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Identification and antifungal activity of novel organic compounds found in cuticular and internal lipids of medically important flies

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A B S T R A C T

Novel organic compounds found in the cuticular and internal lipids of medically important flies were identified. Uracil, 9-tricosene, 1-oleoyl glycerol, dimethyl suberate and butyl stearate were tested for their potential antifungal activity. Minimal inhibitory concentrations of the compounds against reference strains of fungi were determined. Uracil and dimethyl suberate slightly inhibited the growth of entomopathogenic fungi.

The cuticular and internal lipids of *Calliphora vicina*, *Calliphora vomitoria*, *Sarcophaga carnaria* and *Musca domestica* were studied by gas chromatography (GC) combined with mass spectrometry (GC/MS). A comparison of the lipid extracts between the preimaginal and mature stages showed adults flies contained a higher total content of the identified components. Furthermore, their amounts distinctly predominated in the internal lipids of all the species.

The amount of 9-tricosene was the highest in adults of *C. vicina*, while the larvae and pupae had a definitively lower amount of this compound. Uracil was found to be the most abundant component in extracts obtained from *C. vomitoria* especially in the internal lipids of adults. 1-oleoyl glycerol was detected in all of the examined species of flies. It was most abundant in the internal extracts isolated from the larvae of *C. vicina* and the pupae of *C. vomitoria*. Suberic acid dimethyl ester was found in the larval and pupal internal lipids of *C. vicina* and *S. carnaria* in low amounts. Butyl stearate was identified only in the internal lipids of the larvae and adults of houseflies.

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1. Introduction

Insects have a three-layer cuticle that protects the internal tissues against injuries, potential pathogens, toxins and desiccation (Nelson and Blomquist, 1995; Vincent and Wegst, 2004). The external layer (epicuticle) is selectively permeable and contains different classes of lipids including fatty acids, wax esters, fatty acid esters and acylglycerols (Gołębiowski et al. 2011; Cerkowniak et al. 2013). Epicuticular lipid components are proven to inhibit bacterial and fungal growth (Boguś et al. 2010; Urbanek et al. 2012; Gołębiowski et al. 2013a). Moreover, hydrocarbons, esters and free fatty acids released on the epicuticular surface are utilized for chemical communication as pheromones (Jurenka, 2004). Organic components included in the cuticular lipids are obtained directly from the insect's midgut or derived from lipid reserves stored in the fat body (Canavoso et al. 2001).

The fat body of insects is the main storage place of glycogen and internal lipids which are located in the main fat body cells called adipocytes (Van Der Horst and Ryan, 2012). Lipids are mostly accumulated in the form of triacylglycerols (TAG) but lipids, not incorporated in TAG or diacylglycerols (DAG), are also present in the fat body (Arrese and Soulages, 2010). Nutritional components transported from the midgut are incorporated into the adipocytes and used as a metabolic reserve for pupation, development and vitellogenesis (Canavoso et al. 2001).

Synanthropic fly developmental stages and adults of Calliphoridae (*Calliphora vicina*, *Calliphora vomitoria*), Sarcophagidae

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(*Sarcophaga carnaria*) and Muscidae (*Musca domestica*) were analyzed in this study. These flies are medically important as disease vectors and cause myiasis (Uni et al. 1999; Stevens, 2003; Förster et al. 2007; Thyssen et al. 2012). Blowflies and flesh flies are successively applied in forensic entomology and although these groups of insects seem to be the most common subjects of study, significant aspects of their biology are still being contributed (Bunchu et al. 2012). So far, only the internal/cuticular lipids and alcohols of the above mentioned fly species have been investigated. Chemical analyses of *C. vicina* and *C. vomitoria* have revealed different fatty acids and fatty acid methyl esters which demonstrate antifungal activity (Gołębiowski 2012; Gołębiowski et al. 2013b,c,d). Fatty alcohols found in cuticular and fat body extracts of *M. domestica* inhibit bacterial and fungal growth (Gołębiowski et al. 2012).

The results reported here show untypical chemical compounds in cyclorrhaphan flies, including pheromones, lipids and interestingly, uracil. Antifungal and antibacterial assays based on the minimal inhibitor concentration (MIC) have revealed that some of these compounds exhibit low antifungal activity. These data are the first descriptions by gas chromatography–mass spectrometry (GC/MS) of these unique components combined in insects. We discuss the concentration changes of the detected compounds and try to assess their biological significance in the examined species of flies.

2. Materials and methods

2.1. Insects

The developmental (larvae and pupae) and mature stages (females and males) of *C. vicina*, *C. vomitoria*, *S. carnaria* and *M. domestica* were examined. Material was provided by the Institute of Parasitology at The Polish Academy of Science (Warsaw). The colony of the examined flies was raised on beef and other components consisting of a mixture of honey, flour, wheat bran and additionally milk powder.

2.2. Extraction of lipids

Table 1 lists the number of insects, as well as the masses of the insects. The surface lipids from the cuticule were obtained by extraction of each group of insect (*C. vicina*, *C. vomitoria*, *S. carnaria* and *M. domestica*) with petroleum ether for 10 s (extract I) and then a second time in dichloromethane for 5 m (extract II). The third extraction was a long one with dichloromethane and butylated hydroxytoluene (50 mg/L) for 10 days (III extract). The petroleum extract I and dichloromethane extract II contained the cuticular lipids. The third extract contained the internal lipids.

Table 1
Quantitative summary of the experiment: numbers and masses of insect.

Stages	Number of insects	Masses of insects (g)
M. domestica		
Larvae	50	0.6
Pupae	640	11.0
Male	327	2.9
Female	234	3.4
C. vicina		
Larvae	70	4.2
Pupae	151	8.3
Male	23	0.8
Female	26	1.0
C. vomitoria		
Larvae	40	1.6
Pupae	30	0.8
Male	42	0.7
Female	45	0.7
S. carnaria		
Larvae	10	1.2
Pupae	50	3.9
Male	7	0.3
Female	17	0.9

2.3. Derivatization of extracts

The obtained extracts were filtered and collected into a glass vial. 1 mL of these extracts was further taken and placed into a glass flask and then evaporated under the nitrogen. The dried samples were silylized with 100 μL of a mixture of 99% bis(trimethylsilyl) acetamide and 1% chlorotrimethylsilane for 1 h at 100 °C. Trimethylsilyl (TMSi). The derivatives and native compounds were analyzed using GC/MS.

2.4. GC–MS analyses

Gas chromatography–mass spectrometry measurements were carried out by coupling an SSQ 710 (Finnigan Mat) spectrometer to a Hewlett-Packard 5890 gas chromatograph. Compounds were separated in a 30 m × 0.25 mm i.d., HP-5 capillary column (film thickness 0.25 μm). The column temperature was programmed from 80 (held 10 min.) to 320 °C at a rate of 4 °C min^{-1}. The injector and transfer line temperatures were 300 °C. The carrier gas was helium at a flow rate of 1 mL min^{-1}. In mass spectrometry electron-impact ionization was performed at electron energy of 70 eV. The ion source was maintained at 220 °C.

2.5. Identification and quantification

The novel organic compounds were identified by comparing the retention time of the analyzed compounds with standards and on the basis of silyl derivatives or native compound ions. The mass spectra of the standards and the samples were identical. Co-injection of lipid extracts with standards was applied to verify the earlier identification. In order to quantitatively determine each of the analyzed compounds, GC–MS analysis was carried out with an internal standard (19-methylarachidic acid). 19-methylarachidic acid (CAS Number 59708-73-5) was purchased from Sigma–Aldrich, Poland.

2.6. Microbiological activity

Antibacterial activity was assayed against the following bacterial strains: *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* PCM 2118, *Rhodococcus equi* ATCC 6939; *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13882, *Proteus mirabilis* PCM 543, *Proteus vulgaris* ATCC 13315 and *Pseudomonas aeruginosa* ATCC 9027. All of the species of bacteria were obtained from The Polish Collection of Microorganisms (Polish Academy of Science, Wroclaw, Poland). Entomopathogenic fungi cultures used in the antifungal tests were provided by the Institute of Plant Protection (Poznan, Poland). The following strains were used: *Beauveria bassiana* (Dv-1/07), *B. bassiana* (Tve-N39), *Lecanicillium lecanii*, *Metarhizium anisopliae*, *Paecilomyces fumosoroseus* and *Paecilomyces lilacinus*. *Candida albicans* ATCC 10231, and *Candida tropicalis* PCM 2681 were applied as representatives of fungi.

The minimal inhibitory concentration (MIC) of the detected compounds was determined using a standard microbroth dilution method according to the CLSI (Clinical and Laboratory Standards Institute) guidelines. The tested compounds were dissolved in 10% of DMSO in a phosphoric buffer, secondly added to polystyrene 96-well plates (Becton Dickinson) and then serially diluted (range: 2–1024 μg/L) with a Sabouraud Glucose broth (for the MIC test performed on fungi) or a Mueller Hinton II broth (the MIC test

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