Cantharidin analogues and their attractancy for ceratopogonid flies (Diptera: Ceratopogonidae)

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Abstract. Several ceratopogonid flies are attracted to cantharidin and ingest it from both cantharidin-baits and from meloid beetles, one of the few known natural sources for cantharidin. Because meloids are absent in northern Bavaria, and certain cantharidin flies of the genus *Atrichopogon* are temporarily associated with certain plants (*Apiaceae, Aristolochiaceae*), it was suggested that cantharidin ceratopogonids might be generally attracted by chemically similar plant-derived compounds. At first the seasonal fluctuating attractancy, sex ratio and behaviour of *A. oedemerarum* Storå was studied at cantharidin baits. Synthetic cantharidin analogues exhibited an attractancy for *A. oedemerarum* if the exo,exo-7-oxabicyclo[2.2.1]heptane skeleton of cantharidin was associated with a 2,3-dicarboxylic anhydride or a 2,3-γ-lactone. According to structure-activity studies, the analogues seem to fit best into the active site of the receptor if the carbonyl function of the γ-lacton is in the exo- and 2-position. This is the first report indicating that molecules other than cantharidin are attractive for cantharidin flies.

Key words. Cantharidin; Ceratopogonidae; *Atrichopogon oedemerarum*; attractancy; structure – activity relationship.

The terpenoid substance cantharidin represents one of the most famous insect defensive compounds. It is also known as an aphrodisiac. Blister beetles (Meloidae) and false blister beetles (Oedemeridae) are the only known natural sources, and synthesize this unusual vesicant, insecticide and antifeedant[1,2]. However, cantharidin is also a potent attractant for representatives of various insect orders[3–6], including one genus of ceratopogonid flies. During our studies with cantharidin Ceratopogonidae we were mainly interested in research concerning natural sources of cantharidin or potential precursors. Within our collecting area of northern Bavaria, cantharidin baits attract high numbers of various ceratopogonid flies, but cantharidin-containing meloid beetles are virtually absent. False blister beetles, with extremely low cantharidin titres[2], are rare, but they may be potential hosts for the cantharidin fly *Atrichopogon oedemerarum* Storå, which has occasionally been observed feeding on oedemerid beetles[7,8]. In those areas where cantharidin-containing meloids and cantharidin ceratopogonids are sympatric, the flies are attracted to meloids (e.g. *A. lucorum* on *Meloe*), piercing the abdominal intersegmental membranes to feed from haemolymph[9,10].

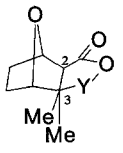
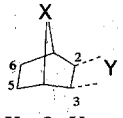
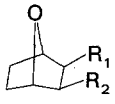
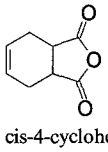
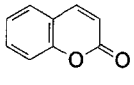
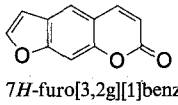
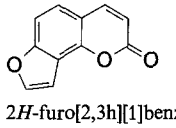
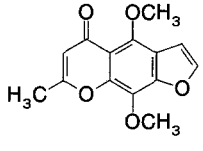
Since meloid beetles are absent in the experimental area, we suggested that this peculiar association of a natural compound with certain ceratopogonid flies might be due to the fact that cantharidin-like substances probably also occur in higher plants or fungi which have nothing to do with meloids or oedemerids. This hypothesis is supported by the fact that the cantharidin ceratopogonid *Atrichopogon lucorum* Meigen was found within flower traps of *Aristolochia clematitis*[11] or blossoms of various Apiaceae (pers. observ.). Moreover, the cantharidin-like compound palasonin (exo,exo-7-oxabicyclo[2.2.1]heptane-3-methyl-2,3-dicarboxylic anhydride), has been isolated from the insecticidal seeds of *Butea frondosa* Roxb., a Fabaceae species from India[12].

In order to test this potential linkage to plant-derived compounds, we investigated whether substances other than cantharidin are attractive for cantharidin ceratopogonids. An additional aim of this study was to obtain information on features of the molecular structure which might be essential for attractancy to the flies. Several typical Apiaceae constituents such as furano- and pyranocoumarins, which like cantharidin are skin-irritating and toxic, were used to test the attractancy of molecular structures which cause effects similar to cantharidin, without being related to it. On the other hand, various synthetic analogues of cantharidin were tested to determine their attractivity for ceratopogonid flies.

Materials and methods

Pyrano- and furanocoumarins, and the cantharidin analogues used are listed in the table. Baits were prepared as follows. A folded filtre paper disk (diam.: 5.5 cm) was impregnated with 100 µl of a 5-mmol solution of cantharidin or corresponding solutions of furano-, or pyranocoumarins or cantharidin analogues in acetone. The bait was placed at the bottom of a capped rectangular plastic box (10 × 10 × 6 cm). Two sides of the plastic box had a hole (diam.: 3.5 cm) covered with plastic gauze (mesh 0.5 mm) to facilitate the escape of volatilized test compounds. Into a hole in each gauze was fitted a square plastic tube (width: 1 cm), through which attracted insects could enter. The traps were mounted on poles (height: 1 m). Frequent checks of field taps (usually five times a week) ensured a low escape of trapped flies. These traps were installed in four test groups at a locality in the vicinity of Bayreuth (Fürsetz; wood habitat). Because of the high attractivity of pure cantharidin, only one group of traps was furnished with a cantharidin bait in 1990.

Tested pyrano- and furanocoumarins, cantharidin and its analogues (No: number; Origin: a: commercially available, s: synthesized; Year: year of testing; Mm: molecular mass; Mp: melting point at 760 torr (°C); R_{temp}: retention temperature (°C)).

No	Compound	Origin	Year	Mm	Mp	R_{temp}
						
1	Y=C=O (Cantharidin)	a	1989/90	196.2	218	184
2	$Y=CH_2$; (racemic)	s	1990	182	-	205
						
3	X=O; Y=−C(O)−O−C(O)−	s	1990	168	108	183
4	X=O; Y=−C(O)−O−C(O)−, 5.6-dehydro	s	1990	166	119	172
5	X=O; Y=−C(O)−O−CH_2; (racemic)	s	1990	154	67	181
6	X=O; Y=−C(O)−O−CH_2; ((+), 80% ee)	s	1990	154	67	181
7	X=O; Y=−C(O)−O−CH_2; ((−), 95% ee)	s	1990	154	67	181
8	X=O; Y=−C(O)−NH−C(O)−	s	1990	167	185	194
9	$X=CH_2$; Y=−C(O)−O−C(O)−	s	1990	166	72	168
						
10	$R_1=R_2$=COOMe	s	1990	214	77	196
11	R_1=COOMe; R_2=COOH; (racemic)	s	1990	200	122	-
						
12	cis-4-cyclohexen-1.2-dicarboxylic acid anhydride	a	1989	152.15	-	160
						
13	5.6-Benzo-2-pyrone (Coumarin)	a	1989	146.15	71	-
						
14	7*H*-furo[3,2g][1]benzopyran-7-one (Psoralen)	a	1989	186.17	160	-
						
15	2*H*-furo[2,3h][1]benzopyrane-2-one (Angelicin)	a	1989	186.17	-	-
						
16	4,9-dimethoxy-7-methyl-5*H*-furo[3,2g][1]benzopyrane-5-one (Khellin)	a	1989	260.25	154	-

The traps of each test group were arranged at distances of about 2 m from one another.

The experiment started on 12 June 1990 and finished on 31 August 1990 (80 days). The baits were changed 22, 48 and 58 days after the beginning of the experiment.

For a rough comparison of volatility data, we determined retention temperatures using gas chromatography. Samples were separated by temperature-programming on a SE 52 capillary column (25 m × 0.32 mm; programme: 60°(1 min)→15°/min→200° → 20°/min→300°→15°/min→320°).

Results and discussion

Attractancy of cantharidin for ceratopogonid flies. In the area of northern Bavaria cantharidin baits are attractive to the following ceratopogonid species: *Atrichopogon*

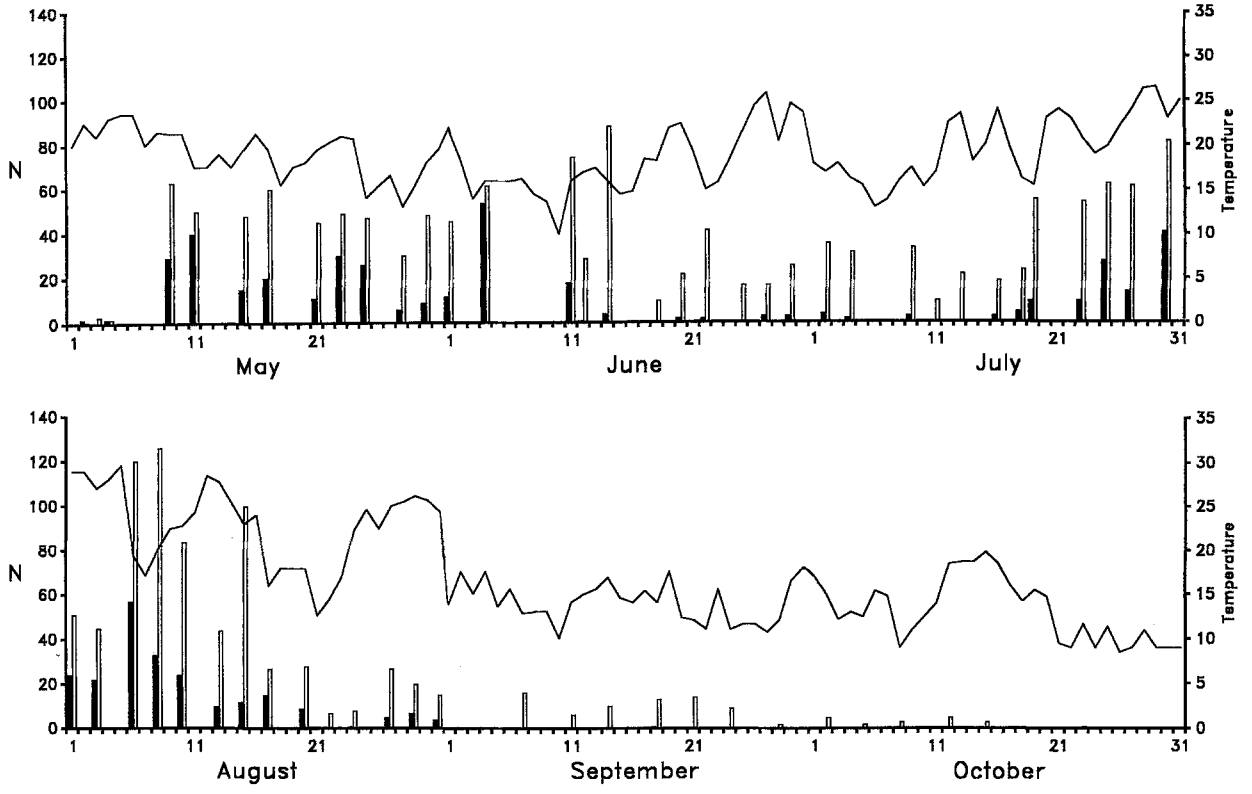


Figure 1. Seasonal abundance of *A. oedemerarum* at cantharidin-traps in the vicinity of Bayreuth (northern Bavaria; black columns: males, open columns: females) and maximal air temperature in °C, registered at one representative site.

oedemerarum Storå, *A. trifasciatus* Kieffer, *A. brunnipes* Meigen and *A. lucorum* (Meigen). Compared to the other three species, *A. oedemerarum* was also the most abundant species within traps baited with analogues of cantharidin, so representatives of this species are considered exclusively in the following account. The number of trapped flies varied significantly from May to September 1990 (fig. 1). The seasonal fluctuation of *A. oedemerarum* is characterized by two maxima at the end of May and in July/August. At present it is not clear whether the sex ratio observed in the traps, with females dominating, corresponds with the actual sex ratio in an area devoid of cantharidin. The seasonal fluctuating maximal air temperature has no evident influence on the number of trapped flies (fig. 1).

Figure 2. Ceratopogonid flies of *Atrichopogon* sucking from a water droplet on a cantharidin-impregnated filtre paper. Faecal droplets of flies are indicated by arrows.

The greediness of cantharidin-philous ceratopogonids for cantharidin could be demonstrated by a simple experiment in the laboratory. If a droplet of distilled water was placed on a cantharidin-impregnated filtre paper beside a water control, *Atrichopogon* females and males immediately appeared and voraciously sucked from the droplet. Even faecal droplets of sucking *Atrichopogon* produced the same behaviour (fig. 2).

Attractancy of cantharidin-analogues and plant-derived compounds for ceratopogonid flies. Several synthetic analogues of cantharidin (**1**) showed a considerable or moderate attractancy for *A. oedemerarum* flies (fig. 3), although the number of trapped flies was always lower as compared to those attracted to authentic cantharidin baits (fig. 4). It is interesting that trapping efficacy of analogues and cantharidin are different with respect to both sex ratio and species. Compared to cantharidin baits with 20% male *A. oedemerarum* only 3% males were found in traps with the most attractive analogue, no. **2**. The other difference lies in the variety of attracted species and their numbers. Only analogues **2** and **3** were attractive for other species (no. **3**: *A. brunnipes* 7 fem., 5 males; *A. trifasciatus* 2 fem., 1 male; *A. lucorum* 3 fem., 1 male; no. **2**: *A. brunnipes* 56 fem., 28 males; *A. lucorum*

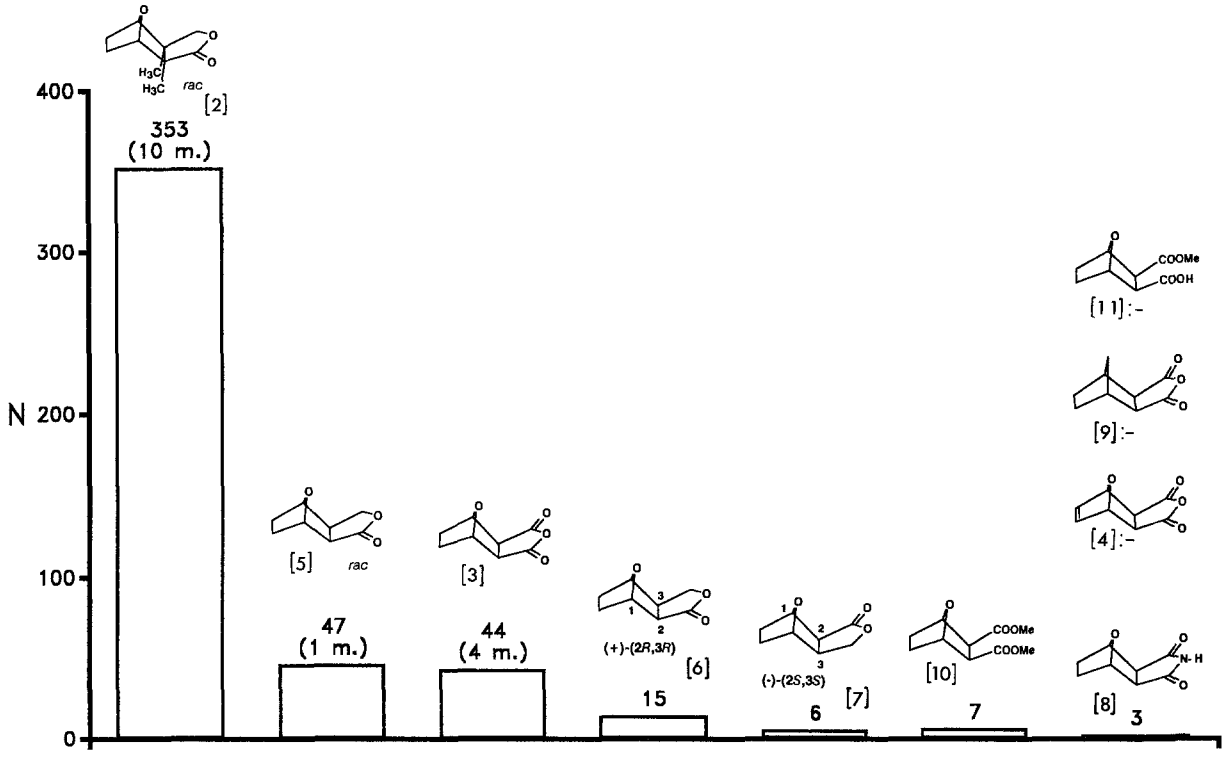


Figure 3. Total number of *A. oedemerarum* trapped from 12.6–31.8.90 using cantharidin analogues (see table) as baits (m.: included males).

3 fem., 1 male). These additional captures were of minor importance compared to those with cantharidin. One can summarize that a distinct shift concerning the sensitivity takes place when cantharidin-related compounds were offered. We suggest that differences in perception between males and females and between the different species are responsible for this behaviour. Moreover, previous experiments during 1989 indicated that none of the plant-derived pyrano- and furanocoumarins (**12–16**) were attractive for ceratopogonid flies. For this reason we only tested those compounds in 1990 which were structurally closely related to cantharidin.

Figure 3 compiles the total captures of *A. oedemerarum* in traps with cantharidin analogues (**2–11**). Non-significant differences (sign test, two-tailed) in attractancy effects (between nos. **3, 5**; **6, 7, 10**; **7, 8, 10**) are not discussed in the following. Compared to figure 4, the data clearly indicate that only cantharidin (**1**), and a few closely-related compounds which have substituents in the *exo*-position, are able to attract the ceratopogonid flies. *Endo*-derivatives (not included in the table) are inactive. Moreover, a particular arrangement of all three or four oxygens atoms within a plane above the C-framework seems to be essential for biological activity. As a matter of fact, the significance of individual oxygen atoms is not equivalent. If, for example, the bridgehead oxygen is replaced by a methylene group (**9**), the attractancy of the molecule is completely lost. This clearly underlines the unique importance of this particular heteroatom for the biological activity. But attractancy is also lost when a 4,5-double bond is introduced into the six-ring system (**4**). On the other hand, if one of the two carbonyl oxygens of the anhydride moiety in cantharidin is replaced by a methylene group (**2**), the biological activity is reduced, but the resulting γ-lactone still retains about 70% of the original attractancy of cantharidin (compare the total captures of *A. oedemerarum* in fig. 3 and fig. 4). Removal of the two angular methyl groups from either the lactone (**5, 6, 7**) or the anhydride (**3**) causes a further drastic loss of attractancy. Interestingly, the truncated molecule is still more active than the bicyclo[2.2.1]-heptane analogue (**9**) where the bridgehead oxygen is missing. Replacement of the central oxygen in the anhydride moiety by a NH-group (**8**) resulted in considerable loss of attractivity compared to no. 3. Based on these findings, we have to assume that in the case of the ceratopogonid flies, the 7-oxabicyclo[2.2.1]heptane moiety in conjunction with an *exo*-annulated five membered γ-lactone is the minimum structural requirement for a highly effective interaction with the receptor. The role of the two angular methyl groups might be seen in an improvement for the ligand/receptor interaction by forcing the cantharidin molecule or its analogues into intimate contact with the corresponding complementary functional groups on the receptor site. At the same time, hydrophobic bonding of these unpolar moieties may also contribute in order to maximize the protein/ligand interaction. It is not yet understood why the two enantiomeric τ-lactones (**6** and **7**) are less active than the corresponding racemate (**5**). This puzzling question will be

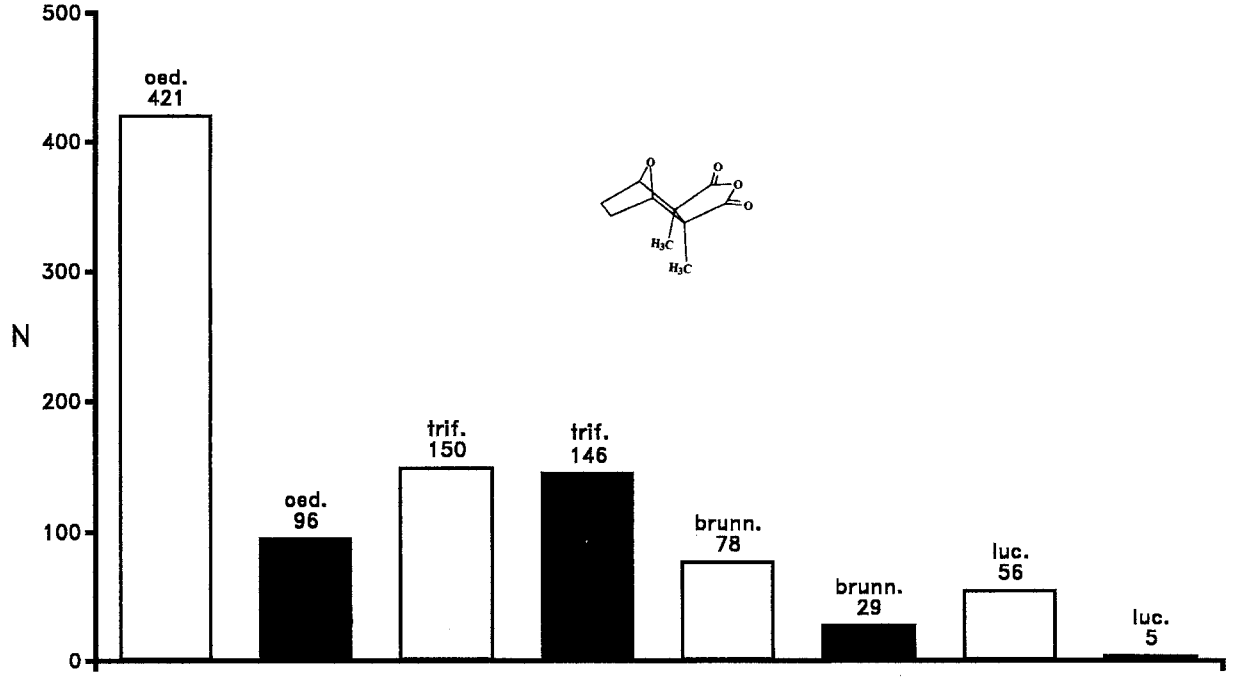


Figure 4. Numbers (N) of all ceratopogonid species trapped by comparable numbers of cantharidin baits from 12.6–31.8.90 (black columns: males; open columns: females; oed: *A. oedemerarum*, trif.: *A. trifasciatus*, brunn.: *A. brunnipes*, luc.: *A. lucorum*).

further addressed in the next season with optically active γ-lactones derived from cantharidin (**1**).

A striking comparison can be made between the above data and those from previous work on the characterization of specific cantharidin-binding sites in mouse tissue[13], and a related study on the herbicidal action of endothal (**3**) on plants[14]. Except for the high activity of the two racemic γ-lactones (**2** and **5**), and the apparently inactive ring-opened analogues (**10** and **11**; fig. 3), our data are in good agreement with those derived from competitive inhibition studies with the cantharidin-binding site in mouse liver cytosol. This coincidence could be even more compelling, if we consider that only those analogues which are sufficiently volatile to be recognized over a distance will reach the chemosensillae of the insects. Since, on the other hand, the diester (**10**), as well as the half-ester (**11**) are clearly less volatile than the corresponding tricyclic γ-lactones and anhydrides, this apparent discrepancy might even be nonexistent. Further experiments, allowing the insects direct access to the loaded baits, followed by analysis of their ovaries, eggs and other tissues, will circumvent the problems encountered with different vapor pressures, and may be helpful in clarifying the very important question of whether or not a common protein or family of proteins is responsible for cantharidin-binding in insects, mammals and plants.

Investigations on the biological significance of cantharidin and its analogues for canthariphilous ceratopogonids are in progress. It could be possible that these flies ingest cantharidin both for their own protection and for the protection of their eggs. This theory may be supported by observations that always more females than males of *A. oedemerarum* were found within the traps. In addition, our observations indicate that cantharidin sources may represent meeting places for the two sexes (lek) and stimulate copulation[15].

Many canthariphilous insects are not only temporarily associated with plants, but show interrelations with fungi too (Beetles: Anthicidae, Endomychidae; flies: Sciaridae)[3, 5, 16, 17]. In the future it may therefore be necessary to look for natural compounds within plants or fungi which resemble the exo,exo-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic anhydride (**3**) or the appropriate lactone structures (**2, 5, 6, 7**). It may even be possible that these kinds of analogues occur in nature and act as key compounds for canthariphilous insects. On the other hand, the attractivity of lactone-analogues (which are probably more widely distributed in plants than cantharidin anhydrides) for canthariphilous ceratopogonids might indicate that these insects were originally feeding on these kinds of plant-lactones, and later specialized on the chemically similar, but more erratically distributed cantharidin from the haemolymph of meloids, false blister beetles or other canthariphilous insects.

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New C_{26} δ-lactones from the Dufour's gland of the urticating ant *Tetramorium aculeatum*

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Abstract. (6R*)-{(2S*)-2-hydroxyheneicos-12-enyl}-5,6-dihydro-2H-pyran-2-one (**1**)° is the major constituent of the secretion of freshly dissected Dufour's gland of the urticating ant *Tetramorium aculeatum.* In solution, compound **1** is slowly transformed into (1S*, 5R*, 7S*)-7-(nonadec-10-enyl)-2,6-dioxabicyclo[3.3.1]nonan-3-one (**2**)° on standing. The structures of compounds **1** and **2** have been established on the basis of their spectral and chemical properties. Compound **1** could be responsible for the urticating properties of the ant.
° IUPAC numbering.
Key words. Tetramorium aculeatum; ant; Dufour's gland; δ-lactone.

Tetramorium aculeatum nests between the leaves of small trees in tropical Africa. It is notorious for its aggressiveness and for causing severe skin irritation by biting and stinging. When abundant, this ant impairs work in coffee plantations, because labourers refuse to work. In Zaire, this ant is named the 'urticating ant'[1, 2]. Dissection of worker ants revealed that they possess a hypertrophied Dufour's gland which reaches the front of the gaster. We report here on the chemical composition of the Dufour's gland secretion of *T. aculeatum.*

Materials and methods

The ants were collected around Yaounde and dipped in methanol, or sent alive to Brussels. The specimens were identified by Dr B. Bolton (British Museum).

Preliminary TLC analysis on silica gel (Macherey-Nagel Sil G/UV 254, 0.25-mm precoated plates; visualization: ceric sulphate; eluent: hexane/acetone 8:2) demonstrated the presence in the methanol extract of dissected Dufour's glands of one major compound and traces of a second one. Both compounds could also be easily detected in the methanolic extract of whole ants.

About 400 ants stored in methanol were extracted with dichloromethane (3 ×) and methanol (3 ×). The extracts were combined and the solvent evaporated under reduced pressure, yielding a solid residue (20.4 mg) that contained the two sought-after compounds. The latter were isolated by chromatography on neutral alumina (activity 1; eluent: hexane 100% to ethyl acetate 100%). This yielded pure **1** (2.2 mg) and **2** (3.8 mg) as oily derivatives whose structures were mainly deduced from their spectral properties.

^{1}H NMR (250 MHz) and ^{13}C NMR (62.8 MHz) spectra were recorded on a Bruker WM 250 spectrometer and are reported in tables 1 and 2. Infrared spectra were taken with a Bruker IFS 25 instrument using NaCl discs on which the compounds had been deposited as a glassy film. The UV spectra were recorded in methanolic solution with a Philips PU 8720 spectrophotometer and the mass spectra with a VG micromass 7070F spectrometer. The CI mass spectra were recorded with ammonia as the reactant gas. The microozonolyses of **1** and **2** were performed as follows: the compound (0.2 mg) was dissolved in methanol/hexane 1:1 (2 ml) and a stream of air enriched with ozone generated by a commercial microozoniser (Litha), was passed through the solution for 5 min. Then, triphenylphosphine (5 mg) was added to the mixture and the resulting solution was analyzed by GLC using a Varian 3700 gas chromatograph equipped with an OV-1 capillary column (25 m × 0.25 mm i.d.), a splitless injector and a flame ionization detector. Nitrogen was the carrier gas and the temperature was programmed from 60° to 140 °C at a rate of 2 °C min^{-1} following an initial delay of 2 min. In these conditions, only one peak, which had the same retention time as *n*-nonanal (coinjection) was observed. This identification was confirmed by