retention time in GLC as the $C_{23}H_{38}O_3$ acetate components of *C. scutellaris*, we may assume that they have the same stereochemistry at the double bonds and thus that the major derivatives possess structures **1a–1c**. Again, we tentatively propose structures **1d–1f** for the minor higher homologs and **1g–1i** for the minor lower homologs. The corresponding dihydroderivatives **4a–4c** were found under peaks A, C and D, respectively (table 2).

Epoxidation of the *C. auberti* secretion followed by preparative TLC and GC-MS as described above for *C. scutellaris* gave the same results, except that the acetylated C_{19} epoxides {$(M + H)^+$ at m/z 351, $(M + H)^+$-AcOH at m/z 291} were detected in this case.

C. sordidula. A freshly collected Dufour gland secretion of *C. sordidula* showed one spot in TLC, slightly more polar than the one displayed by *C. scutellaris* (hexane-ethyl acetate 8:2; eluted twice). The UV spectrum (λ_{max} 233 nm, hexane) indicated the presence of the characteristic cross-conjugated dienone[1]. However, the EIMS of the whole secretion {$M^{+\cdot}$ at m/z 334, fragment ions at m/z 292, 274 ($M^{+\cdot}$-AcOH, calc. for $C_{19}H_{30}O$: 274.2296; found: 274.2290), 266, 247, 235, 209...} showed that the major Dufour gland constituents of this species differed from those of the two others by the length of the carbon chain. Indeed, the $M^{+\cdot}$ ion at m/z 334 ($C_{21}H_{34}O_3$) corresponded to an acetylated C_{19} chain. These data also suggested the absence of any dienic derivative.

GC-MS analysis (fig. 7 and table 3) totally confirmed these views. It showed the presence of at least two acetylated C_{19} long chain compounds with one isolated dou-

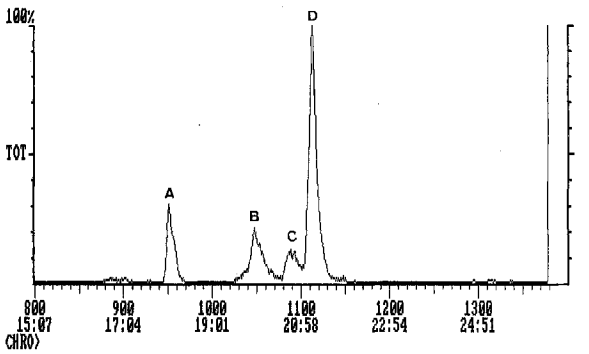


Figure 7. Total ion chromatogram of a fresh sample of *C. sordidula* venom. Letters identify peaks whose compositions are listed in table 3.

ble bond in the chain (peaks C and D in fig. 7, base peak at m/z 275 in CIMS). A corresponding dihydroderivative is also present under peak D (base peak at m/z 277 in CIMS). This was confirmed by the presence in the EI mass spectrum of the whole secretion of small peaks at m/z 294 (336-CH_2CO) and 276 (336-AcOH). As in the other species, these components are accompanied by small amounts of the corresponding alcohols (peak B in fig. 7, base peak at m/z 275), identified as described for *C. scutellaris* and *C. auberti*.

The components of peak A, whose CI mass spectra showed base peaks at m/z 247 and m/z 249, corresponded to lower acetate homologs. They again differed from the major ones by 28 daltons, and are probably based on an acetylated C_{17} chain. Indeed, very small peaks may be seen at m/z 308 and 306 in the EI mass spectrum of the whole secretion. No attempts were made to identify these components fully.

The 600 MHz ^{1}H NMR spectrum of the Dufour gland secretion of *C. sordidula* in $CDCl_3$ (table 4) confirmed the close structural relationship of its constituents with those of the two other species. It allowed the acetate (77%) and aldehyde (15%) components, to be identified, together with a small amount of a Δ^2(Z)-aldehyde isomer (CHO, δ 10.14, J = 6.3 Hz) and an artifact (δ 3.26, m and 5.19, t) arising from cyclization of the latter[2]. These NMR data also allowed an E stereochemistry to be assigned to the cross-conjugated dienone double bonds of the major acetate and aldehyde constituents.

Table 4. ^{1}H NMR spectrum of a venom sample of *C. sordidula* (600 MHz, $CDCl_3$, TMS, δ, J in Hz)

Protons[a]	Acetates (**1g–1i**)	Aldehydes (**3g–3i**)
HC-1		9.79, d, 6.3
H_2C-1	4.76, dd, 4.2, 1.6	
HC-2	6.84, dt, 13.2, 4.2	6.83, dd, 13.2, 6.3
HC-3	6.52, dt, 13.2, 1.6	7.16, d, 13.2
HC-5	6.31, dt, 13.2, 1.2	6.39, dt, 13.2, 1.5
HC-6	6.93, dt, 13.2, 6.5	7.05, dt, 13.2, 6.5
H_2C-7	2.24, q, 7.0	2.24, q, 7.0
HC=CH	5.34, m	5.34, m
H_3C-19	0.87, t, 6.0	0.87, t, 6.0
CH_3COO	2.15, s	
$CH_2CH=$[b]	2.00, bq, 6.0	2.00, bq, 6.0

[a] The signals reported are those of the major constituents of the venom, the acetates **1g–1i** (77%), and the corresponding aldehydes **3g–3i** (15%). Minor signals attibutable to a Δ^2(Z)-aldehyde isomer (4%) and a cyclization artifact[2] (4%) were also detected (see text). [b] The signals of both CH_2 allylic to the isolated double bonds are superimposed.

Microozonolysis afforded pentanal, heptanal and nonanal, in a 5:1:4 ratio. If we admit, by analogy with the two other species, that the isolated double bond stereochemistry is Z as in *C. scutellaris*, we may propose structures **1g–1i** for the major acetate components of the Dufour gland of *C. sordidula*. The corresponding dihydroderivative was assigned structure **4a**, based on the same ^{1}H NMR arguments as above. Accordingly, the major alcohols are formulated as **2g–2i**, and the corresponding aldehydes as **3g–3i** (fig. 1).

The small difference in the relative amounts of alcohol and aldehyde components observed between the two venom samples of *C. sordidula* used for GC-MS and for ^{1}H NMR is due to differences in the relative activities of the two enzymes. The latter may be ascribed to one or several of the following reasons: a) a change in the solvent (CH_2Cl_2 versus $CDCl_3$); b) a change in the time elapsed between the collection of the sample and the analysis; c) a different physiological state of the colony, since these two analyses were performed at different times.

Conclusion

All compounds so far evidenced in the Dufour glands of *Crematogaster* ants are closely related, the major structural differences being 1) the lengths of the carbon chains, since *C. sordidula* produces mostly C_{19} chains, probably accompanied by minor C_{17} homologs, whereas the two other species secrete C_{21} chains, accompanied by small amounts of C_{23} and C_{19} homologs and 2) the presence of dienic derivatives only in *C. scutellaris*. Biogenetically, these compounds seem to derive from the fatty acid pool, in accordance with previous reports on myrmicine Dufour gland constituents[5,6]. However, the biosynthesis of the terminal cross-conjugated dienone common to all these derivatives is still a matter of speculation.

The results presented in this paper show that the defense mechanism first discovered in *C. scutellaris* is quite similar in the three European *Crematogaster* species, although they differ in their biology and ecology. For example, *C. scutellaris* is a far more robust species than the other two, and it nests in dead logs, whereas *C. auberti* and *C. sordidula* nest in the ground.

The genus *Crematogaster* contains numerous species, especially in the tropics, and their taxonomy is poorly understood. *C. sordidula* belongs to the subgenus *Orthocrema*, whereas *C. scutellaris* and *C. auberti* are members of the subgenus *Acrocoelia*. Interestingly, the venom of the first species is clearly distinct from those of the two others, which are more similar. At least two species of the subgenus *Physocrema* from Malaysia exhibit a very different defense mechanism. In *C. inflata* and *C. deformis*,

the metapleural glands are inflated and secrete a sticky and repellent fluid [6, 7]. The defense mechanisms and venom chemistry could offer valuable characteristics to help in clarifying the taxonomy of this large genus. Work along these lines is in progress in our laboratories.

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Flavonoid wing pigments in grasshoppers

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Abstract. Yellow pigments extracted from the hindwings of *Dissoteira carolina* (L.) (Acrididae:Oedipodinae) were identified by HPLC, GLC, MS and absorbance spectra as primarily quercetin and quercetin-β-3-O-glucoside with minor amounts of luteolin. These flavonoids make up about 2% of the hindwing live weight and are also abundant in the yellow hindwings of several related species of band-winged grasshoppers. Fat body UDPG glucosyltransferase preferentially catalyzed glucosylation of the 3-OH of quercetin.
Key words. Cuticle; flavonoids; glucosides; glucosyltransferase; grasshoppers; quercetin; pigments.

The band-winged grasshoppers (Oedipodinae) characteristically have hindwings brightly pigmented with various shades of yellow, orange, red, or blue [1]. Black bands often outline the inner colored areas of the hindwings. However, the Carolina grasshopper, *Dissosteira carolina*, has a large inner area of the fan that is black, while the outer band is light yellow. We have carried out a study of the yellow pigments in the hindwings of *D. carolina* and several related species and found them to be composed primarily of the common plant flavonoid, quercetin (3,3′,4′,5,7-pentahydroxyflavone) and a quercetin conjugate, identified as the β-3-O-glucoside.

We initially extracted the yellow hindwing cuticle by grinding it in a glass tissue grinder in ice cold 10% HCl followed by adsorption of *o*-diphenolic compounds on alumina at pH 8.6 [2]. The *o*-diphenols, recovered from alumina in 1 M acetic acid, were analyzed by high pressure liquid chromatography (HPLC) on a 4.6 × 250 mm reverse phase C18 5 µm spherical particle column at a flow rate of 1 ml/min with an electrochemical detector (+ 0.72 V). The primary mobile phase consisted of 26% acetonitrile, 1.1 mM sodium dodecyl sulfate and 0.05 mM disodium EDTA in 0.1 M phosphate buffer at pH 3.3 [3]. Other mobile phases consisted of 15–20% acetonitrile in 0.1 M phosphate buffer pH 3. Two major unknown electroactive compounds behaving as *o*-diphenols, were observed with retention times of 7.1 and 26.9 min in the primary mobile phase (fig. 1). After an aliquot of the extract in 10% HCl was heated for 10 min at 100 °C, the 7-min peak disappeared, indicating that it was an acid labile conjugate. Additional extracts of yellow wing cuticle were prepared by grinding the samples in 100% methanol followed by paper chromatography with a mobile phase of n-butanol, acetic acid and water [4]. A yellow band, characteristic of certain flavonoids, was observed under ultraviolet light after exposure to ammonia fumes. This material was extracted from the paper with methanol and gave two peaks by HPLC analysis with retention times identical to those for the *o*-diphenolic compounds previously recovered by alumina adsorption. To identify the free compound, a large sample of yellow wing cuticle was extracted in methanol which was evaporated under nitrogen. The extract was then heated in 10% HCl for 10 min at 100 °C to hydrolyze the conjugate and cooled overnight at 5 °C. A yellow pigment precipitated and was collected by centrifugation and washed with distilled water. The pigment was dissolved in 100% methanol and gave an absorbance spectrum identical to that of quercetin (fig. 2). Absorption maxima were observed at 255, 270 shoulder, and 372 nm. The yellow pigment also had a retention time identical to that of standard quercetin (fig. 3), and the two compounds