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Defense chemicals in leaf surface wax synergistically stimulate oviposition by a phytophagous insect

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Summary. Propenylbenzenes, coumarins, and a polyacetylene, identified in the surface wax of carrot leaves, stimulate oviposition in the carrot fly. These compounds are known to be powerful inhibitors of the growth of bacteria, fungi, plants, and nonadapted herbivores. Their co-occurrence appears to be unique to the Umbelliferae, the host plant family of the carrot fly. An artificial mixture of these compounds proved to be synergistic and as stimulatory as the crude carrot leaf extract.
Key words. Leaf surface wax; defense chemicals; insect oviposition; carrot; *Daucus carota;* carrot fly; *Psila rosae.*

The number of defensive plant compounds so far identified is impressive but far from being complete[2]. There is little doubt that these secondary plant metabolites are powerful protective mechanisms against parasitic microorganisms, herbivores and competing plants[3]. By contrast, relatively few plant compounds are known which attract and stimulate herbivores during their search for suitable host plants[4]. This is especially the case for the host selection behavior of female insects that lay their eggs on or near the food-plants of their progeny. Since the larvae emerging from the eggs usually have only limited abilities to locate suitable food plants, their survival depends greatly on appropriate host plant selection by their mothers. It is surprising, therefore, that so little is known about this crucial phase in the life of phytophagous insects[5]. Unsuccessful attempts to isolate and identify the host plant compounds acting as attractants or stimulants have even led some investigators to believe that host plant specificity in oligophagous insects is primarily a consequence of the taxonomic restriction of particular feeding inhibitors[6].

Here we describe the successful identification and quantification of secondary plant components present in the crude extract of leaf surface wax, which act synergistically as a host plant recognition signal during oviposition by the carrot fly, *Psila rosae* (Diptera, Psilidae) and we offer the hypothesis that such complex mixtures of defensive plant metabolites in leaf surfaces may also be involved more generally in host selection and oviposition behavior by oligophagous insects.

The larvae of the carrot fly attack the roots of cultivated and wild Umbelliferae. Prior to oviposition females perform a typical 'run' on the leaf which is terminated as the female descends the stem of the leaf to the soil where the eggs are deposited[7]. Since females have no access to the leaf interior, their host plant selection can only be based on the shape, color and chemistry of the leaf surface[7,8]. Earlier studies showed that leaf surface compounds appeared to be the most significant stimuli for host plant selection[8]. Crude extracts of the leaf surface were prepared by dipping intact carrot *(Daucus carota)* leaves in methylene chloride twice for 30 sec each. The activity of extracts and of their fractions was tested by applying amounts equivalent to the weight extracted from fresh leaf (~ 6 g) to artificial leaves made of filter paper. The measure of activity was the difference between the number of eggs deposited at the base of the extract-treated leaves with that under the solvent treated leaves[8]. Since our extraction procedure does not disrupt the epidermal cells, the resulting crude extract probably contained little material from the leaf interior[9]. Further evidence for the presence of stimulating compounds on the leaf surface was obtained by extracting leaves with 0.1% aqueous Tween detergent, another relatively mild extraction solvent.

To isolate the active components, the crude CH_2Cl_2 extract (dry wt 330 mg) from 1.5 kg carrot leaves was subjected to 2 fractionations on silica gel columns[10]. The first separation yielded one fraction (100% Et_2O) that was active at a level equivalent to the extract of 10 g of leaves (fresh wt). This fraction was further partitioned into 2 active subfractions: 40 and 100% Et_2O. The 40% Et_2O fraction was active at 3-g leaf weight equivalent (LWE) and GC-MS analysis[11] showed the presence of *trans*-methyl-isoeugenol (**1**) and *trans*-asarone (**2**)[12]. Compound **1** has previously been isolated and identified from macerated carrot leaves[13]. Compound **2** has recently been identified in the head space above carrot leaves[14]. Each compound has been found to be attractive to carrot flies in the field[14]. The 100% Et_2O fraction proved to be active at the 1-g LWE and was separated into 20 subfractions using HPLC[15]. All fractions and subfractions were analysed by GC-MS[11].

The compounds identified in the biologically active subfractions were the substituted coumarin osthol (**3**) and the polyacetylene falcarindiol (**6**)[12]. The search for other coumarins and furano-coumarins in the subfractions and in the original silica-gel fraction (100% Et_2O) resulted in the identification of the 2 furanocoumarins bergapten (**4**) and xanthotoxin (**5**), which were active in the bioassay. Another angular furanocoumarin,

oroselone, identified in one of the active HPLC subfractions, was not active at the available level (200 µg per artificial filter paper leaf). The concentrations of the identified active compounds[16] present in the leaf surface extract, along with their biological activities, are listed in table 1.

It is apparent that only 1 compound, the polyacetylene falcarindiol (**6**), representing 0.2% of the dry crude extract, is active at concentrations equal to or less than those found on a leaf weighing 6 g (6-g LWE). Despite this fact and its very low activity threshold of about 0.3 ng per cm^2, this compound alone is not as stimulatory as the complex mixture of all 6 identified compounds, which together were as active as the original purified crude extract (table 2). The systematic comparison of mixtures of falcarindiol (**6**) and the other compounds with pure falcarindiol revealed that the other compounds significantly synergized the activity of falcarindiol, with osthol (**3**) being the most active compound in this respect.

Given the importance of falcarindiol (**6**) as an oviposition stimulant for the carrot fly, it was interesting to compare its activity with that of structurally related compounds. Both falcarinol (same structure but lacking the -OH group at the C8 position) and falcarinone (the corresponding ketone) were found to be significantly less active than falcarindiol (**6**). The corresponding saturated hydrocarbon (heptadecane) was not active at 1 mg whereas the biosynthetic precursor of falcarindiol, oleic acid (*cis*-9-octadecanoic acid[17], was slightly active at 1 mg per artificial leaf. Falcarinol or falcarinone which occur in carrot roots, were not detectable in the leaf surface extracts. According to chemosystematic studies[18] falcarindiol is the only compound so far exclusively identified in the Umbelliferae, the host plants of the carrot fly, whereas the other identified compounds do also occur in other plant families. This seems to be in accordance with the fact that falcarindiol is the active compound with the lowest threshold. The furano- and substituted coumarins were also identified in the Rutaceae[19]. The propenylbenzenes, asarone and methylisoeugenol have been found, also in 2 apparently unrelated plant families, the Araceae and Aristolochiaceae[18]. Since the 3 groups of compounds were identified together only in Umbelliferae it seems likely that the presence of these compounds on the leaf surface and their perception by the carrot fly explain the observed host plant selection of this oligophagous insect.

All the identified compounds are known to be defensive metabolites of different plant species. The propenylbenzene, *cis*-asarone, is a chemosterilant for a bug[20]. The furanocoumarins have long been known to be photosensitizing, toxic and carcinogenic in a variety of organisms[19]. It is interesting to note that the caterpillars of *Papilio polyxenes*, feeding on the leaves of Umbelliferae have been shown to respond with an increased growth to the presence of Xanthotoxin (**5**) in their food[21]. Thus these non-related Umbelliferae feeding insects both respond positively to the presence of this furanocoumarin. Falcarindiol (**6**), in particular, has been identified recently as a potent preformed inhibitor and a phytoalexin for many fungal parasites[22]. In addition, this compound was found to be neurotoxic for mice[23] and a plant resistance factor against nematodes[24]. In conclusion our study supports the hypothesis that plant protective compounds on the leaf surface are not only detoxified by a specialized herbivores[25] but may also be used to locate and identify host plants[26]. It seems likely that insects ovipositing on or near specific host plants may depend mainly on the chemical signature on the leaf surface. Our results indicate that such a signature is more likely to be a complex mixture than an individual 'key compound'. Furthermore it should be kept in mind that the complex sensory stimuli guiding oviposition and host selection also include visual cues[27]. In future studies special attention should be given to plant defense chemicals on the leaf surface as possible recognition signals for specialized herbivores. Future progress along such lines will be the basis for better understanding of host plant selection, sensory physiology of the herbivores and the evolution of insect-plant relationships.

Table 1. Identified active compounds in the surface extract of carrot leaves

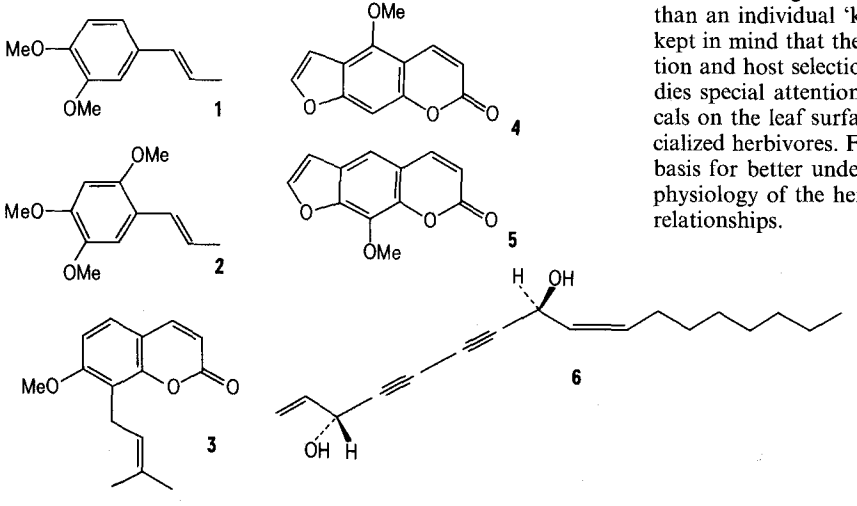
Common name	Amount determined in extract of 6 g leaf*	Estimated threshold** for stimulation of oviposition
1 *Trans*-methylisoeugenol	40 ng	1 mg
2 *Trans*-asarone	920 ng	1 mg
3 Osthol	170 ng	300 µg
4 Bergapten	13 ng	100 µg
5 Xanthotoxin	1 ng	1 mg
6 Falcarindiol	3200 ng	100 ng

* A full-grown carrot leaf weighs about 6 g. ** The given amounts of pure compounds were applied on an artificial filter paper leaf consisting of 2 strips (5 × 15 cm) stapled to a wire (total surface area 300 cm^2).

Table 2. Stimulation of oviposition by the raw extract and a synthetic mixture of the identified compounds

Compared stimuli		No. of counts	Total no. of eggs*	Discrimination coeff.: $\frac{A-B}{A+B} \cdot 100\%$	
I	Raw extract of 6 g leaves (A)	12	1071		
				NS	0.2
II	Synthetic mixture of active compounds** (B)	12	1076		
II	Synthetic mixture of active compounds** (A)	4	607		
				$p < 0.025$	73.2
III	Control: CH_2Cl_2 (B)	4	94		

* Significance of difference between treatments according to Mann-Whitney test. ** Amounts present in 6-g LWE, see table 1.



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10 The 1st column (220 × 20 mm, silicagel 70–230 mesh, Merck) was eluted successively with 200-ml portions of 0, 3, 8, 20% ET_2O in pentane, 100% Et_2O, 20% MeOH in Et_2O and 100% MeOH. The 2nd column (75 × 10 mm) was eluted successively with 15-ml portions of 0, 10, 20, 40% Et_2O in pentane, 100% Et_2O, 100% Et_2O and MeOH.
11 Gas chromatography-mass spectrometry (GC-MS): 25 m SE 54 glass capillary column, 0.31 mm; column temperature 80°C (2 min), 20°C/min to 180°C, 5°C/min to 280°C, vaporizer temp. 240°C, interface temp. 220°C; Finnigan 4000 quadrupole MS operating in electron-impact mode (EI, 70 eV, 240°C) or chemical ionization mode (CI, iso-butane, 0.35 Torr, 180°C), cyclic scanning m/z 35–435 (EI), m/z 85–485 (CI).
12 MS and GC data for the isolated and identified compounds. Most important ions observed are listed from EI data, for compound **6** also from CI data; base peak in italic type: **1**, m/z *178* (M^+), 163, 147, 135, 115, 107, elution temp. 178°C; **2**, m/z *208* (M^+), 193, 165, 163, 150, 147, elution temp. 188°C; **3**, m/z *244* (M^+), 229, 213, 211, 201, 189, elution temp. 216°C; **4**, m/z *216* (M^+), 201, 188, 173, 145, elution temp. 211.5°C; **5**, m/z *216* (M^+), 201, 188, 173, 145, elution temp. 210°C; **6**, m/z (260) (M^+), 242, 171, 157, *129*, 128, 115; CI, m/z 261, 243, 187, *155*, elution temp. 220°C.
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16 **1** and **2** K&K ICN Pharmaceutical Inc., Plainview, N.Y. (USA); **3** E. Lemmich, Danmarks Pharmaceutiske Højskole, Kobenhavn; **4** G. Caporale, Università di Padova, Italy; W. Ivie, College Station, TX (USA); **5** W. Ivie, College Station, TX (USA); Carl Roth, Karlsruhe (FRG); **6** B. Garrod, University of East Anglia, Norwich, England. E. Lemmich, Danmarks Farmaceutiske Højskole, Kobenhavn, K. Munakata, Nagoya University, Japan.
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Inhibition of *Saccharomyces cerevisiae* division by 5-trifluoro-methyl-6-azauracil

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Summary. Cell division, as studied in asynchronous cultures of yeast cells, is sensitive to 5-trifluoromethyl-6-azauracil (F_3CAzU). Under defined conditions (10 mmoles l^{-1} F_3CAzU) this compound blocks immediately and completely the process of cell division. Using synchronized cells, the time-point at which division process of yeast cell can be inhibited by F_3CAzU has been determined. The inhibitory effect of this compound is completely reversed by thymine, thymidine and uracil.
Key words: Saccharomyces cerevisiae; 5-trifluoromethyl-6-azauracil; yeast cell cultures; cell division, inhibition of.

5-Trifluoromethyl-6-azauracil, prepared by Mertes et al.[1,2], Shen et al.[3], and Dipple and Heidelberger[4], was shown[2] to cause 50% inhibition of thymidylate synthetase *(Escherichia coli)* at a concentration of 4 mmoles l^{-1}. However, the in vitro studies carried out with other enzyme preparations[2] as well as in vivo tests in the case of transformed eukaryotic cells[1,2,4] revealed no inhibitory activity or significant cytotoxicity of this compound. In connection with our study of 5-substituted 6-azauracil derivatives as potential fungicides[5] we investigated the effect of F_3CAzU in asynchronous or physiologically synchronized cultures of yeast cells as well as the antagonistic relationship between this compound and preformed pyrimidines.

Material and methods. F_3CAzU was kindly provided by Professor M.P. Mertes of the School of Pharmacy, University of Kansas, Lawrence, Kansas, USA. Thymine, thymidine and uracil were obtained from Calbiochem and hydroxyurea from Serva; lomofungin was kindly provided by Dr. G.B. Whitfield, Upjohn Co., USA. *Saccharomyces cerevisiae* U 92 was from the culture collection of the Prague Institute of Chemical Technology. Difco yeast nitrogen base (B 391) with the addition of 1% glucose was used exclusively as the cultivation medium. The above medium was solidified where necessary with 2% Oxoid agar No.3. Cultivation in liquid medium was carried out under intensive aeration in 10 ml vols at 28°C. The medium was inoculated to a concentration corresponding to an