Infochemicals in a tritrophic system

Interactions between *Brassica*, *Pieris* and *Cotesia*

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Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus
Dr. C.M. Karssen
in het openbaar te verdedigen
op dinsdag 21 juni 1994
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

CIP-gegevens Koninklijke Bibliotheek, Den Haag

ISBN 90-5485-269-0

STELLINGEN

1 Voor het verkrijgen van zuiver dihydroxyaceton fosfaat is de synthese beschreven door Pederson *et al.* niet geschikt.

R. L.Pederson, J. Esker and C-H. Wong, 1991. *Tetrahedron* 47: 2643-2648.

2 Het is niet waarschijnlijk dat de door Takeuchi *et al.* toegewezen cis-ringverknoping van dictamnol juist is.

Takeuchi, N., Fujita, T., Goto, K., Morisaki, N., Osone, N. and Tobinaga, S. 1993. *Chem. Pharm. Bull.* 45: 923-925.
Jenniskens, L.H.D. 1992. Total Synthesis of *cis*-Hydroazulene Sesquiterpenes. Proefschrift, Landbouwuniversiteit Wageningen.

3 De karakterisering van de fase-overgangen van enige 5,15-bis(4'-alkoxyfenyl)-porfyrinatozink(II) verbindingen als overgangen van de kristallijne naar een vloeibare kristallijne, smectische fase is onvoldoende gegrond.

Bruce, D.W., Dunmur, D.A., Santa, L.S. and Wall, M.A. 1992. *J. Mater. Chem.* 2: 363-364
Kugimiya, S. and Takemura, M. 1990. *Tetrahedron Lett.* 31: 3160.
Shimizu, Y., Miya, M., Nagata, A., Ohta, K., Yamamoto, I. and Kusabayashi, S. 1993. *Liquid Crystals* 14: 795-805

4 Onderzoek naar de afgifte van vluchtige signaalstoffen door planten die door herbivoren zijn aangetast moet gebeuren aan de intacte planten en niet aan afgesneden stengels of aan afgesneden bladeren.

5 Ovipositie remmende stoffen worden vaak ten onrechte aangeduid met de term feromoon.

Dit proefschrift.

6 Door Nägele wordt ten onrechte geen rekening gehouden met de verschillen in aantastings mechanisme van verhard cement door ammonium nitraat en ammonium sulfaat bij de interpretatie van de verandering in zeta-potentiaal van cementsteen door aantasting van boven genoemde zouten.

Nägele, E. 1991. *Cem. Concr. Res.* 21: 478-483.

7 Het computertijdperk heeft aan de term discjockey een nieuwe betekenis meegegeven.

8 De uniformiteit binnen de Nederlandse krijgsmacht gaat niet verder dan het GVT (gevechtstenue).

9 De natuurlijke belangstelling voor wetenschap buiten het eigen specialisme bij de deelnemers aan een interdisciplinair project is de bepalende factor voor het welslagen ervan.

10 Het is onwaarschijnlijk dat een honderd pk-grens voor motoren, die wordt nagestreefd door de Europese Commissie, de verkeersveiligheid ten goede komt.

Stellingen behorende bij het proefschrift:"Infochemicals in a tritrophic system, interactions between *Brassica*, *Pieris* and *Cotesia*" door A. Blaakmeer.

Wageningen, 21 juni 1994

Voorwoord

Voordat u begint te lezen of te bladeren in het boekwerk dat voor u ligt, moet ik vermelden dat hoewel er slechts één naam op de voorkant vermeld staat, het proefschrift het resultaat weerspiegelt van de inspanningen van een grotere groep mensen.
Vanaf deze plaats wil ik een ieder, die aan de totstandkoming van dit proefschrift heeft bijgedragen van harte bedanken. Een aantal personen wil ik echter graag met name noemen.

Zonder kool geen vlinders, zonder vlinders geen eieren, zonder eieren geen rupsen en zonder het voorgaande geen onderzoek. Henk Smid, Leo Koopman en André Gidding bedankt voor het kweken van respectievelijk koolplanten en vlinders.
Willem Frentz, één telefoontje (soms twee) naar de Binnenhaven, resulteerde dat logistieke problemen binnen zeer korte tijd werden opgelost. Ook heb je mij de beginselen van MS-DOS bijgebracht. Beide zaken en je belangstelling voor het onderzoek zal ik niet gauw vergeten.
André Stork, ondanks alle problemen tijdens de synthese van miriamide, heb je toch je "gram" gehaald. Daarnaast heb je ook een belangrijk deel van het werk beschreven in hoofdstuk 4 gecoacht.
Frans Griepink, Jan Werkman, Rimko ten Have, Dik Hagenbeek en Dick van de Wal, bedankt voor jullie inzet tijdens doctoraalzoek of afstudeeropdracht.
Gerrit Lelyveld, jouw humor en opgewektheid heeft het lableven aanzienlijk veraangenaamd, daarnaast zorgde je 's morgens altijd voor een "bakkie troost".
Weinig AIO's zullen tijdens hun promotieonderzoek twee begeleiders hebben gehad. Teris van Beek en Joop van Loon, bedankt voor alles wat jullie voor mij hebben gedaan en voor het geen jullie mij hebben bijgebracht.
Aede de Groot en Louis Schoonhoven, bedankt voor de mogelijkheid en de vrijheid die jullie me hebben geboden om dit onderzoek te mogen doen.

Anja, bedankt voor je steun de afgelopen 4½ jaar, speciaal voor al die weekenden dat ik weer zonodig "wilde" werken.

Contents

CHAPTER 1

General introduction

OVERVIEW OF ECOLOGICAL RELATIONSHIPS BETWEEN PLANTS/INSECTS AND INSECTS/INSECTS

For an estimated 250 million years, all plants have been under attack by a diversity of herbivores. Plants have developed a wide range of defence mechanisms, both physical and chemical, against herbivores. The development of spines, prickles, thorns and stinging hairs are examples of morphological adaptations. The reduction of the edibility or nutritive content of the leaves or the evolvement of a toxin, an unpleasant taste or an offensive odour are examples of chemical armoury. It is thought that many insects responded to these changes in plant chemistry by changing their feeding habits thus avoiding the defensive chemicals. Other insects have overcome these changes, using the chemicals as essential cues in host location and selection.

In 1959, Fraenkel was one of the first to voice the suggestion that secondary plant compounds, which were till then regarded by many plant physiologists as waste products of primary metabolism and of no possible use to plants, were directly involved in chemical defence against insects. Six years later, after the influential review of Ehrlich and Raven in 1965, this idea was generally accepted and secondary metabolites became the cornerstone of a new theory of biochemical coevolution between insects and plants.

In 1980, Price *et al.* launched the idea that a theory on insect-plant interactions could not progress realistically without consideration of the third trophic level. Until then, mainly bitrophic systems like herbivore-plant, predator-prey and parasite-host were investigated separately of each other.
An argument in favour of the view that a study of the interactions between plants and insects alone cannot explain the specificity in these interactions is that many processes of resource exploitation are interconnected with each other. In the 1980s, ecologists in cooperation with researchers from other disciplines started studying the more complex tritrophic interactions (Price *et al.*, 1984).

Information exchange between organisms on the three levels of a tritrophic system occurs by means of infochemicals (Dicke and Sabelis, 1988). Infochemicals are categorized into allelochemicals and pheromones. An allelochemical is a chemical that mediates an interaction between two individuals that belong to different species. Pheromones are infochemicals that mediate an interaction between organisms of the same species.

Recently, it has been found that plants damaged by herbivores may produce volatile components which may help parasitoids and predators of the herbivore to locate their herbivorous hosts (Karban and Myers, 1989; Turlings *et al.*, 1990). This can be seen as an indirect defence of the plant against herbivores. A positive effect of this indirect defence mechanism can be a reduced injury of the plant. When this is the case, the infochemicals involved are called synomones.

HOST SELECTION AND OVIPOSITION

Phytophagous insects can be divided in three categories according to their level of specialisation on host plants: polyphagous, oligophagous and monophagous. Polyphagous insects are those that feed on different plant families. Oligophagous insects feed and oviposit on related species belonging to one or only a few taxonomically related plant families with common phytochemical characteristics, and monophagous species feed and oviposit on only one or a few plant species belonging to one genus.

Host selection behaviour is the subject of much research. Host selection by phytophagous insects consists of a sequence of behavioural responses to an array of stimuli associated with host and non-host plants. Insects are equipped with mechano-, visual-, gustatory- and olfactory receptors (Renwick and Radke, 1988). They select their host plants by using a combination of these sensory stimuli (Städler, 1986; Woodhead and Chapman, 1986). Plant odours have been considered to be important cues in host selection for many insects (Visser, 1986).

After being attracted to a plant, several butterflies display a special behaviour. After landing on a leaf, they start drumming, which means that they use their forelegs alternately to drum the leaf surface several times per second. On a host plant, this drumming behaviour is often followed by oviposition and on a non-host plant by taking off.

HOST MARKING PHEROMONES

One of the major activities of an adult female insect is the selection of an oviposition site where her offspring can meet the right conditions for maximal growth. When the insect accepts only a limited number of specific plant species to feed or oviposit on, as is often the case with herbivorous species, there is a fair chance that acceptable oviposition sites are independently discovered by several searching conspecific females. To reduce intraspecific competition, egg-laying females may deposit a chemical substance on or near the eggs. This signals to conspecific females (and also to herself if she happens to visit the same site again) that the site is already occupied. This phenomenon constitutes an important element in foraging strategies of herbivorous insects, since it prompts an even distribution of eggs over available food resources and results in improved resource exploitation (Prokopy *et al.*, 1976; Prokopy, 1981; Roitberg and Prokopy, 1987). Because of their important ecological function these marking substances, often labelled as host marking pheromones (HMP's) or as oviposition deterring pheromones (ODP's), attracted much attention lately, especially since egg-associated substances also may affect related herbivorous species and natural enemies of the herbivores (Prokopy and Webster, 1978; Noldus and van Lenteren, 1985; Schoonhoven *et al.*, 1990; Roitberg and Lalonde, 1991).

A more detailed analysis of the ecological role of a HMP requires its chemical identification. Thus far only a few attempts to identify a HMP have been successful (Hurter *et al.*, 1987; Imai *et al.*, 1990; Thiéry and Le Quéré, 1991). A notable example concerns the cherry fruit fly, *Rhagoletis cerasi*. Females of this species drag their extended ovipositor over the fruit surface after the insertion of an egg. During this dragging, a HMP is deposited which contains N[15(β-glucopyranose)-oxy-8-hydroxypalmitoyl]-taurine as the major biologically active compound (Hurter *et al.*, 1987). Within the Lepidoptera, which comprise the butterflies and moths, several potential uses of a HMP have been reported (Schoonhoven, 1990; Thiéry and Le Quéré, 1991).

The large white butterfly, *Pieris brassicae* L., a specialized insect of cabbage (*Brassica oleracea* L.) and other cruciferous plants, has been studied in great detail (Rothschild and Schoonhoven, 1977; Klijnstra, 1986; Klijnstra and Roessingh, 1986; Klijnstra and Schoonhoven, 1987). Oviposition of *P. brassicae* L. is inhibited when a potential host plant carries conspecific eggs or is sprayed with a methanolic egg wash (Rothschild and Schoonhoven, 1977; Klijnstra, 1986). Inhibition of oviposition is especially pronounced under laboratory conditions when the female butterfly has a choice between HMP-treated plants and control plants. Dispersal activity also appears to increase after contacting HMP (Klijnstra and Schoonhoven, 1987).

HOST PLANT/PARASITOID INTERACTION

One of the large and important groups of natural enemies of herbivorous insects is constituted by the parasitic Hymenoptera, which number about 100,000 species worldwide (Whitman, 1988). One major task faced by a female parasitoid is locating a habitat containing host insects. Initially, the parasitoid may seek a certain environment regardless of the presence or absence of hosts. However, the hosts occur only in specific locations within the environment and a female must locate the micro-habitat where hosts are most likely present. Factors that attract a parasitoid to a plant and retain it in the area have a positive selection value for the plant due to the parasitoid's beneficial effects in reducing herbivore survival and fitness (Karban and Meyers, 1989).

The parasitoids can be attracted by volatiles from different sources like the host-body, frass, scales, honeydew, pheromone gland and so on. However, for long-range attraction, plant chemicals are probably the most important cues for host location by parasitic wasps (Vet and Dicke, 1992). Behavioural bioassays show that parasitoids are often stronger attracted to plants on which their hosts are feeding than to plants without feeding hosts and mechanical damage only. Until now, only a few of the attractive chemicals have been identified (Whitman, 1988). Recent studies (Whitman and Eller, 1990; Turlings *et al.*, 1990; Turlings *et al.*, 1991) show that infested plants release a damage-specific blend of volatiles, which attract natural enemies of the herbivorous insects, possibly functioning as an indirect defence.

The potential value of using natural enemies to control crop pests is great. Especially in glasshouses, in which parasitoids are released, their use has proven to be effective, inexpensive, long-lasting and environmentally sound (van Lenteren and Woets, 1988).

CRUCIFER-INSECT RELATIONSHIPS

Although glucosinolates and their hydrolysis products were early identified as determinants of host plant specificity of cruciferous insects, the specificity of interaction is not exclusively mediated by this group of compounds (Table 1). Other groups of compounds, like flavonoids and cardenolides, also play a role in crucifer-insect interactions. Host plant selection most likely is based on a complex chemosensory balance between stimulants and deterrent compounds, especially in plants containing both types of compounds. Different *Pieris* species can react differently to the same cruciferous plant (Huang *et al.*, 1993).

In general, among crucifer-insect relations, it seems that the volatile isothiocyanates are involved in the attraction of different insects to their host plants (Table 1).

Table 1. Attraction, feeding and oviposition stimulants and deterrents isolated from crucifers for different specialized crucifer feeding insects.

Compound	Behaviour			Species	Reference
Glucosinolates					
different glucosinolates		o		*Delia* spp.	13, 14
		f		*Ceutorhynchus* spp.	20, 27
		f		*Entomoscelis americana* Brown	17
		f/o		*Phyllotreta* spp.	10, 12, 15
		o		*Plutella xylostella* L.	23
sinigrin		f/o		*Pieris* spp.	1, 6, 11
		o		*Plutella xylostella* L.	2, 3
		f		*Ceutorhynchus* spp.	16
		f		*Phaedon cochleariae* Fab.	5
		f		*Phyllotreta armoraciae* Koch	16
		f		*Athalia proxima* Klug	8
		f		*Brevicoryne brassicae* L.	4
glucobrassicin		o		*Pieris* spp.	24
glucoiberin/glucocheirolin		o		*Pieris napi* L.	28
glucobrassicanapin/gluco-napin and glucobrassicin		o		*Delia floralis* Fallen	29
Isothiocyanates					
different isothiocyanates	a			*Ceutorhynchus assimilis* Payk.	19
	a			*Brevicoryne brassicae* L.	25
allylisothiocyanate	a	o		*Delia* spp.	7, 9, 13
	a			*Phyllotreta cruciferae* Goeze	10
Cardenolides			d	*Pieris* spp.	21, 22
Flavonoids		f/o		*Phyllotreta* spp.	15, 18
Cucurbitacin E and I			d	*Phyllotreta nemorum* L.	15
CIF-factor		o		*Delia radicum* L.	26

a: attraction, o: oviposition stimulant, f: feeding stimulant and d: oviposition deterrent.
(1) Verschaffelt, 1910; (2) Thorsteinson, 1953; (3) Gupta and Thorsteinson, 1960; (4) Wensler, 1962; (5) Tanton 1965; (6) Terofal, 1965; (7) Traynier, 1965; (8) Bogawat and Srivastava, 1968; (9) Schnitzler and Muller, 1969; (10) Feeny *et al.*, 1970; (11) Ma and Schoonhoven, 1973; (12) Hicks, 1974; (13) Nair and McEwen, 1976; (14) Nair *et al.*, 1976; (15) Nielsen 1978; (16) Nielsen *et al.*, 1979;(17) Mitchell and Gregory, 1981; (18) Larsen *et al.*, 1982; (19) Kozlowski, 1984; (20) Larsen *et al.*, 1985; (21) Rothschild *et al.*, 1988; (22) Sachdev-gupta *et al.*, 1989; (23) Reed *et al.*, 1989; (24) Traynier and Truscott, 1991; (25) Nottingham *et al.*, 1991; (26) Roessingh *et al.*, 1992a; (27) Larsen, 1992; (28) Huang *et al.*, 1993; (29) Simmonds *et al.*, in prep.

Indole glucosinolates seem to be the strongest oviposition stimulants for butterflies, moths and flies (Table 1). The CIF-factor (CIF means cabbage inducing factor, a newly isolated non-glucosinolate oviposition stimulant with still unknown structure) was isolated from *Brassica oleracea* L. *Delia radicum* L. appears to be a 1000 times more sensitive to this compound than to glucobrassicin, the most active glucosinolate (Roessingh *et al.*, 1992b). This CIF-factor also stimulates the B-type tarsal chemoreceptors of female *Pieris brassicae* L. (J.J.A. van Loon and A. Blaakmeer, unpubl.).

For the monophagous weevil species, other glucosinolates than indole glucosinolates stimulate feeding and oviposition (Table 1).

Studies of artificially damaged or insect infested oilseed rape, mustard and kale plants (Lammerink *et al.*, 1984; Birch *et al.*, 1990; Koritsas *et al.*, 1991; Bodnaryk, 1992) show increased levels of indole glucosinolates. An induced pest-resistance mechanism could be responsible for the observed changes in glucosinolate metabolism. Conceivably the sulphur containing phytoalexins reported in brassicas (Takasugi *et al.*, 1988) are formed from the indole-based glucosinolates.

INSECT PESTS OF CRUCIFERS

Different pests can occur in cabbage crops. The most important pests of Brussels sprouts are various lepidopterous larvae, the cabbage aphid, the cabbage root fly and the Swede midge. The damage not only results in a reduction of the yield but also in a detoriated quality and market value of the crop. In the past, the pests were controlled by preventive sprayings with insecticides. However, total reliance upon pesticides proves in the long run to be counter-effective. Strict adherence to pesticides and regular repetitive spraying may result in an increased resistance of insects to pesticides, detrimental effects on non-target organisms, environmental pollution, potential hazard to the labourers applying the pesticide, phytotoxic residues on crops, spiralling treatment costs and waste of energy. In recent years, the ultimate objective of crop protection extension is the adoption by growers of sound economic crop protection practices, that stress efficient production, and minimize pesticide use (Integrated Pest Management).

Integrated pest control systems are developed for aphids, different caterpillars and cabbage root fly, whereby spraying is only carried out when a given economically relevant density of insects is exceeded (Andaloro *et al.*, 1983). This requires an adequate monitoring of insect density by the growers.

Also, the introduction of seeds coated with insecticides, more selective insecticides and the use of entomopathogens, like granulosis virus, help to reduce the total usage of pesticides and thus to decrease environmental pollution.

In The Netherlands, about 26,800 kg insecticide was used in total for the protection of cabbage crops in 1990 (i.e. 2.4 kg/hectare, Ministerie van Landbouw, Natuurbeheer en Visserij, 1990)

Herbivore/crucifer interactions have been studied extensively compared to the interactions between the complex of cruciferous plants/herbivores and their predators/parasitoids. Until now, only few allelochemicals responsible for the attraction of parasitoids to herbivores (very few of them involved in crucifer-insect relationships) have been isolated and identified (Read *et al.*, 1970; Whitman, 1988; Turlings *et al.*, 1990; Dicke *et al.*, 1990; Turlings *et al.*, 1991; Whitman and Eller, 1992).

PHYTOCHEMISTRY OF CRUCIFERAE

Almost all plants of the Cruciferae, especially those of the genus *Brassica* which comprises the majority of cultivated plants within this family, are characterised by a wide range of secondary plant compounds, known as glucosinolates (Table 2). These glucosinolates and their breakdown products cause the characteristic taste and often pungent odours of cruciferous plants.

The glucosinolates can be hydrolysed by the action of myrosinase. Myrosinase, an enzyme which gets into contact with its substrate when cells are damaged, hydrolyses glucosinolates (I) (Figure 1) by splitting off glucose under formation of an unstable aglucone (II). After rearrangement, different products (isothiocyanates (III), thiocyanates (IV), nitriles (V) or epithionitriles (VI)) are formed, depending on the R-group, reaction conditions and co-factors present in the plant tissue (Figure 1). The hydrolysis products have been extensively studied because some of them can affect the thyroid gland. It is known that goitrin (5-vinyloxazolidine-2-thione)(Figure 2), derived from progoitrin (2-hydroxy-3-butenyl glucosinolate), exerts its effect via interference with thyroxine synthesis. Thiocyanates compete with iodine for uptake by the thyroid gland.

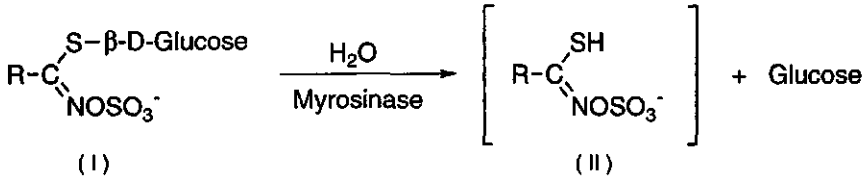
Indole glucosinolates can have carcinogenic properties. The hydrolysis products of these glucosinolates react with nitrites and give carcinogenic N-nitroso compounds, a group of compounds bearing a common functional N-N=O group (Wakabayashi *et al.*, 1985; Tiedink *et al.*, 1991).

Also anti-carcinogenic properties are ascribed to *Brassica* vegetables. This fact is based on epidemiological evidence (Graham, 1983) and results from animal experiments (Wattenberg, 1983; Zhang *et al.*, 1992).

Table 2. Glucosinolates isolated from different *Brassica* species.

Glucosinolate	Source	Reference*
methylthiomethyl	*B. oleracea*	4
3-indolylmethyl	*B. oleracea*	5
1-methoxy-3-indolylmethyl	*B. oleracea*	5
2-phenylethyl	*B. oleracea*	5
propyl	*B. oleracea*	8
isopropyl	*B. alboglabra*	7
1-methylpropyl	*B. nigra*	2
3-methylthiopropyl	*B. oleracea*	3
3-methylsulfinylpropyl	*B. oleracea*	5
2-propenyl	*B. oleracea*	5
butyl	*B. oleracea*	8
4-methylthiobutyl	*B. oleracea*	5
4-methylsulfinylbutyl	*B. oleracea*	5
4-methylsulfonylbutyl	*B. oleracea*	6
2-hydroxy-3-butenyl	*B. oleracea*	5
3-butenyl	*B. oleracea*	5
5-methylthiopentyl	*B. alboglabra*	9
5-methylsulfinylpentyl	*B. alboglabra*	9
4-pentenyl	*B. alboglabra*	9
2-hydroxy-4-pentenyl	*B. alboglabra*	9
hexyl	*B. campestris*	10
6-methylthiohexyl	*B. campestris*	10
benzyl	*B. oleracea*	6
p-hydroxybenzyl	*B. oleracea*	1
indole	*B. alboglabra*	9

* Only the first isolation-report is cited; for author names of *Brassica*'s see Table 3.
(1) Kjaer and Rubenstein, 1954; (2) Nagashima, 1954; (3) Clapp *et al.*, 1959;
(4) Baily *et al.*, 1961; (5) Josefsson, 1967; (6) VanEtten *et al.*, 1976;
(7) Cole, 1976; (8) MacLeod and Nussbaum, 1977; (9) Daxenbichler *et al.*, 1979;
(10) Kameoka and Hashimoto, 1980 .



(III) $R-N=C=S + SO_4^{2-}$

(IV) $R-S-C\equiv N + SO_4^{2-}$

(V) $R-C\equiv N + S + SO_4^{2-}$

(VI) $CH_2-CH-CH_2-C\equiv N + SO_4^{2-}$

S

Figure 1. Enzymatic breakdown of glucosinolates.

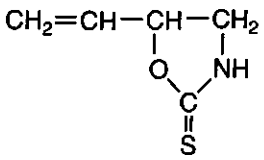


Figure 2. Structure of 5-vinyloxazolidine-2-thione.

THE GENUS *BRASSICA*

The genus *Brassica* comprises a number of vegetables with quite different visual appearances such as red and white cabbage, broccoli, cauliflower, kohlrabi, turnip, swede and Brussels sprouts (Table 3).

Of all *Brassica oleracea* varieties that came about by plant breeding, Brussels sprouts has been developed most recently and is botanically known as *Brassica oleracea* L. var. *gemmifera*. It is an important vegetable in The Netherlands (5,000 hectares in 1989, making up about 50 % of the total cabbage area (IKC-AGV, 1990).

Table 3. List of different *Brassica* species and varieties (adapted from Fenwick et al., 1983).

Brassica oleracea L.	
var. *gongyloides* L.	Kohlrabi
var. *capitata* L.	Red/White cabbage
var. *sabauda* L.	Savoy cabbage
var. *gemmifera* DC.	Brussels sprouts
var. *alba* DC.	Oxheart cabbage
var. *botrytis* L.	
subvar. *cauliflora* DC.	Cauliflower
subvar. *cymosa* Lam.	Sprouting broccoli
var. *acephala DC.*	
subvar. *millecapitata* Thell.	Thousand head kale
subvar. *medullosa* Thell.	Marrowstem kale
subvar. *laciniata* L.	Curley kale
B. alboglabra Baily	Chinese cabbage
B. pekinensis (Lour.) Rupr.	Pe-tsai
B. chinensis L.	
var. *chinensis*	Pak-choi
var. *rosularis* Tsen et Lee	Other oriental greens
B. perviridis Baily	Tendergreen
B. campestris	
spp. *rapifera* (Metzg.) Sinsk	Turnip
spp. *oleifera* (Metzg.) Sinsk	Turnip rape
B. napus L.	
var. *napobrassica* Reichenb.	Swede, rutabaga
var. *napus*	Winter and summer rape
B. nigra (L.) Koch	Black mustard
B. juncea (L.) Czern et Coss	Brown mustard
B. carinata A.Br.	Abyssinian mustard

AIM OF STUDY

This study is part of the ongoing research on insect/plant relationships and tritrophic systems which takes place at the Departments of Entomology and Organic Chemistry. The aim of the study is to isolate and identify infochemicals which are involved in *Cotesia-Pieris*-Crucifer relationships with the prospect of their eventual use in cabbage crop protection. The study focused on two topics: regulation of (1) *Pieris* oviposition behaviour and (2) host selection behaviour of parasitoids of *Pieris* larvae. Both are explained in more detail below.

The host range of *Pieris brassicae* L., the large cabbage white butterfly, and the closely related *Pieris rapae* L., the small cabbage white, is limited to the Cruciferae and a few phytochemically related families (Feltwell, 1982), all of which contain mustard oil glucosides (glucosinolates).

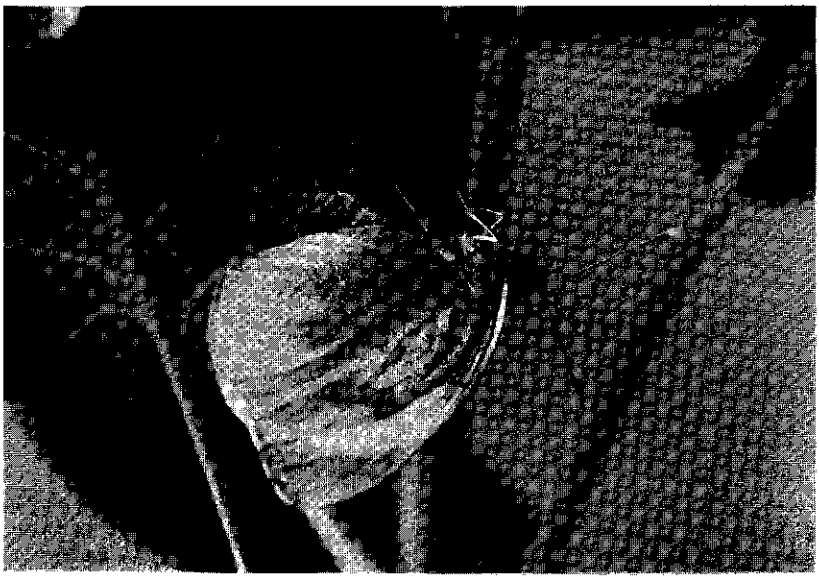


Figure 3. *Pieris brassicae* L. female ovipositing on a leaf of Brussels sprouts.

Glucosinolates are generally considered as making up the primary chemical defence barrier of these plants. Despite the presence of glucosinolates, crucifers are attacked by a wide range of insects (Nair *et al.*, 1976; Nielsen *et al.*, 1989; Landolt, 1989; Reed *et al.*, 1989; Roessingh *et al.*, 1992a).

After being attracted by visual and possibly olfactory cues, *P. brassicae* L. lands on the leaf surface of a plant. Sensilla located on the tarsi contain several specialized chemoreceptors, that can be stimulated by compounds on the leaf surface and/or in the leaf interior.
The identification of the glucosinolate responsible for stimulation of oviposition behaviour is described in chapter 2.
Egg-laying behaviour of *Pieris brassicae* L. females is influenced by previously laid eggs (Rothschild and Schoonhoven, 1977). Females avoid leaves carrying conspecific eggs. It has been suggested that the eggs release chemicals that deter other females from egg-laying at that particular place. These chemicals can be extracted by washing the eggs with water or methanol. An egg wash sprayed onto cabbage leaves was found to be much more deterrent than the presence of an equivalent number of intact eggs. The identification of the substances involved is the subject of chapter 3.
Structure-activity relationships of the isolated compounds and related synthesized structures as oviposition deterrents for *P. brassicae* L. are described in chapter 4.
The question how an ovipositing female, after landing on the upper surface of a cabbage leaf, can perceive the HMP present on the surface of the eggs normally deposited on the underside of the leaf is the subject of chapter 5.

Two *Cotesia* species are natural enemies of *Pieris* larvae. The gregarious endoparasitoid *Cotesia glomerata* L. parasitizes several *Pieris* species. The solitary *Cotesia rubecula* Marshall is considered as a specialized endoparasitoid of the solitarily feeding larvae of *Pieris rapae* L. Chemical information released by plants on which larvae of both *Pieris* species are feeding, may play an important role in host habitat location by these two parasitoids (Steinberg *et al.*, 1992; Kaiser and Cardé, 1992).
Release of volatiles by cabbage plants infested by caterpillars of both *Pieris* species and flight responses of both *Cotesia* parasitoids is the subject of chapter 6.

Figure 4. *Cotesia glomerata* L. female parasitizing a first instar larva of *Pieris brassicae* L..

REFERENCES

Andaloro, J.T., Hoy, C.W., Rose, K.B., Tette, J.P. and Shelton, A.M. 1983. A review of cabbage pest management in New York: from the pilot project to the private sector, 1978-1982. *New York's Food and Life Sciences Bulletin* 105: 1-12.

Baily, S.D., Bazinet, M.L., Driscoll, J.L., McCarthy, A.I. 1961. The volatile sulfur components of cabbage. *J. Food Sci.* 26: 163-170.

Birch, A.N.E., Griffiths, D.W. and MacFarlane Smith, W.H. 1990. Changes in forage and oilseed rape (*Brassica napus*) root glucosinolates in response to attack by turnip root fly (*Delia floralis*). *J. Sci. Food Agric.* 51: 309-320.

Bodnaryk, R.P. 1992. Effects of wounding on glucosinolates in the cotyledons of oilseed rape and mustard. *Phytochemistry* 31: 2671-2677.

Bogawat, J.K. and Srivastava, B.K. 1968. Discovery of sinigrin as a phagostimulant by *Athalia proxima* Klug (Hymenoptera: Tenthredinidae). *Indian J. Entomol.* 30: 89-91.

Clapp, R.C., Long, L.Jr., Dateo, G.P., Bisset, F.H. and Hasselstrom, T. 1959. The volatile isothiocyanates in fresh cabbage. *J. Am. Chem. Soc.* 81: 6278-6281.

Cole, R.A. 1976. Isothiocyanates, nitriles and thiocyanates as products of autolysis of glucosinolates in *Cruciferae*. *Phytochemistry* 15: 759-762.

Daxenbichler, M.E., VanEtten, C.H. and Williams, P.H. 1979. Glucosinolates and derived products in cruciferous vegetables. Analysis of 14 varieties of Chinese cabbage. *J. Agric. Food Chem.* 27:34-37.

Dicke, M. and Sabelis, M.W. 1988. Infochemical terminology: based on cost-benefit analysis rather than origin of compounds? *Funct. Ecol.* 2: 131-139.

Dicke, M., van Beek, T.A., Posthumus, M.A., Ben Dom, N., van Bokhoven, H. and de Groot, Ae. 1990. Isolation and identification of volatile kairomone that affects acarine predator-prey interactions. Involvement of host plant in its production. *J. Chem. Ecol.* 16: 282-396.

Ehrlich, P.R. and Raven, P.H. 1965. Butterflies and plants: a study in co-evolution. *Evolution* 18: 586-608.

Feeny, P., Paauwe, K.L. and Demong, N.J. 1970. Flea beetles and mustard oils: Hostplant specificity of *Phyllotreta cruciferae* and *P. striolata* adults (Coleoptera: Chrysomelidae). *Ann. Entomol. Soc. Am.* 63: 832-841.

Feltwell, J. 1982. The large white butterfly: biology, biochemistry and physiology of *Pieris brassicae* (Linnaeus). Dr. Junk Publishers, The Hague/London.

Fenwick, G.R., Heaney, R.K. and Mullin, W.J. 1983. Glucosinolates and their breakdown products in food and food plants. *CRC Critical Reviews in Food Science and Nutrition* 18: 123-201.

Fraenkel, G. 1959. The raison d'être of secondary plant substances. *Science* 129: 1466-1470.

Graham, S. 1983. Toward a dietary prevention of cancer. *Epidemiol. Rev.* 5: 38-50.

Gupta, P.D. and Thorsteinson, A.J. 1960. Food plant relationships of diamond-back moth (*Plutella maculipennis* (Curt.)) II. Sensory regulation of oviposition of the adult female. *Entomol. Exp. App.* 3: 305-314.

Hicks, K.L. 1974. Mustard oil glucosides: feeding stimulants for adult cabbage flea beetles *Phyllotreta cruciferae* (Coleoptera: Chrysomelidar). *Ann. Entomol. Soc. Am.* 67: 261-264.

Huang, X., Renwick, J.A.A. and Sachdev-Gupta, K. 1993. A chemical basis for differential acceptance of *Erysimum cheiranthoides* by two *Pieris* species. *J. Chem. Ecol.* 19: 195-210.

Hurter, J., Boller, E.F., Städler, E., Blatmann, B., Buser, H.R., Bosshard, N.U., Damn, L., Kozlowski, M.W., Schöni, R., Raschdorf, F., Schlumpf, E., Fritz, H., Richter, W.J. and Schreiber, J. 1987. Oviposition-deterring pheromone in *Rhagoletis cerasi* L.: Purification and determination of the chemical constitution. *Experientia* 43: 157-164.

IKC-PAGV (Informatie- en Kenniscentrum voor de Akkerbouw en de Groenteteelt in de Vollegrond-Proefstation voor de Akkerbouw en de Groenteteelt in de Vollegrond). 1990. Teelt van spruitkool.

Imai, T., Kodama, H., Chuman, T. and Kohno, M. 1990. Female-produced oviposition deterrents of the cigarette beetle, *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae). *J. Chem. Ecol.* 16: 1237-1247.

Josefsson, E. 1967. Distribution of thioglucosides in different parts of *Brassica* plants. *Phytochemistry* 6: 1617-1627.

Kaiser, L. and Cardé, R.T. 1992. In-flight orientation to volatiles from the plant-host complex in *Cotesia rubecula* (Hym.: Braconidae): increased sensitivity through olfactory experience. *Physiol. Entomol.* 17: 62-67.

Kameoka, H. and Hashimoto, S. 1980. Studies on the constituents of the genus *Brassica*. III. The constituents of steam volatile oil from *Brassica rapa* L. var. *laciniifolia* Kitamura. *Nippon Nogei Kagaku Kaishi* 54: 865.

Karban, R. and Myers, J.H. 1989. Induced plant responses to herbivory. *Annu. Rev. Ecol. Syst.* 20: 331-348

Kjaer, A. and Rubenstein, K. 1954. Isothiocyanates. VII. Synthesis of p-hydroxybenzyl isothiocyanate and demonstration of its presence in the glucoside of white mustard (*Sinapis alba* L.). *Acta Chem. Scand.* 8: 598.

Klijnstra, J.W. 1986. The effect of an oviposition deterring pheromone on egg-laying in *Pieris brassicae*. *Entomol. Exp. Appl.* 41: 139-146.

Klijnstra, J.W. and Roessingh, P. 1986. Perception of the oviposition deterring pheromone by tarsal and abdominal contact chemoreceptors in *Pieris brassicae*. *Entomol. Exp. Appl.* 40: 71-79.

Klijnstra, J.W. and Schoonhoven, L.M. 1987. Effectiveness and persistence of the oviposition deterring pheromone of *Pieris brassicae* in the field. *Entomol. Exp. Appl.* 45: 227-235.

Koritsas, V.M., Lewis, J.A. and Fenwick, G.R. 1991. Glucosinolate responses of oilseed rape, mustard and kale to mechanical wounding and infestation by cabbage stem flea beetle (*Psylliodes chrysocephala*). *Ann. Appl. Biol.* 118: 209-221.

Kozlowski, M.W. 1984. Selective responsiveness of the antennal olfactory system in the cabbage seed weevil, *Ceutorhynchus assimilis* towards host plant volatiles. *Acta Physiol. Pol.* 35: 5-6.

Lammerink, J., MacGibbon, D.B. and Wallace, A.R. 1984. Effect of the cabbage aphid (*Brevicoryne brassicae*) on total glucosinolate in the seed of oilseed rape (*Brassica napus*). *New Zealand J. Agric. Res.* 27: 89-92.

Landolt, P.J. 1989. Attraction of the cabbage looper to host plants and host plant odor in the laboratory. *Entomol. Exp. Appl.* 53: 117-124.

Larsen, L.M., Nielsen, J.K. and Sørensen, H. 1982. Identification of 3-0-[2-0-(β-D-xylopyranosyl)-β-D-galactopyranosyl] flavonoids in horseradish leaves acting as feeding stimulants for a flea beetle. *Phytochemistry* 21: 1029-1033.

Larsen, L.M., Nielsen, J.K., Ploger, A. and Sørensen, H. 1985. Responses of some beetle species to varieties of oilseed rape and to pure glucosinolates, pp. 230-244, *in* H. Sørensen (ed.). Advances in the Production and Utilization of Cruciferous Crops. Martinus Nijhoff/Dr. W. Junk Publ., Dordrecht, The Netherlands.

Larsen, L.M., Nielsen, J.K. and Sørensen, H. 1992. Host plant recognition in monphagous weevils: Specialization of *Ceutorhynchus inaffectatus* to glucosinolates from its host plant *Hesperis matronalis*. *Entomol. Exp. Appl.* 64: 49-55.

Ma, W.C. and Schoonhoven, L.M. 1973. Tarsal contact chemosensory hairs of the large white butterfly, *Pieris brassicae*, and their possible role in oviposition behaviour. *Entomol. Exp. Appl.* 16: 343-357.

MacLeod, A.J. and Nussbaum, M.L. 1977. The effects of different horticultural practices on the chemical flavour composition of some cabbage cultivars. *Phytochemistry* 16: 861-865.

Ministerie van Landbouw, Natuurbeheer en Visserij. 1990. Rapportage Werkgroep Vollegrondsgroenteteelt: Achtergronddocument Meerjarenplan Gewasbescherming.

Mitchell, B.K. and Gregory, P. 1981. Physiology of the lateral galeal sensillum in red beetle larvae (*Entomoscelis americana* Brown): responses to NaCl, glucosinolates and other glucosides. *J. Comp. Physiol.* 144: 495-501.

Nagashima, Z. 1954. Studies on wasabi (*Eutrema wasabi*, Maxim)I. An acrid substance of wasabi and mustard (black mustard). *J. Agric. Chem. Soc. Jpn.* 28: 119.

Nair, K.S.S., McEwen, F.L. and Snieckus, V. 1976. The relationship between glucosinolate content of cruciferous plants and oviposition preferences of *Hylemya brassicae* (Diptera: Anthomyiidae). *Can. Entomol.* 108: 1031-1036.

Nair, K.S.S. and McEwen, F.L. 1976. Host selection by the adult cabbage maggot, *Hylemya brassicae* (Dipera: Anthomyiidae): effect of glucosinolates and common nutrients on oviposition. *Can. Entomol.* 108: 1021-1030.

Nielsen, J.K. 1978. Host plant selection of monophagous and oligophagous flea beetles feeding on crucifers. *Entomol. Exp. Appl.* 24: 362-369.

Nielsen, J.K., Larsen, L.M. and Sørensen, H. 1979. Host plant selection of horseradish flea beetle *Phyllotreta aemoraciae* (Coleoptera: Chrysomelidae): identification of two flavonol glucosides stimulating feeding in combination with glucosinolates. *Entomol. Exp. Appl.* 26: 40-48.

Nielsen, J.K., Kirkeby-Thomsen, A.H. and Petersen, M. 1989. Host plant recognition in monophagous weevils: specificity in feeding responses of *Ceutorhynchus constrictus* and the variable effect of sinigrin. *Entomol. Exp. Appl.* 53: 157-166.

Noldus, L.P.J.J. and van Lenteren, J.C. 1985. Kairomones for the egg parasite *Trichogramma evanescens* Westwood: II. Effect of contact chemicals produced by two of its hosts, *Pieris brassicae* L. and *Pieris rapae* L. *J. Chem. Ecol.* 11: 793-800.

Nottingham, S.F., Hardie, J., Dawson, G.W., Hick, A.J., Pickett, J.A., Wadhams, L.J. and Woodcock, C.M. 1991. Behavioral and electrophysiological responses of aphids to host and nonhost plant volatiles. *J. Chem. Ecol.* 17: 1231-1242.

Price, P.W., Bouton, C.E., Gross, P., McPheron, B.A., Thompson, J.N. and Weis, A.E. 1980. Interactions among three trophic levels: influence of plants on interactions between insect herbivores and natural enemies. *Annu. Rev. Ecol. Syst.* 11: 41-65.

Price, P.W., Slobodchikoff, C.N. and Gaud, W.S. 1984. A new ecology. Wiley, New York.

Prokopy, R.J. 1981. Epideictic pheromones that influence spacing patterns of phytophagous insects, pp. 181-213, *in* D.A. Nordlund, R.L. Jones and W.J. Lewis (eds.). Semiochemicals, Their Role in Pest Control. John Wiley & Sons, New York.

Prokopy, R.J., Reissig, W.H. and Moericke, V. 1976. Marking pheromones deterring repeated oviposition in *Rhagoletis* flies. *Entomol. Exp. Appl.* 20: 170-178.

Prokopy, R.J. and Webster, R.P. 1978. Oviposition deterring pheromone of *Rhagoletis pomonella*: A kairomone for its parasitoid *Opius lectus*. *J. Chem. Ecol.* 4: 481-494.

Read, D.P., Feeny, P.P. and Root, R.B. 1970. Habitat selection by the aphid parasite *Diaeretiella rapae* (Hymenoptera: Braconidae) and hyperparasite *Charips brassicae* (Hymenoptera: Cynipidae). *Can. Entomol.* 102: 1567-1578.

Reed, D.W., Pivnick, K.A. and Underhill, E.W. 1989. Identification of chemical oviposition stimulants for the diamondback moth, *Plutella xylostella*, present in three species of Brassicaceae. *Entomol. Exp. Appl.* 53: 277-286.

Renwick, J.A.A. and Radke, C.D. 1988. Sensory cues in host selection for oviposition by the cabbage butterfly, *Pieris rapae*. *J. Insect Physiol.* 34: 252-257.

Roessingh, P., Städler, E., Fenwick, G.R., Lewis, J.A., Nielsen, J.K., Hurter, J. and Ramp, T. 1992a. Oviposition and tarsal chemoreceptors of cabbage root fly are stimulated by glucosinolates and host plant extracts. *Entomol. Exp. Appl.* 65: 267-282.

Roessingh, P., Stadler, E., Hurter, J. and Ramp, T. 1992b. Oviposition stimulant for the cabbage root fly: important new cabbage leaf surface compound and specific tarsal receptors, pp 141-142, *in* S.B.J. Menken, J.H. Visser and P. Harrewijn (eds). Proc. 8th Int. Symp. Insect-Plant Relationships. Kluwer Acad. Publ., Dordrecht.

Roitberg, B.D. and Prokopy, R.J. 1987. Insects that mark host plants. An ecological, ecolutionary perspective on host-marking chemicals. *Bioscience* 37: 400-406.

Roitberg, B.D. and Lalonde, R.G. 1991. Host marking enhances parasitism risk for a fruit-infesting fly *Rhagoletis basiola*. *Oikos* 61: 389-393.

Rothschild, M. and Schoonhoven, L.M. 1977. Assessment of egg load by *Pieris brassicae* (Lepidoptera: Pieridae). *Nature* 266: 532-355.

Rothschild, M., Alborn, H., Stenhagen, G. and Schoonhoven, L.M. 1988. A strophanthidin glycoside in Siberian wallflower: a contact deterrent for the large white butterfly. *Phytochemistry* 27: 101-108.

Sachdev-Gupta, K., Renwick, J.A.A. and Radke, C.D. 1990. Isolation and identification of oviposition deterrents to cabbage butterfly, *Pieris rapae*, from *Erysimum cheiranthoides*. *J. Chem. Ecol.* 16: 1059-1068.

Schnitzler, W.H. and Müller, H.P. 1969. Über die Lockwirkung eines Senföls (Allylisothiocyanat) auf die Große Kohlfliege, *Phorbia floralis* Fallén. *Z. Angew. Entomol.* 63: 1-8.

Schoonhoven, L.M., Beerling, E.A.M., Klijnstra, J.W. and van Vugt, Y. 1990. Two related butterfly species avoid oviposition near each other's egg. *Experientia* 46: 526-528.

Schoonhoven, L.M. 1990. Host-marking pheromones in Lepidoptera, with special reference to two *Pieris* spp. *J. Chem. Ecol.* 16: 3043-3052.

Simmonds, M.S.J., Blaney, W.M., Mithen, R., Birch, A.N. and Fenwick, R. Behavioural and chemosensory responses of turnip root fly (*Delia floralis*) to glucosinolates. In prep.

Städler, E. 1986. Oviposition and feeding stimuli in leaf surface waxes, pp 105-121, in B.E. Juniper and T.R.E. Southwood (eds.). Insects and the Plant Surface. Edward Arnold, London.

Steinberg, S., Dicke, M., Vet, L.E.M. and Wanningen, R. 1992. Response of the braconid parasitoid *Cotesia* (= *Apanteles*) *glomerata* to volatile infochemicals: effects of bioassay set-up, parasitoid age and experience and barometric flux. *Entomol. Exp. Appl.* 63: 163-175.

Takasugi, M., Monde, K., Katsui, N. and Shirata, A. 1988. Novel sulphur-containing phytoalexins from the Chinese cabbage *Brassica campestris* L. spp. *pekinensis* (Cruciferae). *Bull. Chem. Soc. Japan* 61: 285-289.

Tanton, M.T. 1977. Response to food plant stimuli by larvae of the mustard beetle *Phaedon cochleariae*. *Entomol. Exp. Appl.* 22: 113-122.

Terofal, F. 1965. Zum Problem der Wirtsspezifität bei Pieriden (Lep.). *Mitt. Münch. Entomol. Ges.* 55: 1-76.

Thiéry, D. and Le Quéré, J.L. 1991. Identification of an oviposition-deterring pheromone in the eggs of the European Corn Borer. *Naturwissenschaften* 78: 132-133.

Thorsteinson, A.J. 1953. The chemotactic responses that determine host specificity in an oligophagous insect (*Plutella maculipennis* (Curt.) Lepidoptera) *Can. J. Zool.* 31: 52-72.

Tiedink, H.G.M., Malingré, C.E., van Broekhoven, L.W., Jongen, W.M.F., Lewis, J. and Fenwick, G.R. 1991. Role of glucosinolates in the formation of N-nitroso compounds. *J. Agric. Food Chem.* 39: 922-926.

Traynier, R.M.M. and Truscott, R.J.W. 1991. Potent natural egg-laying stimulant for cabbage butterfly *Pieris rapae*. *J. Chem. Ecol.* 17: 1371-1380.

Traynier, R.M.M. 1967. Stimulation of oviposition by the cabbage root fly *Erioischia brassicae*. *Entomol. Exp. Appl.* 10: 401-412.

Turlings, C.J., Tumlinson, J.H., Heath, R.R., Proveaux, A.T. and Doolittle, A.T. 1991. Isolation and identification of allelochemicals that attract the larval parasitoid, *Cotesia marginiventris* (Cresson), to the microhabitat of one of its hosts. *J. Chem. Ecol.* 17: 2235-2251.

Turlings, T.C., Tumlinson, J.H. and Lewis, W.J. 1990. Exploitation of herbivore-induced plant odors by host-seeking wasps. *Science* 250: 1251-1253.

van Lenteren, J.C. and Woets, J. 1998. Biological and integrated pest control in greenhouses. *Annu. Rev. Entomol.* 33: 239-269.

VanEtten, C.H., Daxenbichler, M.E., Williams, P.H. and Kwolek, W.F. 1976. Glucosinolates and derived products in cruciferous vegetables. Analysis of the edible part from twenty-two varieties of cabbage. *J. Agric. Food Chem.* 24: 452-455.

Verschaffelt, E. 1910. The cause determining the selection of food in some herbivorous insects. *Proc. Acad. Sci. Wet. Amsterdam* 13: 536-542.

Vet, L.E.M. and Dicke, M., 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37: 141-172.

Visser, J.H. 1986. Host odor perception in phytophagous insects. *Annu. Rev. Entomol.* 31: 121-144.

Wakabayashi, K., Nagao, M., Tahira, T., Saito, H., Katayama, M., Marumo, S. and Sugimura, T. 1985. 1-Nitrosoindole-3-acetonitrile a mutagen produced by nitrite treatment of indole-3-acetonitrile. *Proc. Jap. Acad.* 61: 199.

Wattenberg. L.W. 1983. Inhibition of neoplasia by minor dietary constituents. *Cancer Res.* 43: 2448-2453.

Wensler, R.J.D. 1962. Mode of host selection by an aphid. *Nature* 195: 830-831.

Whitman, D.W. 1988. Plant natural products as parasitoid cuing agents, pp 386-396 *in* H.G. Cuttler (ed.). Biologically Active Natural Products Potential Use in Agriculture. ACS Symp Ser 380, American Chemical Society, Washington D.C.

Whitman, D.W. and Eller, F.J. 1990. Parasitic wasps orient to green leaf volatiles. *Chemoecology* 1: 69-75.

Whitman D.W. and Eller, F.J. 1992. Orientation of *Microplitis croceipes* (Hymenoptera: Braconidae) to green leaf volatiles: dose-respose curves. *J. Chem. Ecol.* 18: 1743-1753.

Woodhead, S. and Chapman, R.F. 1986. Insect behaviour and the chemistry of plant surface waxes, pp 123-135, *in* B.E. Juniper and T.R.E. Southwood (eds.). Insects and the Plant Surface. Edward Arnold, London.

Zhang, Y., Talalay, P., Cho, C. and Posner, G.H. 1992. A major inducer of anticarcinogenic protective enzymes from broccoli: Isolation and elucidation of structure. *Proc. Natl. Acad. Sci. USA* 89: 2399-2403.

CHAPTER 2

Leaf surface compound from *Brassica oleracea* induces oviposition by *Pieris brassicae*

ABSTRACT

Chemicals present on the surface of cabbage (*Brassica oleracea* L.) leaves were extracted by dipping these leaves for 3 s in dichloromethane followed by a 3 s dip in methanol. When offered in dual choice bioassays using green paper cards as a substrate, the methanol extract stimulated oviposition activity by *Pieris brassicae* L. (Lepidoptera: Pieridae) females. The oviposition stimulant was isolated using medium pressure liquid chromatography, reversed-phase HPLC, ion-pair HPLC and ion exchange chromatography. Using ^{1}H-NMR spectroscopy, the stimulant could be identified as glucobrassicin (3-indolyl-methyl-glucosinolate). When pure glucobrassicin was offered at a dose identical to that in the crude methanol extract, butterflies did not discriminate between these two substrates in a dual choice test. It is argued that a high sensitivity for indole glucosinolates as host recognition factors may confer an adaptive value for these specialist crucifer feeders. The nutritional significance of their precursor tryptophan and the non-volatile nature of the aglycones formed upon enzymic hydrolysis in damaged tissues are proposed as properties of indole glucosinolates that contribute to this possible adaptive advantage.

INTRODUCTION

Most herbivorous insect species accept only a limited number of plant species as hosts. Their behavioural decisions to accept or reject a particular plant species as oviposition substrate or food source are based largely on the perception of the chemical profile of the plant under evaluation (Dethier, 1982). In several cases (but certainly not all, see Jermy, 1984) it has been demonstrated that specific plant chemicals, characteristic for the plant taxon under study, constitute stimuli that induce acceptance. Chemosensory recognition of such 'token stimuli' (Fraenkel, 1959) triggers the behavioural response of oviposition or sustained feeding. One of the first examples of this concept has been the relationship between the cabbage caterpillars *Pieris brassicae* L. and *Pieris rapae* L. and cruciferous plants (Verschaffelt, 1910). Plants belonging to the Cruciferae all contain a class of secondary compounds called glucosinolates

(VanEtten and Tookey, 1979; Fenwick *et al.*, 1983). Schoonhoven (1967) identified chemoreceptors specifically sensitive to glucosinolates on the maxillae of *P. brassicae* L. larvae. As in many herbivorous insects, female *Pieris* adults rather than neonate larvae perform host plant selection. Landing by *Pieris* females is guided mainly by visual cues (Traynier, 1979; Kolb and Scherer, 1982; Renwick and Radke, 1988). After landing on the leaf females drum the leaf surface with their tarsi and it is only after this behavioural step a decision about acceptance or rejection of the plant ensues. Thus, plant selection seems to be based primarily on contact chemoreception (Ma and Schoonhoven, 1973; Renwick and Radke, 1988). Previous studies on both *P. brassicae* L. and *P. rapae* L. adults suggested a role for glucosinolates as host plant specific token stimuli for these butterflies and related species (David and Gardiner, 1962; Ma and Schoonhoven, 1973; Rodman and Chew, 1980; Renwick and Radke, 1983; Traynier, 1984; Traynier and Truscott, 1991). However, none of these studies used an isolation procedure starting with extracts prepared from an intact acceptable host plant, so that definite conclusions about the actual involvement of glucosinolates in host plant recognition by *Pieris* butterflies are not possible. Furthermore, as females seem to evaluate the plant via the leaf surface without contacting the leaf interior (Traynier and Hines, 1987), it is relevant to investigate those compounds that are present on the surface rather than to study total leaf extracts (Städler, 1986; Chapman and Bernays, 1989). This study was designed to isolate and identify oviposition stimulants present on the leaf surface of *Brassica oleracea* L., an acceptable host plant.

MATERIALS AND METHODS

Plant material - *Brassica oleracea* L. var. *gemmifera* cv. Titurel (Cruciferae) plants were reared in a greenhouse until 3 weeks old and then transplanted to a field near Wageningen on April 15, 1990. On each of three occasions, 250 leaves were harvested between October 10 and 17, 1990.

Insects - *P. brassicae* L. adults were obtained from a laboratory colony maintained on *Brassica oleracea* L. This culture was established in 1981 and since then 18 generations were produced each year. Field collected adults have been introduced several times during this period. Rearing conditions were similar to those described by David and Gardiner (1952).

Bioassays - Oviposition preferences were tested in cages measuring 80 × 50 × 100 cm high. The cages were kept in a conditioned greenhouse, with temperatures fluctuating between 22 and 25 °C. In addition to normal daylight, each cage was illuminated from 7.00 till 15.00 h by a 400 Watt mercury vapour lamp hanging 30 cm above the glass roof of the cage.

In each cage 8 females and 4 males were present. Females used in the bioassay had been given the opportunity to oviposit on leaves of *B. oleracea* L. for three days. During the subsequent two days they were offered green paper cards (surface area 80 cm^2) which were sprayed with 1 ml of a 10 mM sinigrin solution in water (obtained from Janssen Pharmaceutica, Tilburg, The Netherlands) on the upper side only, using a chromatographic sprayer (Desaga, Heidelberg, FRG). This two-day training promoted the readiness of females to oviposit on cards. Sinigrin-treated cards were likewise offered during days on which no bioassay was carried out. In the bioassays, cards were sprayed only on the upper surface and the 2 control and 2 treated cards in each cage were placed in diagonally opposite corners, alternated between replicate cages, to minimize positional effects. Butterflies were offered these cards between 9.00 and 15.00 h. Preference of the butterflies was measured by comparing both the number of batches and the total number of eggs deposited on the treated substrates with those on the control substrates. The egg distribution occurring in one cage was considered a replicate. On any one day 6-8 replicates were run. Results of bioassays replicated serially on two consecutive days, were pooled in some cases. The significance of preference was tested with the Wilcoxon's matched pairs signed rank test (Siegel, 1956).

Extraction and fractionation of leaf surface chemicals - Within 30 min after harvest, leaves were dipped in 500 ml dichloromethane. The dichloromethane dip lasted 3 s and was followed by a dip in 500 ml methanol for another 3 s, after a 5 s interval. The choice of this extraction sequence was based on previous experience with a number of apolar and apolar/polar solvent dip sequences (Städler and Roessingh, 1991; van Loon and van Meer, 1991). The three crude methanol dip-volumes prepared on the three harvesting occasions were combined and subsequently divided into batch I, representing 500 leaves and batch II, representing the extracted surface material from 250 leaves. The batch I methanol extract was mixed with water (1:1) and then washed with dichloromethane (2 times 50 ml). The batch II methanol extract was washed with hexane (3 times with 150 ml). The methanol extract was then evaporated to dryness under reduced pressure and dissolved in 10 ml of distilled water. Doses are expressed in gram leaf equivalents (gle), being the amount of surface material extracted from 1 gram of fresh intact leaf. As the average weight of individual leaves was *ca.* 6 g and their average surface area was 80 cm^2 (one side), a dose of 12 gle/artificial leaf corresponded to the amount of surface material extracted from two leaves.

Medium pressure liquid chromatography (MPLC) on Sephasorb - The column (Jobin Yvon, Modulprep compression), diameter 40 mm, containing 300 g Sephasorb HP ultrafine (gel permeation medium separating molecules in the weight range 100-1500; Pharmacia) was used with MeOH-H_2O=80-20 as the mobile phase. Flowrate was 10 ml/min. The detector was a Kratos Spectroflow 773, wavelength of detection was set at 237 nm.

HPLC on C18 - From this point in the separation procedure onwards, the material of batches I and II was processed in different ways. The column used in both cases was an RP C18, 250 × 10 mm, 5 µm particle size, 100 Å pore size (Rainin Instrument Co.). The pumps model 302 and 303, manometric module 802 C, Dynamic mixer 811 and UV-detector 116 (237 nm) were all of Gilson. A software HPLC system manager (model 702) of Gilson was used on an Apple II personal computer. During separation of both batches, the flow rate was 0.9 ml/min. For batch I, the solvent gradient was changed linearly from 0-70% methanol in water during the first 30 min and was then kept at 70% methanol in water from 30-39 min. For batch II, the first 5 min water was used as the solvent, then a linear increase to 100% methanol in 20 min was performed which was maintained during the final 5 min. All separations were performed at 0 °C.

HPLC on C18 using ion-pairing - The same set-up as described above was used for the ion-pair chromatography. The mobile phase contained 0.005 M tetra-butylammonium-sulphate monohydrate (TAS; Betz and Page, 1990). The solvent composition changed in 10 min from 0% MeOH to 50% MeOH in water. This composition was kept constant for another 10 min.

Ion-exchange chromatography - A Lewatit S 1080 (Na^+ form) (Merck) was used as the cation exchange column to remove TAS. Prior to use the column was washed with 15 ml distilled water.

NMR-equipment - The NMR set-up used was a Bruker AC 200E.

RESULTS

The crude methanol-post-dichloromethane extract stimulated oviposition of *P. brassicae* L., as expected (Table 1; van Loon and van Meer, 1991). As a check for possible extraction of interior leaf components, a UV absorption spectrum of the methanolic extract was determined. The absorption maxima typical of chlorophyll were absent, suggesting that disruption of cells in the leaf interior was negligible.

Table 1. Oviposition on artificial leaves by *Pieris brassicae* L. females in a dual choice situation (control *vs.* treated). Treatment consisted of spray-applications of methanolic surface extracts and fractions obtained from these.

Treatment Fraction/extract	dose[c]	control[a] eggs	batches	treated eggs	batches	P[b] eggs	batches	n[d]
Batch I								
methanol, crude[e]	12	195	7	994	27	0.005	0.01	8
methanol, CH_2Cl_2-washed[e]	6	228	8	777	18	0.025	0.025	8
Sephasorb-A	6	0	0	70	2	NS	NS	8
Sephasorb-A	18	20	1	46	1	NS	NS	8
Sephasorb-B	6	315	10	862	21	0.05	0.05	6
Sephasorb-B	12	678	23	2551	62	0.001	0.001	14
B1+B2	12	194	6	1542	49	0.005	0.005	8
B1.11+B2.7	12	131	6	1641	46	0.005	0.005	8
B1.11+B2.8	12	340	10	1035	31	0.01	0.025	8
(B1+B2)-B1.11	12	213	9	287	12	NS	NS	8
glucobrassicin (pure)	12	238	7	934	18	0.01	0.025	8
Batch II								
Sephasorb-A	12	592	14	872	22	NS	NS	6
Sephasorb-A	12	295	12	439	12	NS	NS	6
Sephasorb-B	12	842	29	1514	42	0.005	0.025	8
B1, B3, B4, B5 combined	12	207	11	506	19	0.025	0.05	8
B1, B3, B4, B5 combined	2	346	9	283	8	NS	NS	6
B2.2	12	469	21	949	37	0.05	0.05	10

[a] - controls were sprayed with water, unless indicated otherwise.
[b] - *P* values refer to the maximum one-tailed probability calculated according to Wilcoxon's matched pair signed rank test, under the null hypothesis that total number of eggs or batches were distributed evenly over control and treated artificial leaves. NS means $P > 0.05$.
[c] - gram leaf equivalents of surface extract per artificial leaf
[d] - number of replicate cages used on one day or summed over two consecutive days; each cage contained 8 females
[e] - controls received methanol

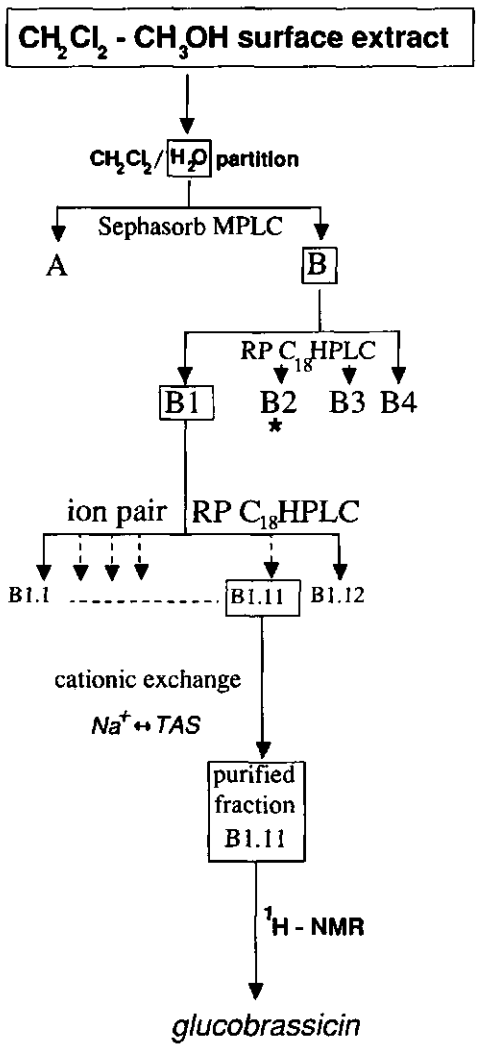


Figure 1. Extraction and purification procedure followed for the isolation of oviposition kairomones for *P. brassicae* L. from *B. oleracea* L. leaves (batch I). * indicates that fraction B2 was further subdivided into 8 fractions (not shown) none of which contained stimulatory activity.

After washing the crude methanol extract to remove apolar material, the dichloromethane (batch I, Table 1) or hexane (batch II, not shown) fractions lacked stimulatory activity. The separation procedure used for batch I is shown in Figure 1. Of the two fractions collected during Sephasorb MPLC, only fraction B stimulated oviposition (Table 1). Fraction B was further separated into four fractions (B1-4) by means of the reversed-phase C18-column.

Fractions B1 and B2 combined showed stimulatory activity (Table 1), while B3 and B4 did not induce oviposition preference for the treated substrate.
Fraction B1 was subdivided into 12 fractions (B1.1 - B1.12) and fraction B2 into 8 fractions (B2.1 - B2.8). Subsequent bioassays of the subfractions containing the material producing the highest extinction values during HPLC (B1.11, B1.12, B2.7 and B2.8), showed that subfraction B1.11 was the most stimulatory, and that pooling of all other 19 subfractions failed to induce preference for the treated substrates.

For batch II, MPLC-fraction B was separated into five fractions: B0-5, of which B0 was the solvent. Subfraction 2 of fraction B2 strongly stimulated oviposition, while the combined fractions B1, B3, B4 and B5 exerted a stimulating effect when applied at a dose of 12 gle/leaf but not at 2 gle/leaf. The activity shown by these fractions was not pursued further. Subfraction B2.1 contained material causing a peak at a retention time identical to that of sinigrin. Its activity was not evaluated.

By means of ^{1}H-NMR analysis following Na^+ - TAS exchange, the fractions B1.11 from batch I and B2.2 from batch II were both identified as pure glucobrassicin. (Table 2; Fig. 2). An authentic reference of glucobrassicin gave an identical NMR spectrum and an identical retention time with ion-pair chromatography on RP C18.

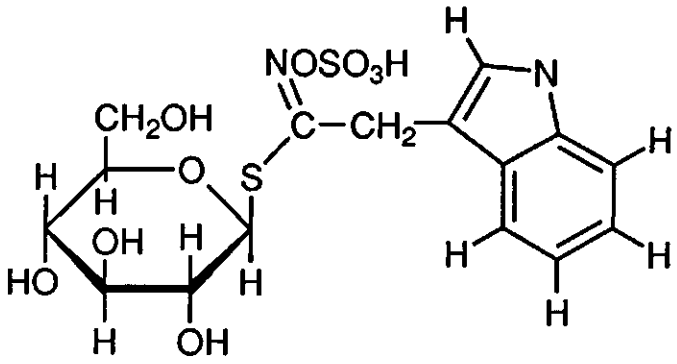


Figure 2. Structural formula of glucobrassicin (3-indolyl-methyl-glucosinolate).

When glucobrassicin was applied in a concentration identical to that in the original methanolic surface extract (based on HPLC-peak areas) and females were offered a choice between these two substrates, they showed no preference (Table 3). The quantity of glucobrassicin extracted was ca. 20 nMol/gle as determined by HPLC.

Table 2. Values of δ from ^{1}H-NMR at 200 MHz in ppm from DSS.

Position	δ	coupling constants
Sugar part		
H2	3.05 (t)	*J* 2-3=9.3 Hz; *J* 2-4=9.3 Hz
H3	2.94 (t)	*J* 3-2=9.3 Hz; *J* 3-4=9.3 Hz
H4	3.11 (t)	*J* 4-3=9.3 Hz; *J* 4-5=9.5 Hz
H5	2.69 (dt)	*J* 5-4=9.5 Hz; *J* 5-6=3.6 Hz
H6	3.33 (d)	*J* 6-5=3.6 Hz
Indole part		
H2	7.21 (s)	
H4	7.39 (br.dd)	
H5/H6	7.00-7.17 (m)	
H7	7.61 (br.dd)	
CH_2	4.09 (AB quartet)	

The average surface area of the cabbage leaves was 160 cm^2, thus these leaves carried on average 0.75 nMol/cm^2 glucobrassicin. A dose of 12 gle/artificial leaf corresponded with a load of glucobrassicin of 3.0 nMol/cm^2. Although the relative effectiveness of sinigrin (applied at 125 nMol/cm^2) and glucobrassicin as oviposition stimulants was not tested in dual choice situations against each other, data on average egg production per female per day in situations where females were sequentially offered one compound at a time suggest that glucobrassicin was at least 20 times more effective than sinigrin on a molar basis.

Table 3. Oviposition on artificial substrates by *Pieris brassicae* L. females in a dual choice situation between artificial leaves sprayed with either a total crude methanolic leaf surface extract or pure glucobrassicin at a dose equal to that in the crude extract.

Experiment	dose[b]	total surface extract		glucobrassicin		P[a]		n[c]
		eggs	batches	eggs	batches	eggs	batches	
1	12	3811	63	2756	50	NS	NS	12
2[d]	2	2120	57	1867	47	NS	NS	9

[a] - *P* values refer to the two-tailed probability calculated according to Wilcoxon's matched pair signed rank test, under the null hypothesis that total number of eggs or batches were distributed evenly over control and treated artificial leaves. NS - $P > 0.05$

[b] - gram leaf equivalents of surface extract per artificial leaf

[c] - number of replicate cages used on one day or summed over two or three consecutive days; each cage contained 8 females

[d] - cage size and number of artificial leaves available were doubled for this experiment

DISCUSSION

This study has revealed that for *P. brassicae* L. a single compound present on the leaf surface may be largely responsible for host plant recognition. Indeed, for the related *P. rapae* L. and *Plutella xylostella* L. (Lepidoptera: Plutellidae) the situation is very similar (Reed *et al.*, 1989; Traynier and Truscott, 1991; Renwick *et al.*, 1992). In contrast, recent studies on three *Papilio* species have shown that the presence of at least two chemically diverse compounds was needed together to obtain stimulation of oviposition activity similar to that of crude total leaf extracts (Honda, 1986; Feeny *et al.*, 1988).

The results of our dual choice bioassay employed, which was preceded by an experience with sinigrin treated artificial leaves must be interpreted cautiously. This is due to the ability for associative learning that has been demonstrated for *P. rapae* L. butterflies (Traynier, 1984; 1986). *P. brassicae* L. females have been found to possess similar learning capabilities (van Loon *et al.*, 1992). The involvement of experience in the measurement of oviposition preference was indicated by the following observation. When a test substrate was highly stimulatory, the control substrate also received many eggs also, a phenomenon reported earlier for *P. rapae* L. (Traynier, 1984 and 1986). In dual choice situations, a significantly positive relationship was observed between the number of eggs deposited on control substrates and substrates sprayed with the crude methanol extract (Spearman's rho = 0.85, $P = 0.007$; van Loon and van Meer, 1991). This phenomenon will lead to an underestimation of stimulatory activity. It also implies that the bioassay possesses a limited sensitivity to demonstrate differences between two highly stimulatory substrates, as is the case with the comparison of the parent surface extract and pure glucobrassicin (Table 3). A no-choice assay method, that measures the probability of oviposition of individual females that were not offered the opportunity to associate the visual and mechanosensory quality of the test substrate with a pure stimulant prior to the bioassay, would be better suited to compare the activity of the parent surface extract with a putative major kairomone like glucobrassicin. Other fractions seemed to have some stimulatory activity but were not pursued further (Table 1). There may yet be other stimulants that we did not detect using our method. If they do exist, however, they play only a minor role (Table 3). Nevertheless it is evident from this study and from the data on *P. rapae* L. reported by Traynier and Truscott (1991) that glucobrassicin is much more powerful stimulant than sinigrin.

The amounts of glucobrassicin calculated to be present on the original cabbage leaf surface must be interpreted as a minimum figure. Apart from the usual losses occurring during fractionation and purification procedures, the extraction of substances from the intact leaf surface has probably been far from exhaustive.

For instance, the yield of apolar waxy material in the dichloromethane fraction using a 3 s dip was only 20% of the amounts of wax reported for a range of glaucous cabbage cultivars (61 µg/cm^2, using 3 dichloromethane dips of 10 s each, Eigenbrode *et al.*, 1991). In this study short dips were used to keep the cuticle intact and prevent leakage of internal leaf components. The amount of glucobrassicin per gle of surface extract is about 34% of that found in a surface extract of a cauliflower cultivar by Roessingh *et al.* (1992).

The forceful tarsal drumming of the leaf surface by exploring butterflies is well known (Feeny *et al.*, 1983; Städler, 1986) and in view of the presence of large spines on butterfly tarsi it is conceivable that a function of this behaviour is penetration of the leaf cuticle. Penetration of stomata by chemosensory sensilla is unlikely (Chapman, 1977). The question of damage done to the leaf by this drumming or other tarsal contacts, in the sense of penetration of the leaf cuticle and the subsequent release of chemicals from damaged cells in the leaf interior has been studied in only a few cases with opposite results (Boppré, 1983; Traynier and Hines, 1987). The approach followed in this study only allows us to conclude that damage is not required to perceive the oviposition kairomone, assuming that leakage from the leaf interior was indeed negligible.

The present results add another case to the list of studies that have demonstrated the presence of polar compounds at the essentially apolar surface of plants (for review, see Städler, 1986). It is of interest to note that partial removal of the apolar wax layer by chloroform or dichloromethane was necessary prior to successful extraction of the oviposition stimulants by methanol washings (van Loon and van Meer, 1991). This suggests that the polar compounds are present at some depth in the waxy surface layer, maybe in a bound form. At present it is unknown how polar compounds that are present in either bound or free form in the apolar leaf surface environment become available to the contact chemosensilla of insects. The sensillum lymph surrounding chemosensory dendrites has a definite polar and lipophobic character (Kaissling and Thorson, 1980), which at first sight seems to make it unadapted for gustation of apolar surfaces. However, a recent study has demonstrated the existence of a considerable outward flow (>3 µm^3/s) of sensillum lymph in contact chemosensilla of the fly, which can dissolve crystalline sodium chloride (Gödde, 1991). A comparable extrusion of sensillar fluid from butterfly tarsal sensilla to the leaf surface is hypothesized in order to understand the perception of surface chemicals.

Evolutionary aspects

A number of recent studies have demonstrated that indole glucosinolates are the most powerful oviposition kairomones among the glucosinolates for *P. brassicae* L., *P.rapae* L. and *Delia radicum* L. (Diptera; Anthomyiidae) and *D. floralis* Fallen, all crucifer specialists (Renwick *et al.*, 1992; Roessingh *et al.*, 1992; Simmonds *et al.*, in prep.). For *Plutella xylostella* L. glucobrassicin was as stimulatory as aromatic and aliphatic glucosinolates (Reed *et al.*, 1989). The high sensitivity for indole glucosinolates of crucifer specialists may be hypothesized to have several evolutionary advantages. Firstly, the glucosinolates are biosynthetically derived from amino acids (Fenwick *et al*, 1983) and indole glucosinolates are derived from the amino acid tryptophan, which is nutritionally essential to insects (Dadd, 1985). The tryptophan content of plant proteins is low and may thereby constitute one of the factors limiting protein synthesis and growth of larvae.
Tryptophan levels in *B. oleracea* L. varieties range between 40 - 72 mg/100 g fresh weight (average 50 mg/100 g) and indole glucosinolate content ranges between 15 and 124 mg/100 g (average 50 mg/100 g; VanEtten and Tookey, 1979; Heany and Fenwick, 1980; Fenwick *et al.*, 1983). It follows that the amount of tryptophan channelled into the biosynthesis of indole glucosinolates is substantial. Thus it seems of functional significance that ovipositing females select plants that contain high levels of tryptophan or its derivatives that may possibly be utilized nutritionally by adapted feeders like *Pieris* larvae. *P. brassicae* L. and *P. rapae* L. larvae both possess an amino acid receptor cell that is sensitive to tryptophan (van Loon and van Eeuwijk, 1989) and glucobrassicin is the most effective stimulus for the lateral glucosinolate receptor of *P. brassicae* L. (Schoonhoven, 1969). In this scenario, glucobrassicin would act as a nutritive signal exposed on the plant surface. Unfortunately, despite the attention paid to glucosinolates in insect-plant research (Chew, 1988a), virtually nothing is known about the metabolic fate of glucosinolates after ingestion by larvae (Chew, 1988b). There is evidence that glucosinolates are sequestered (Aplin *et al.*, 1975). It would certainly be of interest to investigate which biochemical mechanisms enable *Pieris* larvae to deal with the ingested glucosinolates, in view of their documented toxicity and deterrency to non-adapted organisms (Fenwick *et al.*, 1983; Chew, 1988b). An additional positive effect of indole glucosinolates may be attributed to their potential as precursors of indole compounds that have antineoplastic and antimutagenic effects (see for review McDanell *et al.*, 1988).

A second advantage of glucobrassicin as a host recognition factor could be that the indole glucosinolates do not yield volatile aglycones (mustard oils) as products of the enzymatic hydrolysis by myrosinase (Fenwick *et al.*, 1983), while most other groups of glucosinolates do yield such volatiles. This could represent an important advantage as host plant specific volatile products of damage caused by feeding larvae may betray the presence of these larvae

to either more specialized natural enemies such as parasitoids or generalist predators that use these signals to locate their host or prey (Whitman, 1988; Vet and Dicke, 1992). The hymenopterans *Cotesia glomerata* L. and *C. rubecula* Marshall in particular are important parasitoids of *P. brassicae* L. and *P. rapae* L. respectively and can cause considerable mortality under field conditions (Laing and Levin, 1982). Electroantennogram studies indicate that female wasps can smell several isothiocyanates (A. Blaakmeer and J.J.A. van Loon, unpubl. results), although it remains to be shown that the wasps actually use the potential information during host location behaviour.

In conclusion, *P. brassicae* L. females can make use of the single indole glucosinolate glucobrassicin as a host recognition factor, which is present on the surface of cabbage leaves, a favoured oviposition substrate. Improvements in phytochemical separation techniques for glucosinolates have contributed to the recent discoveries of the special role of indole glucosinolates in several insect/crucifer relationships. This justifies renewed interest in the differential effects that glucosinolates may have on specialist herbivores.

REFERENCES

Aplin, R.T., Ward, R.A. and Rothschild, M. 1975. Examination of the large white and small white butterflies (*Pieris* spp.) for the presence of mustard oils and mustard oil glycosides. *J. Entomol.* (A) 50: 73-78.

Betz, J.M. and Page, S.W. 1990. Liquid chromatographic method for the determination of intact, non-derivatized glucosinolates from Brassicaceae, pp 103-104, *in* Proceedings of the Symposium Biology and Chemistry of Active Natural Substances. Bonn.

Boppré, M. 1983. Leaf scratching - a specialized behaviour of danaine butterflies (Lepidoptera) for gathering secondary plant substances. *Oecologia* 59: 414-416.

Chapman, R.F. 1977. The role of the leaf surface in food selection by acridids and other insects. *Colloques Internationaux du C.N.R.S.* 265: 133-149.

Chapman, R.F. and Bernays, E.A. 1989. Insect behavior at the leaf surface and learning as aspects of host plant selection. *Experientia* 45: 215-222.

Chew, F.S. 1988a. Biological effects of glucosinolates, pp 155-181, *in* H.G. Cutler (ed.). Biologically Active Natural Products for Potential Use in Agriculture. ACS Symposium Series.

Chew, F.S. 1988b. Searching for defensive chemistry in the Cruciferae, or, do glucosinolates always control interactions of Cruciferae with their potential herbivores and symbionts? No! Pp 81-112, *in* K.C. Spencer (ed.). Chemical Mediation of Coevolution. Plenum Press, New York.

Dadd, R.H. 1985. Nutrition: organisms, pp 131, *in* G.A. Kerkut and L.A. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 4. Pergamon Press, New York.

David, W.A.L. and Gardiner, B.O.C. 1952. Laboratory breeding of *Pieris brassicae* L. and *Apanteles glomerata* L. *Proc. R. Entomol. Soc. Lond. (A)* 27: 54-56.

David, W.A.L. and Gardiner, B.O.C. 1962. Oviposition behaviour and the hatching of the eggs of *Pieris brassicae* L. in a laboratory culture. *Bull. Entomol. Res.* 53: 91-109.

Dethier, V.G. 1982. Mechanisms of host plant recognition. *Entomol. Exp. Appl.* 31: 49-56.

Eigenbrode, S.D. Stoner, K.A., Shelton, A.M. and Kain, W.C. 1991. Characteristics of glossy leaf waxes associated with resistance to diamond back moth (Lepidoptera: Plutellidae) in *Brassica oleracea*. *J. Econ. Entomol.* 84: 1609-1618.

Feeny, P., Rosenberry, L. and Carter, M. 1983. Chemical aspects of oviposition behaviour in butterflies, pp 27-76, *in* S. Ahmad (ed.). Herbivorous Insects: Host-seeking behavior and mechanisms. Academic Press, New York.

Feeny, P., Sachdev, K., Rosenberry, L. and Carter, M. 1988. Luteolin 7-O-(6''-O-malonyl)-ß-D-glucoside and *trans*-chlorogenic acid: oviposition stimulants for the black swallowtail butterfly. *Phytochemistry* 27: 3439-3448.

Fenwick, G.R., Heany, R.K. and Mullin, W.J. 1983. Glucosinolates and their breakdown products in food and food plants. *CRC Critical Reviews in Food Science and Nutrition* 18: 123-201.

Fraenkel, G.S. 1959. The raison d'être of secondary plant substances. *Science* 129: 1466-1470.

Gödde, J. 1991. Periceptor-events modulate spike responses in labellar "largest" taste hairs of *Protophormia*, *in* Synapse, Transmission, Modulation. Proceedings of the 19th Göttingen Neurobiology Conference. Georg Thieme Verlag, Stuttgart.

Heany, R.K. and Fenwick, G.R. 1980. Glucosinolates in *Brassica* vegetables. Analysis of 22 varieties of Brussels Sprout (*Brassica oleracea* var. *gemmifera*). *J. Sci. Food Agric.* 31: 785-793.

Honda, K. 1986. Flavanone glycosides as oviposition stimulants in a papilionid butterfly, *Papilio protenor*. *J. Chem. Ecol.* 12: 1999-2010.

Kaissling, K.E. and Thorson. 1980. Insect olfactory sensilla: structural, chemical and electrical aspects of the functional organization, pp 261-282, *in* J.B. Sattelle, L.M. Hall and J.G. Hildebrand (eds.). Receptors for Neurotransmitters, Hormones and Pheromones in Insects. Elsevier/North Holland, Amsterdam.

Jermy, T. 1984. Evolution of insect/host plant relationships. *Am. Nat.* 124: 609-630.

Kolb, G.K. and Scherer, C. 1982. Experiments on wavelength-specific behavior of *Pieris brassicae* L. during drumming and egg-laying. *J. Comp. Physiol.* 149: 325-332.

Laing, J.E. and Levin, D.B. 1982. A review of the biology and a bibliography of *Apanteles glomeratus* (L.) (Hymenoptera: Braconidae). *Biocontrol News and Information* 3: 7-23.

Ma, W.C. and Schoonhoven, L.M. 1973. Tarsal contact chemosensory hairs of the large white butterfly *Pieris brassicae* and their possible role in oviposition behaviour. *Entomol. Exp. Appl.* 16: 343-357.

McDanell, R., McLean, A.E.M., Hanley, A.B., Heany, R.K. and Fenwick, G.R. 1988. Chemical and biological properties of indole glucosinolates (glucobrassicins): A review. *Food Chem. Toxicol.* 26: 59-70.

Reed, D.W., Pivnick, K.A. and Underhill, E.W. 1989. Identification of chemical oviposition stimulants for the diamondback moth, *Plutella xylostella*, present in three species of Brassicaceae. *Entomol. Exp. Appl* 53: 277-286.

Renwick, J.A.A. and Radke, C.D. 1983. Chemical recognition of host plants for oviposition by the cabbage butterfly, *Pieris rapae* (Lepidoptera: Pieridae). *Environ. Entomol.* 12: 446-450.

Renwick, J.A.A. and Radke, C.D. 1988. Sensory cues in the host selection for oviposition by the cabbage butterfly, *Pieris rapae*. *J. Insect Physiol.* 34: 251-257.

Renwick, J.A.A., Radke, C.D., Sachdev-Gupta, K. and Städler, E. 1992. Leaf surface chemicals stimulating oviposition by *Pieris rapae* (Lepidoptera: Pieridae) on cabbage. *Chemoecology* 3: 33-38.

Rodman, J.E. and Chew, F.S. 1980. Phytochemical correlates of herbivory in a community of native and naturalized Cruciferae. *Biochem. Syst. Ecol.* 8: 43-50.

Roessingh, P., Städler, E., Fenwick, G.R., Lewis, J.A., Kvist Nielsen, J., Hurter, J. and Ramp, T. 1992. Oviposition and tarsal chemoreceptors of the cabbage root fly are stimulated by glucosinolates and host plant extracts. *Entomol. Exp. Appl.* 65: 267-282.

Schoonhoven, L.M. 1967 Chemoreception of mustard oil glucosides in larvae of *Pieris brassicae*. *Proc. R. Acad. Amsterdam Series C*. 70: 556-568.

Schoonhoven, L.M. 1969. Gustation and foodplant selection in some lepidopterous larvae. *Entomol. Exp. Appl.* 12: 555-564.

Siegel, S. 1956. Nonparametric Statistics for the Behavioral Sciences. John Wiley, New York.

Städler, E. 1986. Oviposition and feeding stimuli in leaf surface waxes, pp 105-121 *in* B.E. Juniper and T.R.E. Southwood (eds.). Insects and the Plant Surface. Edward Arnold, London.

Städler, E. and Roessingh, P. 1991. Perception of surface chemicals by feeding and ovipositing insects, pp 71-86, *in* A. Szentesi and T. Jermy (eds.). Proc. 7th Int. Symp. Insect-Plant Relationships. Symp Biol Hung Vol 39. Akadémia Kiadó, Budapest.

Traynier, R.M.M. 1979. Long term changes in the oviposition behavior of the cabbage butterfly *Pieris rapae* induced by contact with plants. *Physiol. Entomol.* 4: 87-96.

Traynier, R.M.M. 1984. Associative learning in the ovipositional behaviour of the cabbage butterfly, *Pieris rapae. Physiol. Entomol.* 9: 465-472.

Traynier, R.M.M. 1986. Visual learning in assays of sinigrin solution as an oviposition releaser for the cabbage butterfly, *Pieris rapae. Entomol. Exp. Appl.* 40: 25-33.

Traynier, R.M.M. and Hines, E.R. 1987. Probes by aphis indicated by stain induced fluorescence in leaves. *Entomol. Exp. Appl.* 45: 198-201.

Traynier, R.M.M. and Truscott, R.J.W. 1991. Potent natural egg-laying stimulant for cabbage butterfly *Pieris rapae. J. Chem. Ecol.* 17: 1371-1380.

VanEtten, C.H. and Tookey, H.L. 1979. Chemistry and biological effects of glucosinolates, pp 471-500 *in* G.A. Rosenthal and D.H. Janzen (eds.). Herbivores, Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

van Loon, J.J.A. and van Eeuwijk, F.A. 1989. Chemoreception of amino acids in larvae of two species of *Pieris*. *Physiol. Entomol.* 14: 459-469.

van Loon, J.J.A. and Meer, M.M.M. 1991. Chemosensory perception of leaf surface chemicals by ovipositing *Pieris brassicae* L. butterflies. *Proc. Entomol. Exp. Appl.* 2: 56-61.

van Loon, J.J.A., Everaarts, T.C. and Smallegange, R.C. 1992. Associative learning in host-finding by female *Pieris brassicae* butterflies: relearning preferences, pp. 162-164, *in* S.B.J. Menken, J.H. Visser and P. Harrewijn (eds.). Proc. 8th Int. Symp. Insect-Plant Relationships. Acad. Publ., Dordrecht.

Verschaffelt, E. 1910. The cause determining the selection of food in some herbivorous insects. *Proc. Roy. Acad. Amsterdam* 13: 536-542.

Vet, L.E.M. and Dicke, M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37: 141-172.

Whitman, D.C. 1988. Plant natural products as parasitoid foraging cues, pp 386-396, *in* H.G. Cutler (ed.). Biologically Active Natural Products. American Chemical Society, Washington D.C.

CHAPTER 3

Isolation, identification and synthesis of miriamides, new hostmarkers from eggs of *Pieris brassicae*

ABSTRACT

The large white butterfly, *Pieris brassicae* L., a herbivorous pest of crucifers, produces egg-associated chemical markers that inhibit its oviposition. The identification of the marker compounds is reported herein. Separation by means of reversed-phase HPLC demonstrated the presence of three active substances, which were identified as *trans*-2-[3-(3,4,5-trihydroxy-phenylpropenoyl)-amino]-3,5-dihydroxy-benzoic acid {**1**}, *trans*-2-[3-(3,4-dihydroxy-5-β-glucopyranose-phenylpropenoyl)amino]-3,5-dihydroxybenzoic acid {**2**} and *trans*-2-[3-(3,4-dihydroxyphenylpropenoyl)amino]-3,5-dihydroxybenzoic acid {**3**} using mass- and NMR-spectroscopy and chemical synthesis. This group of compounds has not been reported from animal kingdom before. The same compounds are produced by two related *Pieris* species. This is the first report of taxon-specific compounds affecting butterfly oviposition behavior. The availability, stability and inhibitory action on colonisation of cabbage plants by butterflies make application of these compounds in the protection of cabbage crops feasible and comparable with other environmentally safe crop protection strategies.

INTRODUCTION

Females of several herbivorous insect species are known to deposit a marking substance on or near the eggs (Prokopy *et al.*, 1976; Prokopy, 1981; Roitberg and Prokopy, 1987). This substance signals to conspecific females (and also to herself if she happens to visit the same site again) that the site is already occupied. This phenomenon constitutes an important element in foraging strategies of herbivorous insects, because it prompts an even distribution of eggs over the available food resources, results in reduction of intraspecific competition and improves resource exploitation. Because of their important ecological function these host marking pheromones (HMP's), formerly often labelled as oviposition deterring pheromones (ODP's), attract much attention. Egg-associated substances also affect related herbivorous

species and natural enemies of the herbivores (Prokopy and Webster, 1978; Noldus and van Lenteren, 1985; Schoonhoven *et al.*, 1990; Roitberg and Lalonde, 1991). Oviposition deterring activity has also been found in faeces from larvae (Renwick and Radke, 1980; Dittrick *et al.*, 1983; Klein *et al.*, 1990). A more detailed analysis of the ecological role of an HMP requires its chemical identification. Thus far only a few attempts to identify an egg-associated HMP have been successful (Hurter *et al.*, 1987; Imai *et al.*, 1990; Thiéry and Le Quéré, 1991). A notable example concerns the cherry fruit fly, *Rhagoletis cerasi* L. (Hurter *et al.*, 1987).

Within the Lepidoptera, several cases of potential use of an HMP have been reported (Rothschild and Schoonhoven, 1977; Schoonhoven, 1990). The large white butterfly, *Pieris brassicae* L., a specialized herbivore of cabbage (*Brassica oleracea* L.) and other cruciferous plants, has been studied in detail (Rothschild and Schoonhoven, 1977; Klijnstra, 1986; Klijnstra and Roessingh, 1986). Oviposition by *P. brassicae* L. is inhibited when a potential host plant carries conspecific eggs or is sprayed with a methanolic egg wash (Rothschild and Schoonhoven, 1977; Klijnstra, 1986). Inhibition of oviposition is especially pronounced when females have a choice between HMP-treated plants and control plants, or when dispersal activity can be manifested (Klijnstra and Schoonhoven, 1987). Based on a two-choice bioassay the inhibition of oviposition by egg-associated compounds was quantified (Klijnstra and Roessingh, 1986). Herein the identification and synthesis of HMP's isolated from eggs of *Pieris brassicae* L. is presented.

RESULTS

Eggs of *P. brassicae* L., freshly laid on cabbage leaves, *Brassica oleracea* L. var. *gemmifera* cv. Titurel, were collected. Out of 20 HPLC fractions, 4 (9-12) possessed oviposition deterring activity (Figure 1). Progressive bioassay-guided purification showed that three different active compounds were present in these fractions.

Fractions 10 and 11 contained the main component **1**. Compound **1** was very polar and failed to give a molecular ion under normal EI mass spectroscopic conditions. However negative-ion FABMS gave an $[M-H]^-$ peak at *m/z* 346.0572, corresponding with a molecular formula of $C_{16}H_{13}NO_8$. Its nitrogen was expected to be present in the form of a primary or secondary aromatic amine or an amide. No alkaloid reaction with Dragendorff reagent was given by **1**. The high UV absorbance (λ_{max} (MeOH) 353 nm) suggested a highly conjugated system. The ^1H NMR spectrum (Table 1) was very simple and indicated two aromatic rings with two

protons each and one double bond with a trans configuration (J=15.5 Hz). The chemical shifts of the two double bond protons (6.35 and 7.45) are characteristic for a cinnamic acid derivative. One ring contained two equivalent protons, whereas the other ring contained two non-equivalent protons meta to each other (J=2.7 Hz).

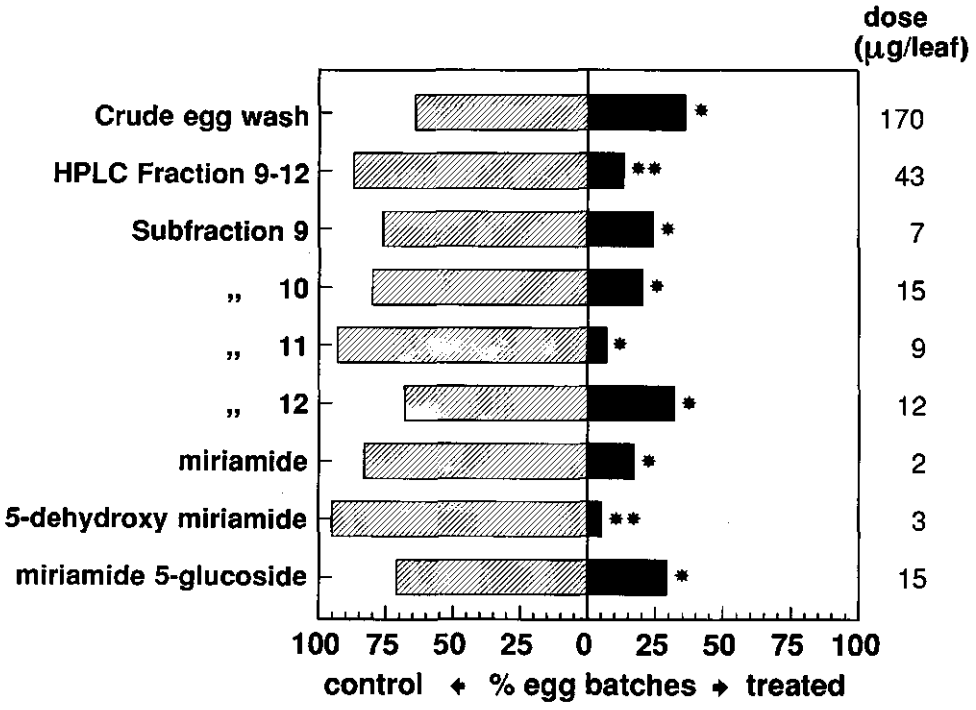


Figure 1. Oviposition preferences displayed by *Pieris brassicae* L. female butterflies in a dual choice situation. Asterisks (*) indicate that treated leaves were significantly less preferred according to Wilcoxon's matched pair signed rank test (two tailed; 23), under the null hypothesis that the total number of batches were distributed evenly over control and treated leaves.
* $0.01 < P < 0.05$; ** $P < 0.01$.

The ^{13}C NMR spectrum (Table 2) showed 16 C atoms and was thus in accordance with the MS data. The two-dimensional (^{1}H-^{13}C) heteronuclear chemical shift correlation (HETCOR) NMR spectrum revealed the proton-carbon correlations, and chemical shifts confirmed the presence of a cinnamic acid structure and another substituted benzene ring.

The COLOC NMR experiment used for ^{1}H-^{13}C couplings, in this case optimised for 4 and 8 Hz, respectively (Figure 3), showed one part of the molecule to consist of a substituted cinnamic acid, in which two equivalent protons were at position 2 and 6 relative to the double bond.

Table 1. ^{1}H-NMR data of the three miriamides **1**, **2** and **3**.[a]

Position	1	2	3
Benzoic acid part			
H4	6.57, d, *J*=2.7	6.60, d, *J*=2.9	6.60, d, *J*=2.7
H6	7.02, d, *J*=2.7	7.03, d, *J*=2.9	7.03, d, *J*=2.7
Cinnamic acid part			
H2	6.62, s	6.83, d, *J*=1.7	7.07, d, *J*=1.9
H5			6.78, d, *J*=8.3
H6	6.62, s	7.11, d, *J*=1.7	6.98, dd, *J*=1.9/8.3
H7	7.45, d, *J*=15.5	7.52, d, *J*=15.5	7.54, d, *J*=15.5
H8	6.50, d, *J*=15.5	6.63, d, *J*=15.5	6.59, d, *J*=15.5
Sugar part			
H1		4.82, d, *J*=7.1	
H2, H3, H4 and H5		3.30-3.55, br m.	
H6		3.97, dd, *J*=2.1/11.9	
H6'		3.74, dd, *J*=5.3/11.9	

[a] - Run at 200 MHz (solvent CD_3OD), with chemical shifts in δ ppm (coupling constants in Hz).

In the other region, two protons were coupled with six carbon atoms, one of which belonged to a carboxylic acid (δ is 170.8). This information combined with that from the ^{1}H NMR spectra indicated a substituted benzoic acid moiety with the two protons at position 2 and 4 relative to the carboxylic acid group. The last structural features that had to be solved was the connection between the two rings and the position of the nitrogen atom in the molecule. A ^{13}C NMR analysis of the compound with a drop of D_2SO_4 added to the CD_3OD gave only small chemical shift changes of a few carbons in the ^{13}C NMR spectra (Table 2), thus excluding the presence of a primary aromatic amine or a primary amide. Only two combinations were still possible, a link of both rings via a secondary amine or via an amide. Interpretation of NMR spectra of different gallic acids, cinnamic acids and anthranilic acids, especially

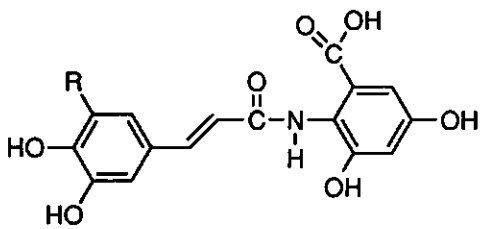
^{13}C NMR spectra of 3,4,5-trihydroxybenzoic acid, 3,4,5-trimethoxybenzoic acid and 3,4,5-trimethoxycinnamic acid, made it clear that the major constituent of the HMP was *trans*-2-[3-(3,4,5-trihydroxyphenylpropenoyl)amino]-3,5-dihydroxybenzoic acid {**1**}, a structure in which a benzoic and cinnamic portions are linked via an amide bond as shown in figure 2; The trivial name miriamide is proposed for **1**, in honour of Miriam Rothschild who was the first to notice the oviposition deterrent activity of compounds associated with the eggs of *P. brassicae* L.

Table 2. ^{13}C-NMR data of **1**, **1**+D_2SO_4, **2** and **3** (synthetic product).[a]

Position	**1**	**1**+D_2SO_4	**2**	**3**
Benzoic acid part				
C1	124.9	126.0	124.9	127.9
C2	120.8	115.4	120.9	121.0
C3	153.2	154.8	153.3	153.1
C4	110.8 +	114.0	110.4	110.9
C5	157.0	158.6	157.2	157.0
C6	110.8 +	114.0	110.9	110.9
C7	170.8	167.6	170.9	170.9
Cinnamic acid part				
C1	127.0	128.7	127.3	128.0
C2	108.6 +	108.5	111.9	115.3
C3	147.1	146.9	147.5	146.7
C4	137.3	138.1	139.2	149.2
C5	147.1	146.9	147.5	122.8
C6	108.6 +	108.5	110.9	116.5
C7	144.7 +	147.4	144.3	144.5
C8	117.7 +	114.0	118.6	117.4
C9	168.1	169.9	168.0	168.0
Sugar part				
C1			104.4	
C2			77.7	
C3			78.5	
C4			71.5	
C5			75.0	
C6			62.5	

[a]- Run at 50 MHz (solvent CD_3OD), with chemical shifts in δ ppm. For miriamide {**1**}, carbons which have a proton attached to them are marked with a '+' sign.

Fraction 9 contained the second compound **2**, whose structure was deduced by comparing its spectral data with those of **1**. The UV spectrum was identical to that of **1**. The ^{1}H NMR spectrum (Table 1) indicated two aromatic rings, a double bond with trans configuration (J=15.5 Hz) and a sugar. The ring protons of the cinnamic acid region had different chemical shifts and were also not equivalent to the corresponding ones in **1**. The chemical shifts of the other protons were almost identical to those of **1**. The ^{13}C NMR (Table 2) indicated 22 C atoms, six of which belong to a sugar unit. Three carbon atoms of the cinnamic acid part (C-2, C-4 and C-6) had chemical shifts different from those in **1**, while the other 13 carbons possessed almost identical chemical shifts as in **1**. This is to be expected when there is an ether rather than a hydroxy substituent on position 5. Enzymatic hydrolysis of fraction 9 with a β-glucosidase gave miriamide and glucopyranose. In contrast, hydrolysis with an α-glucosidase only gave the starting material back. Thus a β-glucopyranose had to be attached to the ring next to a proton in the cinnamic part. This led to the conclusion that the second compound was *trans*-2-[3-(3,4-dihydroxy-5-β-glucopyranose-phenylpropenoyl)amino]-3,5-dihydroxybenzoic acid (miriamide 5-glucoside) {**2**}.



1 R = OH
2 R = O-β-Glucopyranose
3 R = H

Figure 2. Molecular stuctures of miriamide (R = OH), 5-dehydroxy miriamide (R = H) and miriamide 5-glucoside (R = O-ß-glucopyranose).

Fraction 12 contained the third component **3**. The ^{1}H NMR spectrum (Table 1), showed the presence of two aromatic rings and a trans double bond (J=15.5 Hz). The chemical shifts of the two protons of the benzoic acid unit were identical to the shifts of the analogous region of **1**. The cinnamic acid part contained three protons (δ is 6.78, 6.98 and 7.07). From the coupling constants, the chemical shifts and the lack of symmetry they were deduced to be at position 2, 5 and 6, relative to the side chain. It was concluded that this compound was *trans*-2-[3-(3,4-dihydroxyphenylpropenoyl)amino]-3,5-dihydroxybenzoic acid (5-dehydroxy miriamide) {**3**}, a structure wherein the hydroxy group at C-5 in the cinnamic acid part was replaced by a proton.

The structural assignments of miriamide {**1**} and 5-dehydroxy miriamide {**3**} were confirmed by synthesis (Scheme 1).

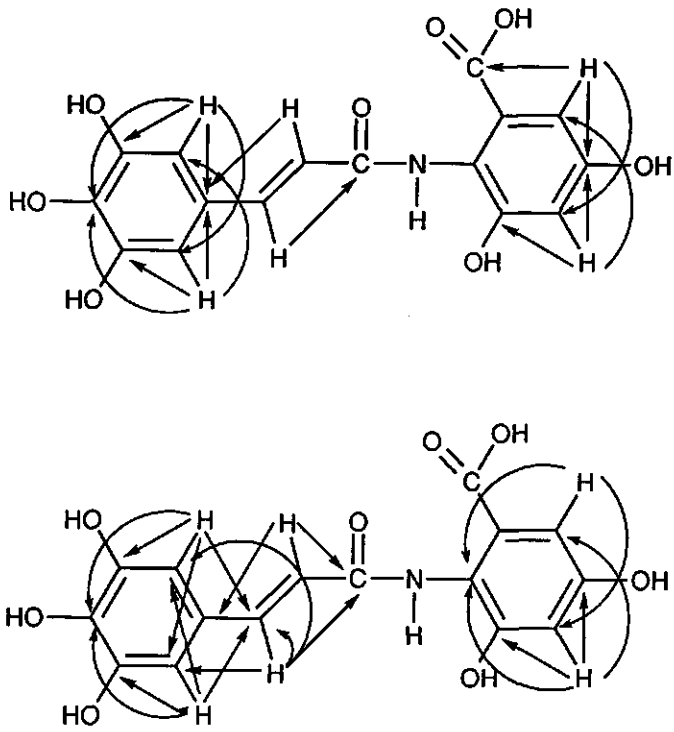
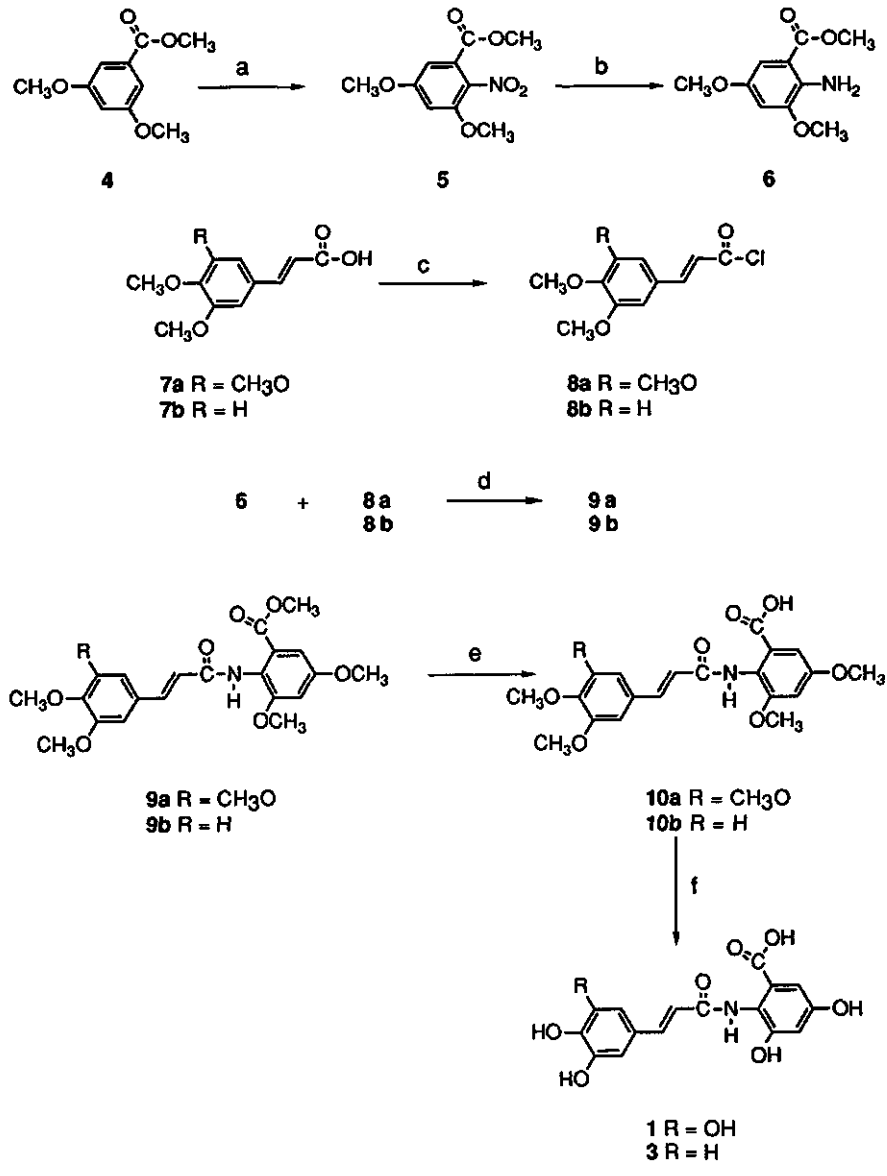


Figure 3. Longe-range $^{1}H/^{13}C$ connectivities observed in **1** optimised for 4 Hz couplings (above) and 8 Hz couplings (below).

Compound **1** was prepared starting from methyl-3,5-dimethoxybenzoate {**4**} and *trans*-(3,4,5-trimethoxy)cinnamic acid {**7a**}. Nitration of **4** gave methyl-2-nitro-3,5-dimethoxybenzoate {**5**}, and reduction of the nitro group resulted in methyl-2-amino-3,5-dimethoxybenzoate {**6**}. *trans*-(3,4,5-Trimethoxy)cinnamic acid {**7a**} was converted into its acid chloride **8a** with thionyl chloride. Reaction of **8a** with the amine **6** then gave *trans*-2-[3-(3,4,5-trimethoxyphenylpropenoyl)amino]-3,5-dimethoxybenzoic acid methyl ester {**9a**}. Saponification of the methyl ester **9a** with KOH resulted in *trans*-2-[3-(3,4,5-trimethoxyphenylpropenoyl)amino]-3,5-dimethoxybenzoic acid {**10a**} and demethylation this acid with BBr_3 gave miriamide {**1**}. The synthesis of 5-dehydroxy miriamide **3** was accomplished in the same way starting from *trans*-(3,4-dimethoxy)cinnamic acid {**7b**} and methyl-3,5-dimethoxybenzoate {**4**}.



Scheme 1. Syntheses of **1** and **3**. Reagents and conditions: (a) HNO_3/Ac_2O; (b) 10% Pd/C/MeOH/THF; (c) $SOCl_2/C_6H_6$; (d) CH_2Cl_2/Et_3N; (e) KOH/MeOH; (f) BBr_3/CH_2Cl_2.

DISCUSSION

Structures of the type described are, to the best of our knowledge, unknown from the animal kingdom. Structurally related compounds have been documented from plants and were found in *Avena* coleoptiles (Collins, 1989).

When miriamide {1} is applied to cabbage leaves at doses of 2.2 µg/leaf and higher (Figure 4), complete inhibition of oviposition on the treated leaf occured in a large number of replicates. In this dose range the average percentage deterrence is 80%, which means that the control leaf receives ten times as many batches as the treated leaf in this relatively crowded bioassay set-up. It is also clear (Figure 1 and 4) that **2** is less active then the other two miriamides.

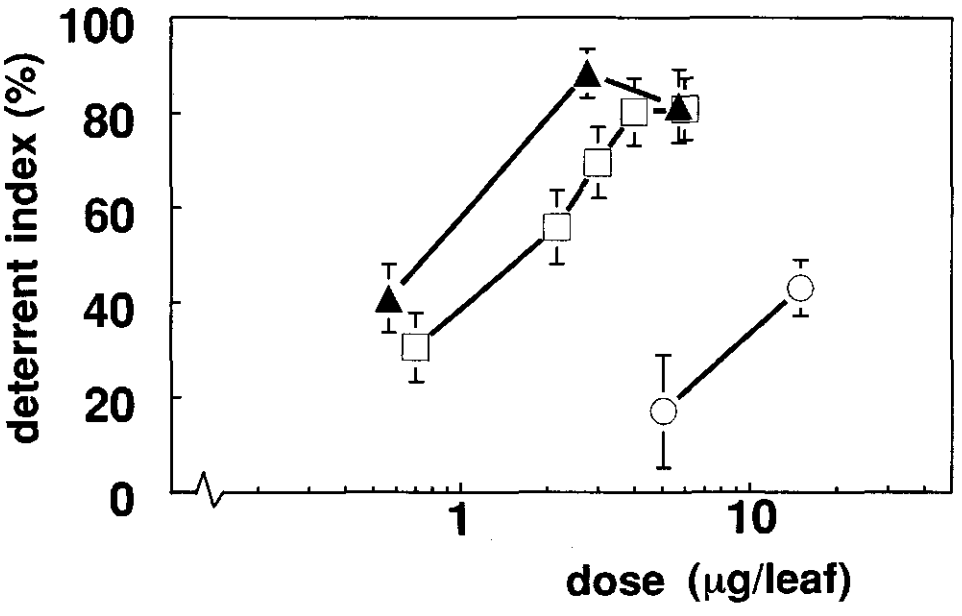


Figure 4. Oviposition deterrent index in the dual choice oviposition assay as a function of the doses of the three pure miriamides. Deterrent index was calculated as: (C - T) x 100 /(C + T), in which C is the number of egg batches on control leaf and T the number of egg batches on the treated leaf. Means ± standard error of the mean (SEM) of 6-12 replicates are shown. Triangles: 5-dehydroxy miriamide; Rectangles: miriamide; Circles: miriamide 5-glucoside.

Egg washes made of eggs that were laid on glass also contained the three miriamides proving that these are genuinely associated with the eggs. When *P. brassicae* L. females were offered a choice between a leaf treated with a crude wash of 25 eggs (yielding 28 µg of dry matter) and a leaf sprayed with the three miriamides in the ratio similar to that in the eggs (1 µg together, corresponding to the amount obtained from 25 eggs), no significant preference for either leaf was exhibited. This proves that all the three miriamides are together responsible for the inhibitory effect of the crude egg wash.
Analysis of the accessory glands shows that they contain only the less active miriamide 5-glucoside, whereas the eggs contain all the three miriamides. Behan and Schoonhoven (1978) already suggested that the accessory glands contained an inactive or less active form of the pheromone. It is likely that after secretion of miramide 5-glucoside onto the egg surface, miriamide 5-glucoside is partially converted (enzymatically ?) to the two other more active miriamides.

Previous results indicated that the related *Pieris rapae* L., a cosmopolitan pest species, also produces an HMP (Schoonhoven *et al.*, 1990). Interestingly, the HMP's produced by these two butterfly species not only deter oviposition by conspecific females but also by females of the other *Pieris* species, thus reducing interspecific competition for common food resources. HPLC-chromatograms and UV-spectra of *P. rapae* L. and *P. napi* L. egg washes indicated the presence of the miriamides in these eggs as well. Dual choice bioassays with *P. rapae* L. showed that oviposition of this species is also inhibited by miriamide. Eggs of the Pierids *Aporia crataegi* L. and *Colias philodice* Latreille lacked these compounds. Eggs of five other species of Lepidoptera [*Spodoptera exempta* Walker, *S. exigua* Hbn., *Mamestra brassicae* L. (all three Noctuidae); *Smerinthus ocellata* L. (Sphingidae) and *Cerura vinula* L. (Notodontidae)] were also screened for the presence of these compounds but no miriamides could be detected. It is to be stressed that both taxonomic specificity and effectiveness of the miriamides are distinctly higher than those of the generally occurring methylated fatty acids with HMP activity reported recently from the European corn borer *Ostrinia nubilalis* Hbn. ovipositing on an artificial substrate (Thiéry and Le Quéré, 1991). The latter compounds have previously been documented as semiochemicals from hymenopteran insects (Shimron *et al.*, 1985). With the European corn borer, absolute inhibition was not observed and the maximum level of inhibition induced by the latter compounds is low compared to that caused by equivalent amounts of miriamides.

In small-scale field experiments with crude egg washes the oviposition behaviour of *Pieris brassicae* L. was altered (Klijnstra and Schoonhoven, 1987). These experiments also showed the very high persistence of the HMP on plant surfaces (more than one week in the field and

greenhouses). Interference with butterfly behaviour in the initial phase of plant colonisation is a logical option that can be implemented now that the relevant semiochemicals have become available. The high stability and useful biological activity of the miriamides open new possibilities for the protection of cabbage crops against *Pieris* caterpillars in a way comparable with other environmentally safe crop protection strategies.

MATERIAL AND METHODS

General experimental methods - HPLC: The pumps, model 302 and 303, manometric module 802C, Dynamic mixer 811 and UV-detector 116 were all of Gilson. A software HPLC system manager (model 702) from Gilson was used on an Apple II personal computer. The columns used were a Microsorb RP C18 250 × 10 mm (flowrate 3 ml/min) and a Microsorb RP C18 250 × 4 mm (flowrate 1 ml/min), both 5 µm particle size and 100 Å pore size (Rainin Instrument Co.).

MR: All ^{1}H NMR spectra were recorded at 200 MHz (Bruker AC-E 200) in CD_3OD , $CDCl_3$ or DMSO-*d6*, and all ^{13}C NMR spectra were recorded at 50.3 MHz (same apparatus).

MS: Three different mass spectroscopic methods, electron ionization (EI), field desorption (FD) and direct chemical ionization (DCI) failed to produce the mol wt of the main component **1**. However, it was possible to measure a positive fast bombardment mass spectrum (FABMS) ($[M+H]^+$, $[M+H+glycerol]^+$) and a negative FABMS ($[M-H]^-$,$[M-H+glycerol]^-$) of **1** at a Finnigan MAT 95 mass spectrometer, *m/z* $[M-H]^-$ is 346.0572 (calculated for $C_{16}H_{12}NO_8$ 346.0563).

UV spectra were recorded in MeOH on a Beckman DU-7 spectrophotometer (λ_{max} **1** = 353 nm, λ_{max} **2** = 353 nm and λ_{max} **3** = 350 nm).

Plant material - *Brassica oleracea* L. var. *gemmifera* cv. Titurel plants were reared in a greenhouse (20–30°C, 50–80%RH, 16L:8D) in standard potting soil. Illumination consisted of daylight supplemented by high-pressure sodium/mercury vapour lamps hanging 0.75 m above pot level.

Insects - *P. brassicae* L. adults were obtained from a laboratory colony maintained on *Brassica oleracea* L. This culture was established in 1981 and since then, 18 generations have been produced each year. Field collected adults have been introduced several times during this period. Rearing conditions were similar to those described by David and Gardiner (1952).

Bioassays - Oviposition preferences were tested in cages measuring 80 x 50 x 100 cm high. The cages were kept in a conditioned greenhouse, with temperatures fluctuating between 22 and 25°C. In addition to normal daylight, each cage was illuminated from 7 A.M.—3 P.M. by a 400 Watt mercury vapor lamp hanging 30 cm above the glass roof of the cage. Each cage held 8 females and 4 males. In the bioassay, leaves were sprayed only on the upper surface, and one control and one treated leaf were placed in diagonal opposite corners, alternated between replicates, to minimize positional effects. Females could oviposit on the leaves during 6 h (8 A.M.—2 P.M.) periods. Preference of the butterflies was measured by comparing the number of egg batches on the treated leaves and the controls. A repilcate was considered the egg distribution occurring in one cage. On any one day 6—8 replicates were run. The significance of preference was tested with the Wilcoxon's matched pairs signed rank test (Siegel, 1956).

Extraction and isolation - About 150,000 eggs (30 g) were washed during five periods of five minutes each with pure methanol. The methanolic egg washes were evaporated to dryness and dissolved in a small volume of pure MeOH. This yellow crude egg extract was separated into twenty fractions using reversed phase C18 HPLC. The mobile phase contained 0.05% TFA. The solvent composition changed in 30 min linearly from MeCN-H_2O (8:92) to MeCN-H_2O (80:20), and was kept at that composition for 10 min. The flowrate was 3.0 ml/min. A fraction was collected every 2 minutes.

Fraction 9, 10/11 and 12 were further separated with HPLC using the same column with different solvent compositions. For 9, 10/11 and 12 solvent compositions were MeCN-H_2O (12:88), MeCN-H_2O (20:80) and MeCN-H_2O (25:75) respectively. Flowrate of the solvents (containing 0.05% TFA) was 3.0 ml/min.

Hydrolysis of Miriamide 5-glucoside was performed with α-glucosidase (Sigma NO G-6136) in 10 mM KH_2PO_4 (pH=6) and with β-glucosidase (Sigma NO G-4511) in 10 mM KH_2PO_4 (pH=5), both for one hour at 37°C.

SYNTHESIS

Methyl-2-nitro-3,5-dimethoxybenzoate {5} - Concentrated HNO_3 (40 ml) was added dropwise to a stirred solution of methyl-3,5-dimethoxybenzoate {4} (9.8 g, 50 mmol) in Ac_2O (100 ml) (8°C). The temperature was maintained between 8° and 15°C. After the addition, stirring was continued for 1 h and H_2O (500 ml) was added. The precipitate was filtered off and was washed 3 times with 100 ml of H_2O. After crystallisation from MeOH, methyl-2-nitro-3,5-dimethoxybenzoate {5} (10.6 g, 88 %) was isolated. ^{1}H-NMR spectrum ($CDCl_3$, 200 MHz);

δ 6.71 (d, *J*=2.5 Hz, H-4), 6.99 (d, *J*=2.5 Hz, H-6). ^{13}C NMR-spectrum ($CDCl_3$, 50 MHz); δ 103.0 (C-4), 105.5 (C-6), 125.3 (C-1), 134.7 (C-2), 152.3 (C-3), 160.8 (C-5), 163.6 (C-7).

Methyl-2-amino-3,5-dimethoxybenzoate {**6**} - Methyl-2-amino-3,5-dimethoxybenzoate {**6**} was prepared from methyl-2-nitro-3,5-dimethoxybenzoate {**5**} according to the procedure described by Klaubert *et al.* (1981). After reacting for 120 h, the amine was isolated (Yield was 89 %). ^{1}H-NMR spectrum ($CDCl_3$, 200 MHz); δ 5.72 (s, NH_2), 6.53 (d, *J*=2.7 Hz, H-4), 6.89 (d, *J*=2.6 Hz, H-6). ^{13}C-NMR spectrum ($CDCl_3$, 50 MHz); δ 102.1 (C-4), 104.0 (C-6), 108.7 (C-1), 136.6 (C-2), 147.9 (C-3), 149.3 (C-5), 168.1 (C-7).

trans-(3,4,5-Trimethoxy)cinnamoylchloride {**8a**} - $SOCl_2$ (15 ml) was added to a solution of *trans*-(3,4,5-trimethoxy)cinnamic acid {**7a**} (7.14 g, 30 mmol) in C_6H_6 (25 ml). The mixture was refluxed for 2 h. Removal of the excess $SOCl_2$ by azeotropic distillation followed by bulb to bulb distillation of the residue gave *trans*-(3,4,5-trimethoxy)cinnamoylchloride {**8a**} (7.05 g, 92 %). ^{1}H-NMR spectrum ($CDCl_3$, 200 MHz); δ 6.58 (d, *J*=15.4 Hz, H-8), 6.83 (s, H2/H6), 7.79 (d, *J*=15.5 Hz, H-7). ^{13}C-NMR spectrum ($CDCl_3$, 50 MHz); δ 106.1 (C-2/C-6), 121.0 (C-8), 128.1 (C-1), 141.6 (C-4), 150.5 (C-7), 153.3 (C-3/C-5), 165.9 (C-9).

trans-2-[3-(3,4,5-Trimethoxyphenylpropenoyl)amino]-3,5-dimethoxybenzoic acid methyl ester {**9a**} - A solution of methyl-2-amino-3,5-dimethoxybenzoate {**6**} (1.27 g, 6 mmol) in CH_2Cl_2 (10 ml) was added dropwise at 0°C to a stirred solution of *trans*-(3,4,5-trimethoxy)cinnamoyl chloride {**8a**} (1.7 g, 6.6 mmol) in CH_2Cl_2 (15 ml) and Et_3N (758 mg, 7.5 mmol). After stirring for 16 h at room temperature, the mixture was poured into 75 ml of a satd. $NaHCO_3$ solution. The aqueous layer was extracted three times with CH_2Cl_2 (25 ml). After drying and evaporation, *trans*-2-[3-(3,4,5-trimethoxyphenylpropenoyl)amino]-3,5-dimethoxybenzoic acid methyl ester {**9a**} (1.95 g, 75 %) was crystallized from EtOAc. Mp. 173°-174°C. ^{1}H-NMR spectrum ($CDCl_3$, 200 MHz); δ 6.51 (d, *J*=15.5 Hz, H-8), 6.65 (d, *J*=2.8 Hz, H-4), 6.73 (s, H-2/H-6), 6.93 (d, *J*=2.8 Hz, H-6), 7.58 (d, *J*=15.5 Hz, H-7), 8.09 (s, NH). ^{13}C-NMR spectrum ($CDCl_3$, 50 MHz); Benzoic acid part: δ 103.1 (C-4), 104.8 (C-6), 119.7 (C-2), 126.6 (C-1), 154.2 (C-3), 157.6 (C-5), 167.4 (C-7). Cinnamic acid part: δ 105.0 (C-2/C-6), 120.0 (C-8), 130.3 (C-1), 139.6 (C-4), 141.8 (C-7), 153.3 (C-3/C-5), 164.2 (C-9). 52.3, 55.6, 56.1 and 60.9 (OCH_3). *Anal.* found; C 61.0, H 5.7, N 3.1; calcd for $C_{22}H_{25}NO_8$, C 61.2, H 5.8, N 3.3.

^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) of **9b**; Benzoic acid part: δ 6.63 (d, *J*=2.8 Hz, H-4), 6.91 (d, *J*=2.6 Hz, H-6). Cinnamic acid part: δ 6.47 (d, *J*=15.5 Hz, H-8), 6.81 (d, *J*=8.2 Hz, H-5), 7.03 (dd, *J*=1.6/3.7 Hz, H-6), 7.08 (d, *J*=1.9 Hz, H-2), 7.60 (d, *J*=15.5 Hz, H-7), 8.05 (s, NH).

^{13}C-NMR spectrum ($CDCl_3$, 50 MHz) of **9b**; Benzoic acid part: δ 103.1 (C-4), 104.8 (C-6), 119.8 (C-2), 126.5 (C-1), 154.1 (C-3), 157.5 (C-5), 167.5 (C-7). Cinnamic acid part; δ 109.6 (C-2), 110.9 (C-6), 118.5 (C-8), 122.2 (C-5), 127.8 (C-1), 141.7 (C-7), 149.0 (C-3), 150.6 (C-4). 164.5 (C-9). 52.3, 55.7, 55.8 and 56.1 (OCH_3). *Anal.* found; C 62.5, H 5.6, N 3.3; calcd for $C_{21}H_{23}NO_7$, C 62.8, H 5.8, N 3.5. Mp. 168°-168.5°C.

trans-2-[3-(3,4,5-Trimethoxyphenylpropenoyl)amino]-3,5-dimethoxybenzoic acid {**10a**} - A solution of *trans*-2-[3-(3,4,5-trimethoxyphenylpropenoyl)amino]-3,5-dimethoxybenzoic acid methyl ester {**9a**} (862 mg, 2.0 mmol) in MeOH (10 ml) was stirred with 1 M KOH (5 ml) for 4 h and then poured into 1 M HCl (10 ml). The precipitate was filtered off and was washed successively with H_2O (15 ml) and $CHCl_3$ (25 ml). After drying *trans*-2-[3-(3,4,5-trimethoxyphenylpropenoyl)amino]-3,5-dimethoxybenzoic acid {**10a**} (760 mg, 90 %) was collected. ^{1}H-NMR spectrum (CD_3OD, 200 MHz) of **10a**; Benzoic acid part: δ 6.71 (d, *J*=2.8 Hz, H-4), 6.95 (d, *J*=2.6 Hz, H-6). Cinnamic acid part: δ 6.78 (d, *J*=15.7 Hz, H-8), 6.92 (s, H-2/H-6), 7.50 (d, *J*=15.7 Hz, H-7).

trans-2-[3-(3,4,5-Trihydroxyphenylpropenoyl)amino]-3,5-dihydroxybenzoic acid {**1**} A solution of 1M BBr_3 (8ml, 8 mmol) in CH_2Cl_2 was added dropwise to a stirred suspension of *trans*-2-[3-(3,4,5-trimethoxyphenylpropenoyl)amino]-3,5-dimethoxybenzoic acid {**10a**} (510 mg, 1.22 mmol) in dry CH_2Cl_2 (15 ml) at − 78°C. After stirring for 2 h at 0°C, the reaction was quenched with 1 M HCl (15 ml). The mixture was centrifuged and the residue was washed with 0.02 M HCl (2 × 5 ml) and H_2O (3 × 5 ml). After drying *trans*-2-[3-(3,4,5-trihydroxyphenylpropenoyl)amino]-3,5-dihydroxybenzoic acid (Miriamide) {**1**} was isolated (395 mg, 90 %). ^{1}H-NMR spectrum (DMSO-d6, 200 MHz); δ 6.48 (d, *J*=2.4 Hz, H-4), 6.52 (d, *J*=15.5 Hz, H-8), 6.53 (s, H-2/H-6), 6.70 (d, *J*=2.4 Hz, H-6), 7.21 (d, *J*=15.5 Hz, H-7), 8.64 (s, OH), 9.11 (s, OH), 9.50 (s, OH), 9.86 (s, OH). ^{1}H-NMR spectrum (CD_3OD, 200 MHz) of **1**; Benzoic acid part: δ 6.60 (d, *J*=2.8 Hz, H-4), 7.03 (d, *J*=2.8 Hz, H-6). Cinnamic acid part: δ 6.54 (d, *J*=15.6 Hz, H-8), 6.65 (s, H-2/H-6), 7.47 (d, *J*=15.5 Hz, H-7). ^{13}C-NMR spectrum (CD_3OD, 50 MHz) of **1**; Benzoic acid part: δ 110.8 (C-4/C-6), 120.9 (C-2), 124.9 (C-1), 153.2 (C-3), 157.1 (C-5), 170.9 C-7). Cinnamic acid part: δ 108.6 (C-2/C-6), 117.7 (C-8), 127.0 (C-1), 137.4 (C-4), 144.8 (C-7), 147.2 (C-3/C-5), 168.2 (C-9). *Anal.* found; C 54.5, H 3.7, N 3.8; calcd for $C_{16}H_{13}NO_8$, C 55.3, H 3.8, N 4.0

^{1}H-NMR spectrum (CD_3OD, 200 MHz) of **3**; Benzoic acid part: δ 6.60 (d, *J*=2.8 Hz, H-4), 7.03 (d, *J*=2.9 Hz, H-6). Cinnamic acid part: δ 6.56 (d, *J*=15.5 Hz, H-8), 6.77 (d, *J*=8.2 Hz, H-5), 6.96 (dd, *J*=1.8/8.2 Hz, H-6), 7.05 (d, *J*=1.8 Hz, H-2), 7.53 (d, *J*=15.5 Hz, H-7). ^{13}C-NMR spectrum (CD_3OD, 50 MHz) of **3**; Benzoic acid part: δ 110.9 (C-2/C-4), 121.0 (C-2), 127.9 (C-1), 153.1 (C-3), 170.9 (C-7). Cinnamic acid part: δ 115.3 (C-2), 116.5 (C-6), 117.4

(C-8), 122.8 (C-5), 128.0 (C-1), 144.5 (C-7), 146.7 (C-3), 149.2 (C-4), 168.0 (C-9). *Anal.* found; C 58.1, H 4.0, N 4.0; calcd for $C_{16}H_{13}NO_7$, C 58.0, H 3.9, N 4.2.

REFERENCES

Behan, M. and Schoonhoven, L.M. 1978. Chemoreception of an oviposition deterrent associated with eggs in *Pieris brassicae*. *Entomol. Exp. Appl.* 24: 163-179.

Collins, F.W. 1989. Oat phenolics: Avenanthramides, novel substituted N-cinnamoylanthranilate alkaloids from oat groats and hulls. *J. Agric. Food Chem.* 37: 60-66.

David, W.A.L. and Gardiner, B.O.C. 1952. Laboratory breeding of *Pieris brassicae* L. and *Apanteles glomerata* L. *Proc. R. Entomol. Soc. Lond. (A)* 27: 54-56.

Dittrick, L.E., Jones, R.L. and Chiang, H.C. 1983. An oviposition deterrent for the european corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae), extracted from larval frass. *J. Insect Physiol.* 29: 119-121.

Hurter, J., Boller, E.F., Städler, E., Blattmann, B., Buser, H.-R., Bosshard, N.U., Damm, L., Kozlowski, M.W., Schöni, R., Raschdorf, F., Dahinden, R., Schlumpf, E., Fritz, H., Richter, W.J. and Schreiber, J. 1987. Oviposition-deterring pheromone in *Rhagoletis cerasi* L.: Purification and determination of the chemical constitution. *Experientia* 43: 157-164.

Imai, T., Kodama, H., Chuman, T. and Kohno, M. 1990. Female-produced oviposition deterrents of the cigarette beetle, *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae). *J. Chem. Ecol.* 16: 1237-1247.

Klaubert, D.H., Sellstedt, J.H., Guinosso, C.J., Capetola, R.J. and Bell, S.C. 1981. *N*-(aminophenyl)oxamic acids and esters as potent orally active antiallergy agents. *J. Med. Chem.* 24: 742.

Klein, B., Schildknecht, H., Hilker, H. and Bombosch, S. 1990. Eiablagehemmende wirkstoffe aus dem larvenkot von *Spodoptera littoralis* (Boisd). *Z. Naturforsch.* 45c: 895-901.

Klijnstra, J.W. 1986. The effect of an oviposition deterring pheromone on egg-laying in *Pieris brassicae*. *Entomol. Exp. Appl.* 41: 139-146.

Klijnstra, J.W. and Roessingh, P. 1986. Perception of the oviposition deterring pheromone by tarsal and abdominal contact chemoreceptors in *Pieris brassicae*. *Entomol. Exp. Appl.* 40: 71-79.

Klijnstra, J.W. and Schoonhoven, L.M. 1987. Effectiveness and persistence of the oviposition deterring pheromone of *Pieris brassicae* in the field. *Entomol. Exp. Appl.* 45: 227-235.

Noldus, L.P.J.J. and Lenteren, J.C. van. 1985. Kairomones for the egg parasite *Trichogramma evanescens* Westwood. Effect of contact chemicals produced by two of its hosts, *Pieris brassicae* L. and *Pieris rapae* L. *J. Chem. Ecol.* 11: 793-800.

Prokopy, R.J. 1981. Epideictic pheromones that influence spacing patterns of phytophagous insects, pp. 181-213, *in* D.A. Nordlund, R.L. Jones and W.J. Lewis (eds.). Semiochemicals, Their Role in Pest Control. John Wiley & Sons, New York.

Prokopy, R.J., Reissig, W.H. and Moericke, V. 1976. Marking pheromones deterring repeated oviposition in *Rhagoletis* flies. *Entomol. Exp. Appl.* 20: 170-178.

Prokopy, R.J. and Webster, R.P. 1978. Oviposition deterring pheromone of *Rhagoletis pomonella*: A kairomone for its parasitoid *Opius lectus*. *J. Chem. Ecol.* 4: 481-494.

Renwick, J.A.A. and Radke, C.D. 1980. An oviposition deterrent associated with frass from feeding larvae of the cabbage looper, *Trichoplusia ni* (lepidoptera: Noctuidae). *Environ. Entomol.* 9: 318-320.

Roitberg, B.D. and Prokopy, R.J. 1987. Insects that mark host plants; an ecological, evolutionary perspective on host-marking chemicals. *BioScience* 37: 400-406.

Roitberg, B.D. and Lalonde, R.G. 1991. Host marking enhances parasitism risk for a fruit-infesting fly *Rhagoletis basiola*. *Oikos* 61: 389-393.

Rothschild, M. and Schoonhoven, L.M. 1977. Assesment of egg load by *Pieris brassicae* (Lepidoptera: Pieridae). *Nature* 266: 532-535.

Schoonhoven, L.M., Beerling, E.A.M., Klijnstra, J.W. and Vugt, Y. 1990. Two related butterfly species avoid oviposition near each other's eggs. *Experientia* 46: 526-528.

Schoonhoven, L.M. 1990. Host-marking pheromones in Lepidoptera, with special reference to two *Pieris* spp. *J. Chem. Ecol.* 16: 3043-3052.

Shimron, O., Hefetz, A. and Tengo, J. 1985. Structural and communicative functions of Dufour's gland secretion in *Eucera palestinae* (Hymenoptera: Anthophoridae). *Insect Biochem.* 15: 635-638.

Siegel, S. 1956. Nonparametric Statistics for the Behavioral Sciences. John Wiley, New York.

Thiéry, D. and Le Quéré, J.L. 1991. Identification of an oviposition-deterring pheromone in the eggs of the European Corn Borer. *Naturwissenschaften* 78: 132-133.

CHAPTER 4

Structure-activity relationship of isolated avenanthramide alkaloids and synthesized related compounds as oviposition deterrents for *Pieris brassicae*

ABSTRACT

The structure-activity relationship of compounds isolated from the eggs of *Pieris brassicae* L., the large cabbage white butterfly, and eight synthesized related compounds as oviposition deterrents for this insect was studied. The activity of all structures was tested in a dual choice bioassay. The two most active oviposition deterrents for *P. brassicae* L. were *trans*-2-[3-(4-hydroxyphenylpropenoyl)amino]-3,5-dihydroxybenzoic acid {**8**} and *trans*-2-[3-(3,4-dihydroxy-phenylpropenoyl)amino]-3,5-dihydroxybenzoic acid {**2**}. Substituents of the cinnamic acid part of the molecule affected the oviposition deterring activity more profoundly than changes in the way both ring systems were connected. Changes in the anthranilic acid part of the molecule resulted into a lower oviposition deterring activity.

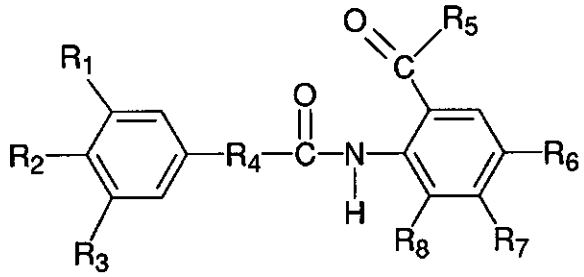
INTRODUCTION

The cabbage white butterflies, *Pieris brassicae* L. and *P. rapae* L., herbivorous pests of crucifers, produce egg-associated chemicals that can inhibit their oviposition (Rothschild and Schoonhoven, 1977). These chemicals can be collected by washing the eggs with water or methanol. Oviposition by *P. brassicae* L. and *P. rapae* L. is inhibited when a potential host plant is sprayed with such an egg wash (Schoonhoven *et al.*, 1990; Schoonhoven, 1990). Inhibition of oviposition is especially pronounced when females have a choice between treated plants and control plants, or when dispersal activity can be manifested (Klijnstra and Schoonhoven, 1987). Recently the compounds responsible for the oviposition deterring effect of a crude egg wash, were isolated and identified as *trans*-2-[3-(3,4,5-trihydroxyphenylpropenoyl)amino]-3,5-dihydroxybenzoic acid {**1**}, *trans*-2-[3-(3,4-dihydroxyphenylpropenoyl)amino]-3,5-dihydroxybenzoic acid {**2**} and *trans*-2-[3-(3,4-dihydroxy-5-glucopyranose-phenylpropenoyl)amino]-3,5-dihydroxybenzoic acid {**3**} (Blaakmeer *et al.*, 1994).

These were three previously unknown avenanthramide alkaloids (amides of cinnamic and anthranilic acids) (Collins, 1989; Niemann *et al.*, 1992; Niemann, 1993). Substances that modify the oviposition behaviour of *Pieris* butterflies might have practical value in preventing colonization of cabbage by these specialized insects (Klijnstra and Schoonhoven, 1987). In order to determine whether there are more active or simpler structures than the natural deterrents identified so far, a limited SAR study was undertaken. Eight structurally related compounds, with changes in either both ring systems or the way they are coupled, were synthesized and their oviposition deterring activity was measured quantitatively. In this paper, we describe the results of these studies.

RESULTS AND DISCUSSION

The tested compounds were *trans*-2-[3-(3,4,5-trihydroxyphenylpropenoyl)amino]-3,5-dihydroxybenzoic acid {**1**} (syn.: miriamide), *trans*-2-[3-(3,4-dihydroxyphenylpropenoyl)amino]-3,5-dihydroxybenzoic acid {**2**}, *trans*-2-[3-(3,4-dihydroxy-5-glucopyranose-phenylpropenoyl)-amino]-3,5-dihydroxybenzoic acid {**3**}, *trans*-2-[3-(3,4,5-trimethoxyphenylpropenoyl)amino-3,5-dimethoxybenzoic acid methyl ester {**4**}, *trans*-2-[3-(3,4,5-trihydroxyphenylpropenoyl)-amino]-3-hydroxy-5-methoxybenzoic acid {**5**}, *trans*-2-[3-(3,4,5-trihydroxyphenylpropenoyl)-amino]-3,5-dihydroxybenzoic acid methyl ester {**6**}, *trans*-2-[3-(3,4,5-trihydroxyphenylpropenoyl)amino]-4,5-dihydroxybenzoic acid {**7**}, *trans*-2-[3-(4-hydroxyphenylpropenoyl)amino]-3,5-dihydroxybenzoic acid {**8**}, *trans*-2-[3-(4-hydroxyphenylpropenoyl)amino]-4,5-dihydroxybenzoic acid {**9**}, 2-[3-(3,4,5-trihydroxyphenylpropionyl)amino]-3,5-dihydroxybenzoic acid {**10**} and 2-[(3,4,5-trihydroxybenzoyl)amino]-3,5-dihydroxybenzoic acid {**11**} (Figure 1). Oviposition deterrency was quantified by means of an oviposition deterrent index (ODI) (see experimental). The dose-response curves are given in Figure 2. The concentration at which ODI equals 50% (ED_{50}) was calculated for each compound by the method of Spearman and Kärber (Spearman, 1908; Finney, 1978). The ED_{50} values with their 95 % confidence intervals are given in Figure 3. We considered compounds to exert a significantly different deterrent effect when there was no overlap between the confidence intervals of their ED_{50} values. When comparing the ED_{50} value of compound **1** with the ED_{50} values of the other compounds, three groups with different ED_{50} could be distinguished (Figure 3). The ED_{50} of compound 4 could not be calculated because the oviposition deterring activity was only 28% at the highest dose tested (10 µg/leaf).



	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
1	OH	OH	OH	CH=CH	OH	OH	H	OH
2	OH	OH	H	CH=CH	OH	OH	H	OH
3	OH	OH	glucose	CH=CH	OH	OH	H	OH
4	OCH_3	OCH_3	OCH_3	CH=CH	OCH_3	OCH_3	H	OCH_3
5	OH	OH	OH	CH=CH	OH	OCH_3	H	OH
6	OH	OH	OH	CH=CH	OCH_3	OH	H	OH
7	OH	OH	OH	CH=CH	OH	OH	OH	H
8	H	OH	H	CH=CH	OH	OH	H	OH
9	H	OH	H	CH=CH	OH	OH	OH	H
10	OH	OH	OH	CH_2-CH_2	OH	OH	H	OH
11	OH	OH	OH	---	OH	OH	H	OH

Figure 1. Molecular structures of the isolated and synthesized compounds.

Removal of one or two hydroxy-groups (from position 3 or from positions 3 and 5) of the cinnamic part of the parent molecule **1** (structures **2** and **8**) increases deterrency (Figure 3). Neither reduction nor removal of the double bond (**10** and **11**) affects activity compared to **1**. When at the same time two hydroxy-groups are removed from positions 3 and 5 of the cinnamic part of the molecule and one hydroxy-group is shifted from position 3 to 4 at the anthranilic part (compound **9**), deterrency remains equal to that of compound **1**. Methylation of one of the hydroxy-groups of the anthranilic part of the parent molecule **1**, which gives compounds **5** or **6**, or changing the position of one hydroxy-group from position 3 to 4 at the anthranilic part of the molecule (structure **7**), reduces effectiveness compared to **1**. When a glucose-group is linked to the cinnamic part of the molecule (**3**) deterrency is drastically reduced relative to that of **1**.

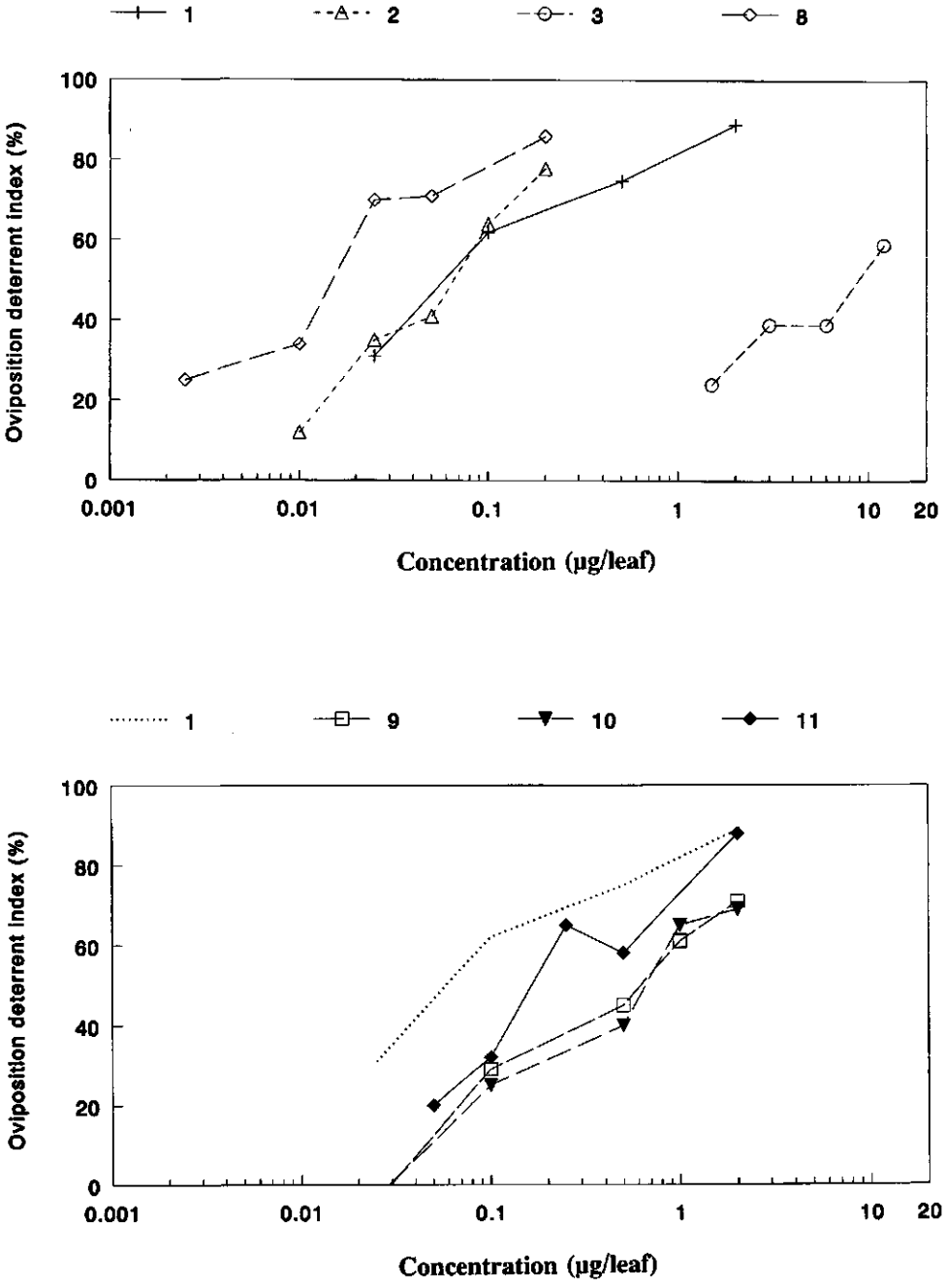


Figure 2. Dose response curves of the tested compounds.

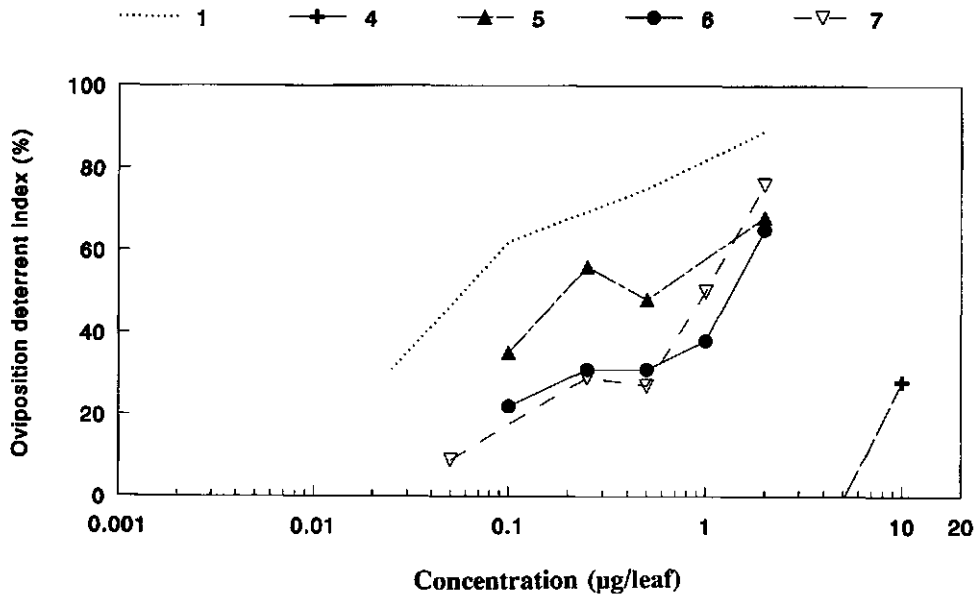


Figure 2. Dose response curves of the tested compounds (continued).

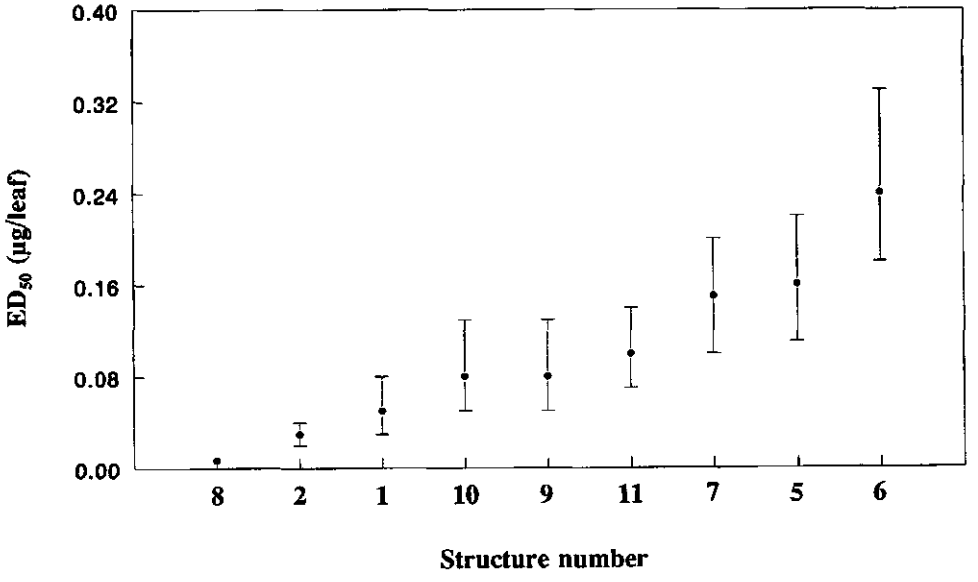


Figure 3. The calculated ED_{50} with its 95 % confidence interval for 9 of the 11 tested compounds. The interval for compound **3** runs from 1.37 to 2.16 µg/leaf. The ED_{50} of compound **4** could not be calculated (see text).

In conclusion, modification of the groups linked to the anthranilic part of the molecule leads to a lower effectiveness compared to **1**. Methylation of **1** and glucosylation of the 5-OH of the cinnamic part likewise reduce deterrency, while it was found that changes in the way both ring systems were linked has no influence on effectiveness. In contrast, mono- and dihydroxy substituted cinnamic parts of the molecule increase its deterrent activity.
A study of the chemoreception of phenolic acids by larvae of *P. brassicae* L. and *P. rapae* L. showed that ortho dihydroxyphenolic acids were the most active stimulants for lateral and medial sensilla (van Loon, 1990). It will be of interest to test monohydroxybenzoic acids or monohydroxycinnamic acids, which have not been tested by van Loon (1990), on both larvae and adults to see if these compounds have a higher activity.
In field experiments, carried out at the same time as this SAR study, with cabbage plants sprayed with pure miriamide **1** no oviposition deterring or dispersal activity could be measured (van Loon, pers. comm.). Additional laboratory experiments demonstrated that compound **1** was unstable when exposing it to direct sunlight. The instability is probably due to the high UV absorption which is caused by the strongly conjugated structure of **1**.
In order to find more simple compounds which can be used effectively to prevent colonization of cabbage by cabbage butterflies, the synthesis of less conjugated derivatives like compound **10** or less substituted compounds or esters of cinnamic and benzoic acids will be the subject of further research. Photostability of the other compounds described in the present study and the compounds that will be synthesized should be investigated.

MATERIAL AND METHODS

General experimental methods - All ^{1}H and ^{13}C NMR spectra were recorded at a Bruker AC-E 200. Microanalyses were carried out on a Carlo erba elemental analyzer mod. 1106.
UV spectra were recorded on a Beckman DU-7 spectrophotometer.

Plant material - *Brassica oleracea* L. var. *gemmifera* cv. Titurel plants were reared in a greenhouse (20-30°C, 50-80%RH, 16L:8D) in standard potting soil. Illumination consisted of natural daylight supplemented by high pressure sodium vapour lamps hanging 0.75 m above pot level. A voucher specimen (van Setten 1073) has been deposited at the Herbarium Vadense (WAG), Wageningen, The Netherlands.

Insects - *Pieris brassicae* L. adults were obtained from a laboratory colony maintained on *Brassica oleracea* L. This culture was established in 1981 and since then 18 generations have been produced each year. Field collected adults have been introduced several times during this

period. Rearing conditions were similar to those described by David and Gardiner (1952). Voucher specimen 378,421 has been deposited at the insect collection of the Department of Entomology, Wageningen Agricultural University.

Bioassays - The bioassay was the same as described by Blaakmeer *et al.* (1994), except that there were 2 males and 4 females present in each cage and on any one day 12-16 replicates were run. The oviposition deterrent index was calculated in the following way: ODI = (C-T) × 100 / (C+T), where C and T represent the number of egg batches laid on control and treated leaves. All doses of each compound were tested in an increasing concentration series on one generation of butterflies.

ISOLATION AND SYNTHESIS

The isolation of **1**, **2** and **3** and the synthesis of **1** and **2** have already been described (5). Compound **4** (*trans*-2-[3-(3,4,5-trimethoxyphenylpropenoyl)amino-3,5-dimethoxybenzoic acid methyl ester) was an intermediate in the synthesis of **1** (for spectral data of **1**, **2**, **3** and **4** see Blaakmeer *et al.* (1994)).

trans-2-[3-(3,4,5-Trihydroxyphenylpropenoyl)amino]-3-hydroxy-5-methoxybenzoic acid {**5**}. Compound **5** was a by-product in the synthesis of **1** (Blaakmeer *et al.*, 1994). After treatment of *trans*-2-[3-(3,4,5-trimethoxyphenylpropenoyl)amino]-3,5-dimethoxybenzoic acid with BBr_3 90 % of **1** and 10 % of **5** was isolated. ^{1}H NMR-spectrum (CD_3OD, 200 MHz) of **5**; Benzoic acid part: δ 3.73 (OCH_3), 6.67 (d, *J*=2.7 Hz, H-4), 7.08 (d, *J*=2.7 Hz, H-6). Cinnamic acid part: δ 6.48 (d, *J*=15.5 Hz, H-8), 6.60 (s, H-2/H-6), 7.42 (d, *J*=15.5 Hz, H-7). ^{13}C-NMR spectrum (CD_3OD, 50 Mhz) of **5**; Benzoic acid part: δ 56.0 (OCH_3), 109.3 (C-4/C-6), 122.0 (C-2), 124.9 (C-1), 153.3 (C-3), 159.3 (C-5), 170.8 C-7). Cinnamic acid part: δ 108.6 (C-2/C-6), 117.6 (C-8), 126.9 (C-1), 137.4 (C-4), 145.0 (C-7), 147.2 (C-3/C-5), 168.1 (C-9). *Anal.* found; C 51.5, H 4.5, N 3.6; calcd for $C_{17}H_{15}NO_8$×2(H_2O), C 51.4, H 4.8, N 3.5. UV λ_{max} (MeOH) = 355 nm.

trans-2-[3-(3,4,5-Trihydroxyphenylpropenoyl)amino]-3,5-dihydroxybenzoic acid methyl ester {**6**} - A solution of 1 M BBr_3 (8 ml, 8 mmol) in CH_2Cl_2 was added by drops to a stirred suspension of *trans*-2-[3-(3,4,5-trimethoxyphenylpropenoyl)amino]-3,5-dimethoxybenzoic acid methyl ester {**4**} (216 mg, 0.5 mmol) in dry CH_2Cl_2 (15 ml) at −78°C. After stirring for 2 h at 0°C, the reaction was quenched with 1 M HCl (15 ml). The mixture was centrifuged and the residue was washed with 0.02 M HCl (2 × 5 ml) and H_2O (3 x 5 ml).

After drying *trans*-2-[3-(3,4,5-trihydroxyphenylpropenoyl)amino]-3,5-dihydroxybenzoic acid methyl ester (**6**) (179 mg, 99 %) was collected. ^{1}H-NMR spectrum (CD_3OD, 200 Mhz) of **6**; Benzoic acid part: δ 3.83 (OCH_3), 6.59 (d, *J*=2.6 Hz, H-4), 6.90 (d, *J*=2.8 Hz, H-6). Cinnamic acid part: δ 6.56 (d, *J*=15.9 Hz, H-8), 6.64 (s, H-2/H-6), 7.44 (d, *J*=15.5 Hz, H-7). ^{13}C-NMR spectrum (CD_3OD, 50 Mhz) of **6**; Benzoic acid part: δ 52.9 (OCH_3), 110.1 (C-4/C-6), 119.7 (C-2), 126.1 (C-1), 153.7 (C-3), 157.2 (C-5), 169.3 (C-7). Cinnamic acid part: δ 108.7 (C-2/C-6), 117.8 (C-8), 127.1 (C-1), 137.3 (C-4), 144.6 (C-7), 147.1 (C-3/C-5), 168.3 (C-9).
Anal. found; C 51.8, H 4.5, N 3.4; calcd for $C_{17}H_{15}NO_8 \times 1.5(H_2O)$, C 52.8, H 4.7, N 3.6.
UV λ_{max} (MeOH) = 343 nm.

Compounds **7-9**. These compounds were prepared in the same way as described for **1** (Blaakmeer *et al.*, 1994). For **7** the starting material was 3,4-dimethoxybenzoic acid methyl ester and *trans*-(3,4,5-trimethoxy)cinnamic acid. Nitration of 3,4-dimethoxybenzoic acid methyl ester gave 2-nitro-4,5-dimethoxybenzoic acid methyl ester (yield 92 %) and reduction of the nitro group resulted in 2-amino-4,5-dimethoxybenzoic acid methyl ester (yield 94 %). For **8** the starting material was *trans*-(4-methoxy)cinnamic acid and 3,5-dimethoxybenzoic acid methyl ester. *trans*-(4-Methoxy)cinnamic acid was converted into its acid chloride (yield 98 %) with thionyl chloride. *trans*-(4-Methoxy)cinnamic acid and 3,4-dimethoxybenzoic acid methyl ester were the starting materials for the synthesis of **9**.

trans-2-[3-(3,4,5-Trihydroxyphenylpropenoyl)amino]-4,5-dihydroxybenzoic acid (**7**) - Yield of the coupling of the acid chloride with the amine, yield of the saponification of the methyl ester and yield of the demethylation were respectively 25, 84 and 48 %. ^{1}H-NMR spectrum (CD_3OD, 200 Mhz); Benzoic acid part: δ 7.50 (s, H-3), 8.22 (s, H-6). Cinnamic acid part: δ 6.38 (d, *J*=15.8 Hz, H-8), 6.63 (s, H-2/H-6), 7.42 (d, *J*=15.6 Hz, H-7). ^{13}C-NMR spectrum (CD_3OD, 50 Mhz); Benzoic acid part: δ 108.4 (C-3), 119.5 (C-6), 131.0 (C-2), 141.9 (C-5), 152.3 (C-4), 171.5 (C-7), (C-1 not observed). Cinnamic acid part: δ 108.5 (C-2/C-6), 118.4 (C-8), 127.0 (C-1), 137.2 (C-4), 143.7 (C-7), 147.1 (C-3/C-5), 166.7 (C-9). *Anal.* found; C 50.5, H 4.4, N 3.6; calcd for $C_{16}H_{13}NO_8 \times 1.9(H_2O)$, C 50.4, H 4.4, N 3.7.
UV λ_{max} (MeOH) = 349 nm.

trans-2-[3-(4-Hydroxyphenylpropenoyl)amino]-3,5-dihydroxybenzoic acid (**8**) - Yield of the coupling of the acid chloride with the amine, yield of the hydrolysis of the methyl ester and yield of the demethylation were respectively 71, 95 and 98 %.
^{1}H-NMR spectrum (CD_3OD, 200 Mhz); Benzoic acid part: δ 6.60 (d, *J*=2.9 Hz, H-4), 7.04 (d, *J*=2.8 Hz, H-6). Cinnamic acid part: δ 6.63 (d, *J*=15.6 Hz, H-8), 6.80 (d, *J*=8.6 Hz,

H-3/H-5), 7.47 (d, *J*=8.6 Hz, H-2/H-6), 7.60 (d, *J*=15.6 Hz, H-7). ^{13}C-NMR spectrum (CD_3OD, 50 Mhz); Benzoic acid part: δ 110.8 (C-4/C-6), 121.0 (C-2), 124.6 (C-1), 153.1 (C-3), 157.0 (C-5), 170.7 C-7). Cinnamic acid part: δ 116.8 (C-3/C-5), 117.4 (C-8), 127.4 (C-1), 131.1 (C-2/C-6), 144.1 (C-7), 161.0 (C-4), 168.1 (C-9). *Anal.* found: C 56.8, H 4.4, N 4.1; calcd for $C_{16}H_{13}NO_6 \times 1.3(H_2O)$, C 56.7, H 4.6, N 4.1. UV λ_{max} (MeOH) = 319 nm.

trans-2-[3-(4-Hydroxyphenylpropenoyl)amino]-4,5-dihydroxybenzoic acid {**9**} -Yield of the coupling of the acid chloride with the amine, yield of the hydrolysis of the methyl ester and yield of the demethylation were respectively 78, 94 and 83 %.
^{1}H-NMR spectrum (CD_3OD, 200 Mhz) Benzoic acid part: δ 7.51 (s, H-3), 8.22 (s, H-6). Cinnamic acid part: δ 6.48 (d, *J*=15.6 Hz, H-8), 6.81 (d, *J*=8.3 Hz, H-3/H-5), 7.50 (d, *J*=9.8 Hz, H-2/H-6), 7.55 (d, *J*=15.6 Hz, H-7). ^{13}C-NMR spectrum (CD_3OD, 50 Mhz); Benzoic acid part: δ 115.4 (C-1), 108.5 (C-3), 119.5 (C-6), 137.2 (C-2), 141.9 (C-5), 152.3 (C-4), 171.5 (C-7). Cinnamic acid part: δ 116.8 (C-3/C-5), 118.4 (C-8), 127.5 (C-1), 130.9 (C-2/C-6), 143.0 (C-7), 160.9 (C-4), 166.7 (C-9). *Anal.* found; C 61.3, H 4.2, N 4.2; calcd for $C_{16}H_{13}NO_6$, C 61.0, H 4.2, N 4.4. UV λ_{max} (MeOH) = 336, 315 nm.

Synthesis of *2-[3-(3,4,5-trihydroxyphenylpropionyl)amino]-3,5-dihydroxybenzoic acid* {**10**}.
2-[3-(3,4,5-Trimethoxyphenylpropionyl)amino]-3,5-dimethoxybenzoic acid methyl ester - DCC (1.44 g, 7 mmol) was added to a stirred solution of 3,4,5-trimethoxyphenylpropionic acid in DMF (25 ml) and the mixture was stirred at room temperature under N_2. After 1 h, 2-amino-3,5-dimethoxybenzoic acid methyl ester (1.12 g, 5.3 mmol) in DMF (10 ml) was added to the solution. After 48 h, the mixture was poured into 1M HCl (50 ml). The aqueous layer was extracted three times with EtOAc (75 ml). After drying and evaporation, 2-[3-(3,4,5-trimethoxyphenylpropionyl)amino]-3,5-dimethoxybenzoic acid methyl ester (290 mg, 13 %) was isolated.
2-[3-(3,4,5-Trimethoxyphenylpropionyl)amino]-3,5-dimethoxybenzoic acid - A solution of 2-[3-(3,4,5-trimethoxyphenylpropionyl)amino]-3,5-dimethoxybenzoic acid methyl ester (270 mg, 0.62 mmol) in H_2O (25 ml) and MeOH (25 ml) was stirred with KOH (140 mg, 2.5 mmol) at room temperature. After 24 h, the reaction was quenched with 1 M HCl (3 ml). The precipitate was filtered off and was washed with H_2O (5 ml). After drying 2-[3-(3,4,5-trimethoxyphenylpropionyl)amino-3,5-dimethoxybenzoic acid (190 mg, 73 %) was collected.
2-[3-(3,4,5-Trihydroxyphenylpropionyl)amino]-3,5-dihydroxybenzoic acid {**10**} - A solution of 1M BBr_3 (6.5 ml) in CH_2Cl_2 was added dropwise to a stirred suspension of 2-[3-(3,4,5-trimethoxyphenylpropionyl)amino]-3,5-dimethoxybenzoic acid (180 mg, 0.43 mmol) in dry CH_2Cl_2 (15 ml) at −78°C. After stirring for 2 h at 0°C, the reaction was quenched with 1 M HCl (10 ml). The mixture was centrifuged and the residue was washed with 0.02 M HCl

(2 × 5 ml) and H_2O (3 × 5 ml). After drying 2-[3-(3,4,5-trihydroxyphenylpropionyl)amino]-3,5-dihydroxybenzoic acid {**10**} was isolated (40 mg, 27 %). ^{1}H-NMR spectrum (CD_3OD, 200 Mhz); Benzoic acid part: δ 6.56 (d, *J*=2.8 Hz, H-4), 6.96 (d, *J*=2.7 Hz, H-6). Cinnamic acid part: δ 2.66 (2H), 2.77 (2H), 6.24 (s, H-2/H-6). *Anal.* found; C 46.4, H 4.8, N 3.1; calcd for $C_{16}H_{15}NO_8 \times 3(H_2O)$, C 47.6, H 5.3, N 3.5. UV λ_{max} (MeOH) = 321 nm.

Synthesis of *2-[(3,4,5-trihydroxybenzoyl)amino]-3,5-dihydroxybenzoic acid* {**11**}.

3,4,5-Trimethoxybenzoyl chloride - $SOCl_2$ (15 ml) was added to a solution of 3,4,5-trimethoxybenzoic acid (5.1, 21.1 mmol) in C_6H_6 (25 ml). The mixture was refluxed for 1.5 h. Removal of the excess $SOCl_2$ by azeotropic distillation followed by bulb to bulb destillation of the residue gave 3,4,5-trimethoxybenzoyl chloride (4.6 g, 83 %).

2-[(3,4,5-Trimethoxybenzoyl)amino]-3,5-dimethoxybenzoic acid methyl ester - A solution of 2-amino-3,5-dimethoxybenzoic acid methyl ester (1.27 g, 6 mmol) in CH_2Cl_2 (10 ml) was added dropwise at 0°C to a stirred solution of 3,4,5-trimethoxybenzoyl chloride (1.27 g, 5.5 mmol) in CH_2Cl_2 (15 ml) and Et_3N (758 mg, 7.5 mmol). After stirring for 48 h at room temperature, the mixture was washed with 1M HCl (50 ml), satd. $NaHCO_3$ solution (50 ml) and satd. NaCl solution (50 ml). After drying and evaporation, 2-[(3,4,5-trimethoxybenzoyl)-amino]-3,5-dimethoxybenzoic acid methyl ester (1.6 g, 79 %) was crystallized from EtOAc.

2-[(3,4,5-Trimethoxybenzoyl)amino]-3,5-dimethoxybenzoic acid - A solution of 2-[(3,4,5-trimethoxybenzoyl)amino]-3,5-dimethoxybenzoic acid methyl ester (1.22 g, 3.0 mmol) in H_2O (25 ml) and MeOH (25 ml) was stirred with KOH (674 mg, 12 mmol) for 4 h at 40°C and then poured into 1 M HCl (12 ml). The precipitate was filtered off and was washed successively with H_2O (15 ml) and $CHCl_3$ (25 ml). After drying 2-[(3,4,5-trimethoxybenzoyl)amino]-3,5-dimethoxybenzoic acid (1.1 g, 95 %) was collected.

2-[(3,4,5-Trihydroxybenzoyl)amino]-3,5-dihydroxybenzoic acid {**11**} - A solution of 1M BBr_3 (8 ml, 8 mmol) in CH_2Cl_2 was added dropwise to a stirred suspension of 2-[(3,4,5-trimethoxybenzoyl)amino]-3,5-dimethoxybenzoic acid (810 mg, 2.07 mmol) in dry CH_2Cl_2 (15 ml) at −78°C. After stirring for 2 h at 0°C, the reaction was quenched with 4 M HCl (7.5 ml). The mixture was centrifuged and the residue was washed with 0.02 M HCl (2 × 5 ml) and H_2O (3 × 5 ml). After drying 2-[(3,4,5-trihydroxybenzoyl)amino]-3,5-dihydroxybenzoic acid {**11**} was isolated (630 mg, 95 %). ^{1}H-NMR spectrum (CD_3OD, 200 Mhz); Benzoyl part: δ 7.07 (H-2/H-6), Benzoic acid part: δ 6.63 (d, *J*=3.0 Hz, H-4), 7.10 (d, *J*=3.0 Hz, H-6). ^{13}C-NMR spectrum (CD_3OD, 50 Mhz); Benzoyl part: δ 108.9 (C-2/C-6), 125.2 (C-1), 139.6 (C-4), 147.4 (C-3/C-5), 169.3 (C-7). Benzoic acid part: δ 111.6 (C-6), 112.1 (C-4), 122.4 (C-2), 124.0 (C-1), 153.2 (C-3), 157.3 (C-5), 171.8 (C-7). *Anal.* found; C 47.8, H 3.7, N 4.1; calcd for $C_{14}H_{11}NO_8 \times 1.7(H_2O)$, C 47.8, H 4.1, N 4.0. UV λ_{max} (MeOH) = 340, 297, 264 nm.

REFERENCES

Blaakmeer, A., Stork, A., van Veldhuizen, A., van Beek, T.A., de Groot, Ae., van Loon, J.J.A. and Schoonhoven, L.M. 1994. Isolation, identification and synthesis of miriamides, new host markers from eggs of *Pieris brassicae* (Lepidoptera: Pieridae). *J. Nat. Prod.* 57: 90-99.

Collins, F.W. 1989. Oat phenolics: Avenanthramides, novel substituted *n*-cinnamoylanthranilate alkaloids from oat groats and hulls. *J. Agric. Food Chem.* 37:60-66.

David, W.A.L. and Gardiner, B.O.C. 1952. Laboratory breeding of *Pieris brassicae* L. and *Apanteles glomerata* L. *Proc. R. Entomol. Soc. Lond.* (A) 27: 54-56.

Finney, D.J. 1978. Statistical Method in Biological Assay. Charles Griffin and Company LTD, London and High Wycombe.

Klijnstra, J.W. and Schoonhoven, L.W. 1987. Effectiveness and persistence of the oviposition deterring pheromone *Pieris brassicae* in the field. *Entomol. Exp. Appl.* 45: 227-235.

Niemann, G.J., Liem, J., van der Kerk-van Hoof, A. and Niessen, W.M.A. 1992. Phytoalexins, benzoxazinones, *n*-aroylanthranilates and *n*-aroylanilines, from *Fusarium*-infected carnations stems. *Phytochemistry* 31: 3761-3767.

Niemann, G.J., 1993. The anthranilamide phytoalexins of the Caryophyllaceae and related compounds. *Phytochemistry* 34: 319-328.

Rothschild, M. and Schoonhoven, L.M. 1977. Assessment of egg load by *Pieris brassicae* (Lepidoptera: Pieridae). *Nature* 266: 532-535.

Schoonhoven, L.M., E.A.M. Beerling, J.W. Klijnstra and Y. van Vugt. 1990. Two related butterfly species avoid oviposition near each other's eggs. *Experientia* 46: 526-528.

Schoonhoven, L.M. 1990. Host-marking pheromones in Lepidoptera with special reference to two *Pieris* spp. *J. Chem. Ecol.* 16: 3043-3052.

Spearman, C. 1908. The method of 'right and wrong cases' ('constant stimuli') without Gauss's formulae. *Br. J. Psychol.* 2: 277-242.

van Loon, J.J.A. 1990. Chemoreception of phenolic acids and flavonoids in larvae of two species of *Pieris*. *J. Comp. Physiol.* (A) 166: 889-899.

CHAPTER 5

Plant response to eggs *vs.* host marking pheromone as factors inhibiting oviposition by *Pieris brassicae*

ABSTRACT

Pieris brassicae L. butterflies secrete miriamides onto their eggs. These avenanthramide alkaloids are strong oviposition deterrents when sprayed onto a cabbage leaf. However, these compounds could not be detected in cabbage leaves from which egg batches had been removed 2 days after deposition and that still showed oviposition deterrency. It was concluded that the miriamides were not directly responsible for the avoidance by females of occupied leaves while searching for an oviposition site. Evidence was obtained that cabbage leaves themselves produce oviposition deterrents in response to egg batches. Fractions containing potent oviposition deterrents could be isolated from surface extracts of leaves from which previously laid egg batches had been removed. The term Host Marking Pheromone that was used previously is not applicable in this case.

INTRODUCTION

Several phytophagous insect species belonging to different orders, deposit chemical markers on or around their eggs. These markers are called Host Marking Pheromones (HMP's) and constitute a chemical signal that deters conspecific females from egg-laying at that site (Prokopy *et al.*, 1976; Roitberg and Prokopy, 1987). The chemical nature of the substances involved has been established in only a few cases and was found to be profoundly different for different insect species (Hurter *et al.*, 1987; Imai *et al.*, 1990).

Already more than a century ago (Kirby and Spence, 1863) observed that oviposition by *Pieris brassicae* L., commonly occurring on cruciferous plants, was influenced by the presence of previously laid eggs. Rothschild and Schoonhoven (1977) confirmed this observation under more controlled conditions. In a choice situation between cabbage leaves with or without conspecific eggs, the butterfly prefers to oviposit on the latter.

An oviposition deterring mixture can be collected by washing the eggs with water or methanol. A methanolic egg wash of 100 eggs, when sprayed onto cabbage leaves, is much more deterrent to females than the presence of 100 intact eggs (Klijnstra and Schoonhoven, 1987). Recently Blaakmeer *et al.* (1994) isolated and identified three novel avenanthramide alkaloids, which together explained the oviposition deterring effect of a crude egg wash. The compounds were found to be secreted onto the eggs by the accessory gland of the female during oviposition (Blaakmeer, unpubl. results). The question arises how the deterrent signal is spread over the leaf, as from the behaviour it is clear that a female perceives the presence of an egg batch without actually contacting it. Moreover, the miriamides are not volatile at all (Blaakmeer *et al.*, 1994). This study was aimed at investigating putative translocation of these molecules through cabbage leaves. At the onset it already became clear that no such translocation occurred. This led us to look into the possible involvement of chemicals in the leaf surface, induced by previously laid egg batches, that deter *P. brassicae* L. females from oviposition.

MATERIAL AND METHODS

Plant material - *Brassica oleracea* L. var. *gemmifera* cv. Titurel plants were reared in a greenhouse (20-30°C, 50-80%RH, 16L:8D) in standard potting soil. Illumination consisted of natural daylight supplemented by high pressure sodium vapour lamps hanging 0.75 m above pot level.

Insects - *Pieris brassicae* L. adults were obtained from a laboratory colony maintained on *Brassica oleracea* L. This culture was established in 1981 and since then 18 generations have been produced each year. Field collected adults have been introduced several times during this period. Rearing conditions were similar to those described by David and Gardiner (1952).

Bioassays - Oviposition preferences were tested in wooden cages with walls of muslin and doors of glass measuring 80 × 50 × 100 cm high. The cages were placed in a conditioned greenhouse, with temperatures fluctuating between 22 and 25°C. In addition to natural daylight, each cage was illuminated from 7.00 till 15.00 h by a 400 Watt sodium vapour lamp hanging 30 cm above the glass roof of the cage. In each cage 8 females and 4 males were introduced just after eclosion. The butterflies were repeatedly used for bioassays during 10 days. In the bioassays, 1 ml of solvent or fraction was sprayed on the upper leaf surface only using a chromatographic solvent sprayer. One control and one treated leaf were placed in diagonally opposite corners and positions were alternated between replicate cages to minimize

positional effects. In the first isolation attempt of putative deterrents, control leaves were sprayed with leaf surface extracts of control plants and fractions obtained from it. In the other attempts control leaves were sprayed with methanol only. Females could oviposit on the leaves during 5 h (8 AM - 1 PM). The preference of the butterflies was measured by comparing the number of egg batches deposited on the leaves sprayed with different fractions with that on the control leaves. On any one day 8 replicates were run. The significance of preference was tested using the Wilcoxon's matched pairs signed rank test (Siegel, 1956).

Deterrency of leaves that had carried egg batches - To determine the degree of avoidance of leaves that had carried egg batches (previously documented by Rothschild and Schoonhoven (1977)), we used a bioassay in which the oviposition preference of about 40 individuals was measured. In this bioassay, females were given a choice between control leaves and leaves from which egg batches (3-15 batches per leaf, an average batch consists of 45 eggs) that had adhered to the leaf during 24, 48 or 72 h since oviposition, were removed just prior to the bioassay. These leaves had been on intact plants and were excised just prior to removal of the eggs. After a female butterfly had made a choice for one of the leaves and had started to lay eggs, the female and the one or two eggs she had already deposited were immediately removed. Significance of preference for control leaves was tested by a Chi-square test for expected frequencies (Sokal and Rohlf, 1981).

HPLC - The pump models 302 and 303, manometric module 802C, Dynamic mixer 811 and UV-detector 116 were all of Gilson. A software HPLC system manager (model 702) from Gilson was used on an Apple II personal computer. The column used was a Microsorb RP C18 250 × 10 mm, 5 µm particle size and 100 Å pore size (Rainin Instrument Co.).

Extraction and fractionation of surface of leaves which had carried egg batches - Eggs were laid by *P. brassicae* L. on leaves of intact *B. oleracea* L. var *gemmifera* cv. Titurel plants (8-10 weeks old). After 48 h, leaves carrying 4 to 6 egg batches were harvested. The egg batches were gently removed with a brush and the leaves were dipped in 500 ml dichloromethane for 3 s followed by a dip in 500 ml methanol, also for 3 s. The crude methanol dips of 1,000 (*ca.* 6000 g) leaves collected during the months June, July and August were combined.
The methanol extract was then evaporated to dryness and redissolved in 10 ml of methanol. This crude methanol extract was separated into 6 fractions using reversed phase C18 HPLC. The mobile phase contained 0.05% trifluoroacetic acid (TFA). The solvent composition changed in 20 min linearly from MeCN/H_2O: 10/90% to MeCN/H_2O: 70/30% and was kept at that composition for 5 min, at a flow rate of 3.0 ml/min. After four min, a fraction was collected every 3 min (in total 6) .

Fractions 4, 5 and 6 were further separated by means of HPLC using the same column but different gradients. The flow rate of the solvents (containing 0.05% TFA) was 3.0 ml/min. For fraction 4 the solvent composition changed in 30 min linearly from MeCN/H_2O: 20/80% to MeCN/H_2O: 35/65%. After 4 min, a fraction (in total 4) was collected every 5 min. For fraction 5 the solvent composition changed in 30 min linearly from MeCN/H_2O: 25/75% to MeCN/H_2O: 55/45%. After 4 min, 2 fractions were collected each for 8 min. For fraction 6 the solvent composition changed in 25 min linearly from MeCN/H_2O: 35/65% to MeCN/H_2O: 65/35%. After 4 min, 3 fractions were collected each for 6 min.

RESULTS

Cabbage leaves from which egg batches had been removed after 24, 48 or 72 h of egg-laying were avoided as an oviposition substrate in favour of clean cabbage leaves in dual choice situations (Table 1). In dual choice situations the crude methanol extract of the leaf surface still deterred oviposition (Figure 1).

Table 1. Oviposition deterrence of leaves that had carried egg batches during the time indicated (treated leaves). Groups of 6-8 females were observed individually while having a choice between treated and control leaves. As soon as a female started to oviposit, she was removed from the cage. This was repeated for seven groups. Numbers of ovipositions are totals over seven groups.

Number of egg batches	Residence time (h) of egg batches on leaves	number of ovi-positions on treated[a] leaves	number of ovi-positions on control leaves
7	24	15	33*
15	24	4	36*
5	24[b]	14	30*
8	48	11	29*
3	72	14	36*

[a] - treatment signifies the adherence of egg batches (numbers indicated in the first column) during the periods indicated in the second column

[b] - egg batches were removed after 24 hours and leaf was tested 24 hours later. In all other cases, leaves were offerd directly after removal of the egg batches.

* - Number of ovipositions on treated leaves significantly lower than that on control leaves (Chi-square test).

Surprisingly, HPLC analysis of the crude methanol extract of the leaf surface failed to demonstrate the presence of the three miriamides which are the only oviposition deterring compounds obtained from the egg washes of *P. brassicae* L. (Blaakmeer *et al.*, 1994).

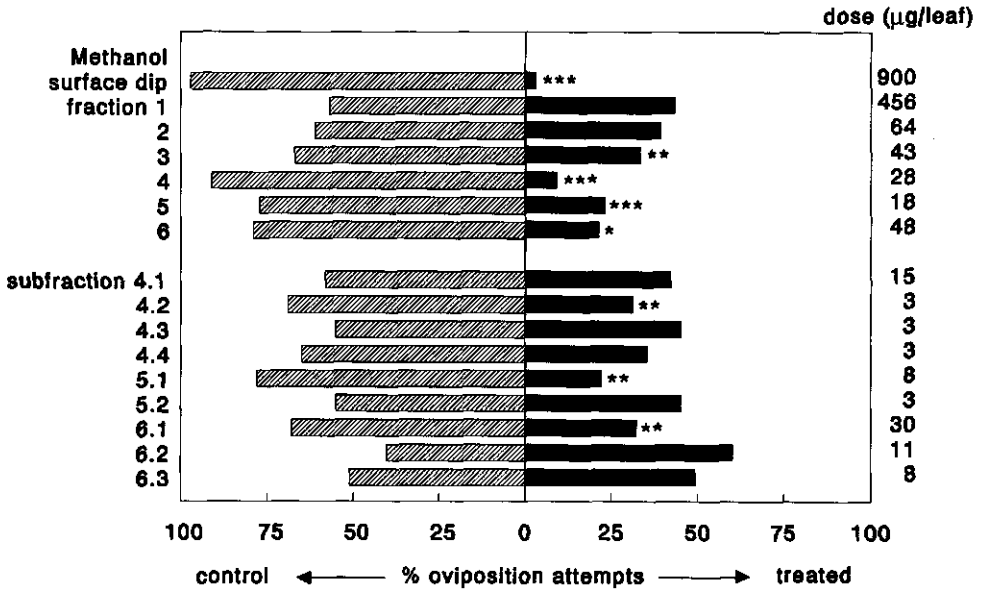


Figure 1. Oviposition preferences displayed by *Pieris brassicae* L. female butterflies in a dual choice situation. Results obtained during the first isolation attempt. Asterisks (*) indicate that treated leaves were significantly less preferred according to Wilcoxon's matched pair signed rank test (two tailed; Siegel, 1956), under the null hypothesis that egg batches were distributed evenly over control and treated leaves. The amount of dry material applied to test the activity of a certain fraction originated from fractionation of dry material present in the original methanolic leaf dip of two cabbage leaves. * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$.

The separation procedure used to isolate oviposition deterring fractions/compounds of the crude methanol extract is shown in Figure 2. The methanol extract was separated into six fractions using reversed phase C18 HPLC. Fraction 3, 4, 5 and 6 contained oviposition deterring activity (Figure 1), while fractions 1 and 2 did not. Fraction 3 was not further analyzed because of the low oviposition deterring activity compared to the three other fractions. Fraction 4 was further subdivided into four fractions and only fraction 4.2 showed oviposition deterring activity. Fraction 5 was further separated into two fractions, of which only 5.1 contained oviposition deterring activity. Fraction 6 was separated into three fractions of which only 6.1 contained oviposition deterring activity. The oviposition deterring activity was lost by further purification of the three active subfractions. Two other attempts to isolate and identify components responsible for the oviposition deterring activity in the methanol extracts of leaves which had carried egg batches for two days (removed prior to extraction), gave exactly the same result, *i.e.* activity was reproducibly found in the same sub-fractions but vanished when further purification was undertaken.

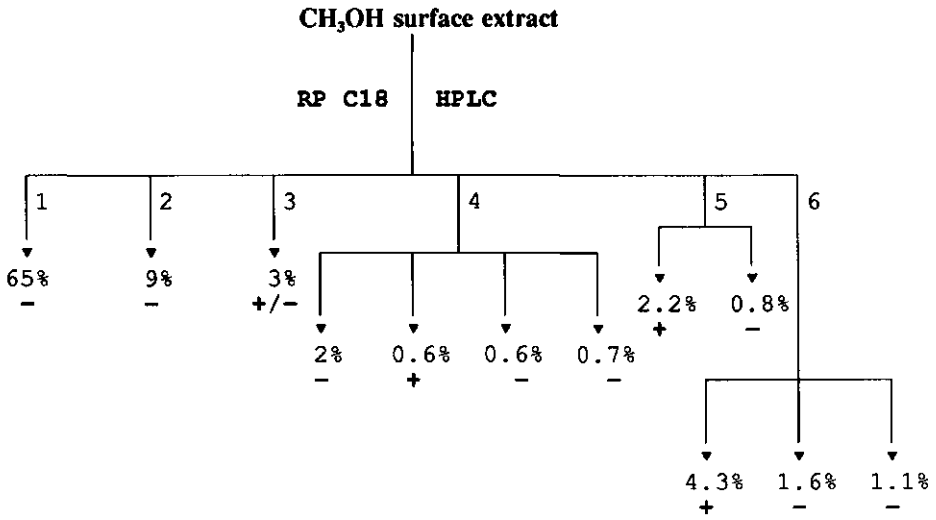


Figure 2. Purification scheme of the methanol surface extract and the dry material distribution (%) in the fractions obtained. Fractions significantly deterring oviposition are marked with a "+" sign. The 9% loss of dry material during fractionation was caused by sampling errors in weighings.

DISCUSSION

The strongly oviposition deterring miriamides, which are constituents of eggs of *P. brassicae* L. (Blaakmeer *et al.*, 1994), could not be detected in surface extracts from leaves from which egg batches had been removed (detection limit of the miriamides is 0.15 µg/leaf). Because at least 1.5 µg/leaf of one of the two most active miriamides is necessary to get an oviposition deterring activity comparable to that reported here (Blaakmeer *et al.*, 1994), we conclude that the miriamides are not responsible for the oviposition deterring activity of a leaf after oviposition by *P. brassicae* L. It is doubtful whether the three miriamides are involved in the avoidance of leaves carrying an egg batch under natural conditions. It may be possible that the miriamides associated with an egg batch remain tightly bound to the leaf surface after removal of egg batches. However, in separate experiments, application of a droplet containing the three pure miriamides in a dose equivalent to 100 eggs at the lower side of a leaf at 5 different spots did not render this leaf less acceptable to the females when tested two days later. This is additional evidence for the absence of a role for the miriamides in inducing the apparent changes in leaf surface chemistry. Therefore the term HMP does not correctly describe the phenomenon of *Pieris* butterflies avoiding hosts plants already carrying conspecific eggs (Schoonhoven, 1990).

The loss of oviposition deterring activity after purification of the subfractions 4.2, 5.1 and 6.1 could be due to instability of the active compounds. Lack of synergism can be excluded as a cause for the loss of activity because recombination of fractions of the subfractions 4.2, 5.1 and 6.1 did not show any oviposition deterring activity.

We interprete these behavioural effects of leaf surface fractions as deterrency caused by plant compounds and not by compounds of insect origin. We can exclude the possibility that the adherence of eggs to the leaf surface reduces the concentrations of glucosinolates, known to be the major oviposition stimulants to *P. brassicae* L. (van Loon *et al.*, 1992). We first assured that spray application of exogenous glucosinolates, doubling the amount present on the surface of a normal cabbage leaf, did not induce a preference for leaves thus treated. This justified that in the second and third isolation attempts we sprayed the control leaves in the dual choice assay with methanol only. Nevertheless, females significantly preferred the latter. This proves that the glucosinolates applied only on the treated leaves, albeit in reduced amounts, cannot account for the preference for the control leaves.

The HPLC procedure used was identical to that described by Blaakmeer *et al.* (1994) for the separation of the crude egg wash. The HPLC fractions of the crude surface leaf dip that contained the activity was compared with the corresponding fractions of an egg wash. The latter was found to contain only the three miriamides as active compounds.

In the bioassay used, the reaction to the egg batches was studied only in those leaves that actually had carried eggs, but not in other leaves of the same plant. However, when a more sensitive bioassay was used in which individual females were followed, other leaves of the same plant were found to become less acceptable than control leaves from a plant which never received any eggs (van Loon, unpublished observations).

A hypersensitivity reaction to eggs is also found for other *Brassica* species (Shapiro and DeVay, 1987). Some individual plants of *B. nigra* L. produce a necrotic zone at the base of freshly laid eggs of *P. rapae* L. and *P. napi* L., thereby desiccating them.

In contrast to what we suggested previously (Blaakmeer *et al.*, 1994) the ecological function of the miriamides on the egg surface of *P. brassicae* L. eggs remains unclear. The avenanthramides, compounds related to miriamides, isolated from oat groats and hulls (Collins, 1989) and from infected carnation stems (Niemann, 1993), have strong anti-fungal activity. Miriamides could possibly protect the eggs of *P. brassicae* L. against various fungal diseases or against certain predators. The egg-induced changes in leaf surface chemistry documented here are to our knowledge the first example of a plant response to an insect product and a subsequent effect on insect behaviour without prior injury being inflicted to the plant.

In conclusion, the three miriamides, isolated from the eggs of *P. brassicae* L. (Blaakmeer *et al.*, 1994) are not responsible for the oviposition deterring effect of leaves which carry egg batches. Instead, evidence was obtained that the leaves react to contact with eggs or to compounds emanating from the eggs, which then act as elicitors. The elicitors of insect origin and the mechanism via which they operate to cause chemical changes in the plant surface will be subject to future studies.

REFERENCES

Blaakmeer, A., Stork, A., van Veldhuizen, A., van Beek, T.A., de Groot, Ae., van Loon, J.J.A. and Schoonhoven, L.M. 1994. Isolation, identification and synthesis of miriamides, new host markers from eggs of *Pieris brassicae* (Lepidoptera: Pieridae). *J. Nat. Prod.* 57: 90-99.

Collins, F.W. 1989. Oat phenolics: Avenanthramides, novel substituted *N*-cinnamoylanthranilate alkaloids from oat groats and hulls. *J. Agric. Food Chem.* 37: 60-66.

David, W.A.L. and Gardiner, B.O.C. 1952. Laboratory breeding of *Pieris brassicae* L. and *Apanteles glomerata* L. *Proc. R. Entomol. Soc. Lond.* (A) 27: 54-56.

Hurter, J., Boller, E.F., Städler, E., Blattmann, B., Buser, H.-R., Bosshard, N.U., Damm, L., Kozlowski, M.W., Schöni, R., Raschdorf, F., Dahinden, R., Schlumpf, E., Fritz, H., Richter, W.J. and Schreiber, J. 1987. Oviposition-deterring pheromone in *Rhagoletis cerasi* L.: Purification and determination of the chemical constitution. *Experientia* 43: 157-164.

Imai, T., Kodama, H., Chuman, T. and Kohno, M. 1990. Female-produced oviposition deterrents of the cigarette beetle, *Lasioderma serricorne* (F.). *J. Chem. Ecol.* 16: 1237-1247.

Kirby, W. and Spence, W. 1863. An introduction to Entomology, 7th ed. Longman, Green, Longman, Roberts and Green, London.

Klijnstra, J.W. and Schoonhoven, L.M. 1987. Effectiveness and persistence of the oviposition deterring pheromone of *Pieris brassicae* in the field. *Entomol. Exp. Appl.* 45: 227-235.

Niemann, G.J., 1993. The anthranilamide phytoalexins of the Caryophyllaceae and related compounds. *Phytochemistry* 34: 319-328.

Prokopy, R.J., Reissig, W.H. and Moericke, V. 1976. Marking pheromones deterring repeated oviposition in *Rhagoletis* flies. *Entomol. Exp. Appl.* 20: 170-178.

Roitberg, B.D. and Prokopy, R.J. 1987. Insects that mark host plants; an ecological, evolutionary perspective on host-marking chemicals. *BioScience* 37: 400-406.

Rothschild, M. and Schoonhoven, L.M. 1977. Assessment of egg load by *Pieris brassicae* (Lepidoptera: Pieridae). *Nature* 266: 532-535.

Schoonhoven, L.M. 1990. Host-marking pheromones in Lepidoptera with special references to two *Pieris* spp. *J. Chem. Ecol.* 16: 3043-3052.

Shapiro A.M. and DeVay J.E. 1987. Hypersensitivity reaction of *Brassica nigra* L. (Cruciferae) kills eggs of *Pieris* butterflies (Lepidoptera: Pieridae). *Oecologia* 71: 631-632.

Siegel, S. 1956. Nonparametric Statistics for the Behavioral Sciences. John Wiley, New York.

Sokal, R.R. and Rohlf, F.J. 1981. Biometry. The principles and practice of statistics in biological research. 2nd ed. W.H. Freeman & Comp., New York.

van Loon, J.J.A., Blaakmeer, A., Griepink, F.C., van Beek, T.A., Schoonhoven, L.M. and de Groot, Ae. 1992. Leaf surface compound from *Brassica oleracea* (Cruciferae) induces oviposition by *Pieris brassicae* (Lepidoptera: Pieridae). *Chemoecology* 3: 1-6.

CHAPTER 6

Comparative headspace analysis of cabbage plants damaged by two species of *Pieris* caterpillars: Consequences for in-flight host location by *Cotesia* parasitoids

ABSTRACT

Headspace composition, collected from intact cabbage plants and cabbage plants infested with either *Pieris brassicae* L. or *P. rapae* L. (Lepidoptera: Pieridae) first instar larvae, was determined by GC-MS. Twenty-one volatiles were identified in the headspace of intact plants. Twenty-two volatiles were identified in the headspace of plants infested by *P. brassicae* L. larvae, 2 of which, *cis*-3-hexenyl butyrate and *cis*-3-hexenyl isovalerate, were not detected in the headspace of either intact or *P. rapae* L. damaged plants. In the headspace of the latter, 21 compounds were identified, all of which were also produced by intact plants. No significant quantitative differences were found between headspace composition of the plants damaged by one or the other caterpillar species. Major differences between intact and caterpillar damaged plants in contribution to the headspace profile were revealed for hexyl acetate, *cis*-3-hexenyl acetate, myrcene, sabinene and 1,8-cineole. The larval endoparasitoid *Cotesia glomerata* L. was attracted by the volatiles emanating from *Brassica oleracea* L. damaged by *P. brassicae* L. first instar larvae. *C. rubecula* Marshall, a specialized larval endoparasitoids of *P. rapae* L., was attracted by the volatiles released from the *B. oleracea* - *P. rapae* L. plant-host complex. This shows that cabbage plants kept under the conditions of headspace collection produce attractive volatiles for both parasitoids.

INTRODUCTION

Chemical communication between plants and natural enemies of plant feeding arthropods receives increasing attention (Dicke and Sabelis, 1988; Turlings *et al.*, 1990a; Whitman and Eller, 1990). Plants damaged by herbivores release volatiles at higher rates than intact plants (Dicke *et al.*, 1990; Turlings *et al.*, 1990a; Whitman and Eller, 1990). Especially the green leaf odours (six-carbon alcohols, their esters and aldehydes) become more abundant when

plant tissue is damaged (Saijo and Takeo, 1975; Wallbank and Wheatly, 1976; Buttery *et al.*, 1985; Tollsten and Bergström, 1988). Volatiles released upon damage may guide carnivorous insects during their search for herbivorous hosts. This active release has been viewed as an indirect defence response of the plant, the herbivore-induced volatiles thus functioning as synomones (Whitman, 1988; Dicke *et al.*, 1990; Turlings *et al.*, 1990a; Whitman and Eller, 1990; Takabayashi *et al.*, 1991).

Chemical information from the plant is considered to be more easy to detect than volatile cues emanated from the host larvae, while the latter provide the parasitoid with more reliable information about host presence (Vet and Dicke, 1992). Behavioural studies have led to the suggestion that herbivore-induced synomone production by the plant is affected by the species of herbivore feeding (Sabelis and van de Baan, 1983; Dicke, 1988; Turlings *et al.*, 1993).

The gregarious *Cotesia glomerata* L. and the solitary *C. rubecula* Marshall (Hymenoptera: Braconidae) are important larval parasitoids of *P. brassicae* L. and *P. rapae* L. caterpillars, both specialized herbivores of cruciferous plants (Laing and Levin, 1982). *C. glomerata* L. accepts both caterpillar species (Richards, 1940; Laing and Levin, 1982), while *C. rubecula* Marshall is considered to be a specialized parasitoid of *P. rapae* L. (Nealis, 1986, 1990; Wiskerke and Vet, 1991; Kaiser and Cardé, 1992; Geervliet *et al.*, 1993).

The present study was aimed at chemical analysis of headspace composition of volatiles released by (1) intact cabbage plants over the different seasons and (2) cabbage plants damaged by either caterpillar species. The first set of analyses was performed to determine an optimal period for sampling headspace volatiles from greenhouse-reared plants. The latter part served to assess to what extent the parasitoids could use differential chemical information emanating from the two plant-host complexes when discriminating between hosts.

MATERIAL AND METHODS

Plant material - *Brassica oleracea* L. var *gemmifera* cv. Titurel plants were reared in a greenhouse (20-30°C, 50-80% RH, 16L:8D) from seed till 8 weeks old in potting soil. Illumination consisted of natural daylight supplemented by 400 Watt high pressure sodium vapour lamps hanging 0.75 m above pot level. Lights were turned on (9 W/m^2, maximal 16 h) when daylight intensity was lower than 6 W/m^2. Plants were fertilized weekly. Intact individually potted plants (pot volume 0.8 l) and potted plants infested with known numbers of first instar larvae (24 h post-hatching) of either *P. brassicae* L. or *P. rapae* L. were used.

Insects

Caterpillars - First instar larvae of *P. brassicae* L. and *P. rapae* L. had hatched from eggs deposited on *B. oleracea* L. plants from which samples were obtained. Eggs originated from laboratory reared female butterflies of both species maintained on the Brussels sprout cultivar mentioned above. Colonies of both species have been maintained in our laboratory since 1981 and approximately 18 generations were reared each year. Rearing conditions were similar to those described by David and Gardiner (1952).

Parasitoids - Parasitoid colonies were based on field material collected in the vicinity of Wageningen. *C. glomerata* L. were reared in a greenhouse compartment at 22-26°C, 50-70% RH and a light regime 16L:8H. *C. rubecula* Marshall were reared in a climatic room under the same conditions. Both parasitoid species were offered first instar larvae of their preferred host species, *P. brassicae* L. for *C. glomerata* L. and *P. rapae* L. for *C. rubecula* Marshall, for parasitization. The parasitized larvae were placed in cages with ample amount of Brussels sprouts plants. Cocoons were collected in petri-dishes and emergence of the adults took place in a nylon gauze cage (35 × 40 × 30 cm), where mating occurred. The wasps were supplied with honey and water.

Collection of headspace volatiles - Plants were put in a 56 l stainless steel vessel with a perspex cap, in which a rotor, propelled by a magnetic stirrer outside the vessel, was mounted for perturbing the air. Technical air which was cleaned at the inlet of the vessel by passage through 275 g potassium hydroxide, 175 g molecular sieves 4A and 13X (Linde) and 180 g activated charcoal, was used for generating the air stream. After the plants were put in their position, the vessel was purged with purified air (200 l) during 1 h. The plants were constantly illuminated by a 400 Watt sodium vapour lamp hanging 75 cm above the cap, yielding 11 W/m^2 at plant level. Under these conditions, volatiles by intact plants were accumulated overnight (17 h) in the vessel. The next morning, the headspace volatiles were trapped in a 160 × 4 mm glass tube containing 130 mg Tenax TA at the outlet of the vessel, with a flowrate of 300 ml/min. Forty five litres of air was passed through the trap during 2.5 h, resulting in entrainment of 58 percent of the volatiles accumulated overnight (van 't Riet and Tramper, 1991). When a second trap was placed after the first one, no breakthrough occurred. In the set-up described above, only Teflon tubing was used. Samples from uninfested plants were collected throughout the year. Samples from caterpillar infested plants were collected during day-time in June 1993. After the plants with first instar larvae were put in their position, the vessel was also purged with purified air (200 l) during 1 h. The volatiles were trapped during the subsequent 2.5 h (flow: 300 ml/min), which resulted in entrainment of 32 percent of the volatiles produced (van 't Riet and Tramper, 1991).

Analysis of headspace volatiles - The collected volatiles were released from the Tenax by heating the trap in a Thermodesorption Cold Trap Unit (Chrompack) at 250°C for 10 min and flushing with a He-flow of 10 ml/min. The released compounds were cryofocused in a cold trap (0.52 mm i.d. deactivated fused silica) at an approximate temperature of −85°C. The cold trap was connected to a Supelcowax 10 fused silica capillary column (60 m × 0.25 mm i.d., 0.25 µm film thickness), which was connected in a Pye 204 gas chromatograph. The column was mounted to a VG MM7070 F mass spectrometer, working in the 70 eV EI-mode. The compounds were transferred to the capillary column by ballistically heating the cold trap to 220°C. Starting at a temperature of 40°C (4 min), the GC-oven was programmed at an initial rate of 2°/min during 30 min, followed by a rate of 6°/min to a final temperature of 270°C (4 min). The GC was calibrated with a sample containing a known amount of the 7 main components to measure an average response factor, which was used to calculate the amount of all volatiles in the headspace samples.

Windtunnel set-up - The windtunnel used (200 × 60 × 60 cm) was constructed according to Takken (1991), with some modifications. An air-humidifier was constructed between the glasswool filter and the activated charcoal filter. The light source used was a half-round frame, hanging over the flight-compartment, with the inner side covered with light-reflecting material (aluminium). Inside the frame 8 fluorescent tubes (Philips TLD 32W/84HF) and 4 bulbs (Philips Softtone 200W) were mounted. Light intensity was 2 W/m^2 at the release site of the wasps. The temperature in the windtunnel was 21°C and the relative humidity fluctuated between 40 and 60%. Wind speed at the release site was 0.2 m/s.

Bioassays - For bioassays mated females, 3-5 days old, were used. As a pre-flight treatment the wasps were allowed to walk over a host-damaged leaf, containing fresh host damage (including host by-products, such as silk and faeces) but without caterpillars, 16 h before testing. After this experience, wasps were kept individually in vials (5.5 cm, 1.5 cm i.d.), supplied with honey, in a climate chamber at 15°C until needed.
In the windtunnel a dual-choice situation was offered to the wasps, consisting of two separate air-streams. Filtered air (as described in collection of headspace volatiles) was split into two air-streams and each of them was led through a metal vessel (described above) (flow 2 l/min), one containing an intact plant and the other a plant with 50 first instar larvae of the respective host species of the parasitoid. Subsequently, the volatile-containing air was led into the tunnel through vertical glass-tubes. T-shaped glass-tubes (21 cm, 4.5 cm i.d.) were placed over the vertical glass-tubes to offer the wasps a landing site. For visual orientation of the wasps an undamaged cabbage leaf, in a vial closed with Parafilm, was placed into each T-shape glass-tube. The release site was a large glass tube (30 cm, 15 cm i.d.) on a socket (10 cm high),

with both ends open, placed at a distance of 1 m from the odour sources. The vial containing the individual wasp was placed on the bottom of the release tube. The test lastel until the parasitoid landed, with a maximum of 5 minutes after flight initiation. Wasps that did not choose for one of the alternative odour sources were counted under 'no oriented flight'. Both parasitoid species were tested on the same day, replicates being carried out on subsequent days. Preference of the responding parasitoids for one of the offered air-streams was tested by a Chi-square test for expected frequencies ($P < 0.05$) (Sokal and Rohlf, 1981).

RESULTS

Seasonal fluctuations in volatile production

The total amount of volatiles released during the combined accumulation and collection period by intact uninfested cabbage plants shows a clear seasonal trend (Figure 1). During September 1990 a steep decline in volatile release occurred.

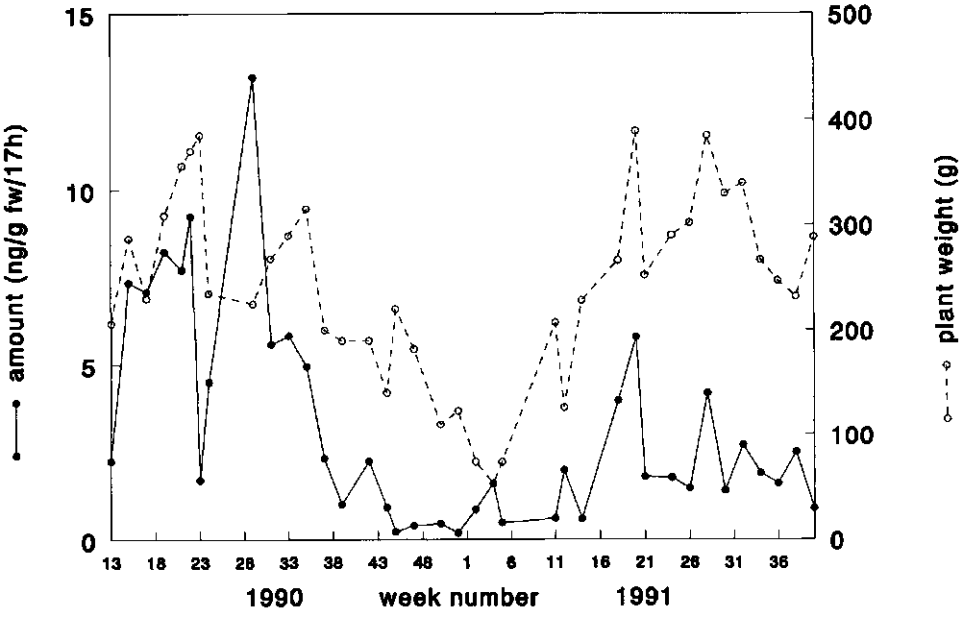


Figure 1. Emission of total headspace volatiles (ng/g fw) over a collection period of 17 h by 4 intact 8 week old potted *B. oleracea* L. plants and fresh weight of aerial parts of the 4 plants over time.

Release rates remained on average a factor 10 lower during winter till an increase occurred at the end of March 1991. Although exhibiting quite some variation in the amount emitted, thereafter release rates showed an upward trend again, but did not reach the level of 1990 (range 9-22 ng/g fw/17 h in spring and summer 1990, versus 3-9 ng/g fw/17 h in spring and summer 1991). This trend in volatile production concurs with a similar trend in the fresh weight of aerial parts of 8 weeks old plants, which is clearly lower in the winter season (Figure 1).

Headspace composition of uninfested and infested plants

The average headspace compositions of intact plants and plants infested with larvae of either *P. brassicae* L. and *P. rapae* L. are given in Table 1. Volatile profiles of *P. brassicae* L. infested plants were made up of 22 compounds. Qualitative differences were found compared to the composition of intact plants, as they produced *cis*-3-hexenyl butyrate and *cis*-3-hexenyl isovalerate, that were not detected in the headspace samples from intact plants. Both compounds likewise cause a qualitative difference between *P. brassicae* L. and *P. rapae* L. infested plants (Table 1). On the other hand, ß-phellandrene was not detected in headspace collections from *P. brassicae* L. infested plants, while this compound was present in both intact and *P. rapae* L. infested plants. The five main headspace-components (*cis*-3-hexenol, *cis*-3-hexenyl acetate, sabinene, limonene, 1,8-cineole) from intact plants and caterpillar infested plants were similar. Although quantitative differences in these main components were observed between intact plants and caterpillar infested plants, only for 1,8-cineole the contribution to the headspace of intact plants was significantly higher than that of plants infested by either one caterpillar species (Student's *t*-test on arcsine sqaure root transformed percentages, $P < 0.05$). The contribution of *cis*-3-hexenol and *cis*-3-hexenyl acetate was lower in intact headspace of the former, while this was the reverse for the other three components. The headspace odours emitted by *P. rapae* L. infested cabbage plants contained 21 compounds which were also released by intact plants (Table 1). Absolute release rates of volatiles by both plant-host complexes did not differ significantly (Figure 2).

Bioassays

The percentages of females that showed completed flights are 80% for *C. glomerata* L. and 69% for *C. rubecula* Marshall. In Figure 3 the distribution of choices for either one of the two odour sources (Plant Host Complex versus Clean Cabbage) is given for both parasitoid species. Females of both species do have a preference for the volatiles emitted by caterpillar infested plants.

Table 1 . Composition of headspace volatile mixtures (as average percentages ± SEM (Standard Error of the Mean) of total amount of volatiles trapped (total area under GC peaks)) collected for 2.5 h (during day-time) from intact potted *B. oleracea* L. plants, and plants infested by first instar larvae of either *P. brassicae* L. or *P. rapae* L.

Compound	Intact plants n=2	Plants infested with *P. brassicae* n=4	Plants infested with *P. rapae* n=4
Alcohols			
1-penten-3-ol	0.5 ± 0.3	1.4 ± 0.6	0.8 ± 0.5
cis-3-hexenol	5.1 ± 0.1	7.0 ± 1.6	5.4 ± 1.3
2-ethyl-1-hexanol	4.2 ± 0.4	3.8 ± 0.4	2.8 ± 0.4
Aldehydes			
hexanal	0.7 ± 0.5	1.2 ± 0.8	0.6 ± 0.2
octanal	0.7 ± 0.5	1.1 ± 0.4	0.2 ± 0.2
Esters			
hexyl acetate	1.2 ± 0.1	3.1 ± 0.9	3.0 ± 0.5
cis-3-hexenyl acetate	17.6 ± 2.9	33.3 ± 5.3	31.9 ± 4.1
cis-3-hexenyl butyrate	-	0.3 ± 0.3	-
cis-3-hexenyl isovalerate	-	0.2 ± 0.2	-
Ketones			
3-pentanone	2.1 ± 0.1	2.6 ± 0.9	1.8 ± 1.1
Sulphides			
dimethyldisulphide	1.6 ± 0.3	0.1 ± 0.1	0.3 ± 0.1
dimethyltrisulphide	0.9 ± 0.3	0.1 ± 0.1	0.3 ± 0.2
Terpenoids			
α-pinene	1.4 ± 0.1	0.4 ± 0.4	0.7 ± 0.4
α-thujene	4.9 ± 0.3	4.4 ± 0.6	5.0 ± 0.5
β-pinene	1.9 ± 0.2	0.8 ± 0.4	0.9 ± 0.5
sabinene	21.1 ± 0.5	12.9 ± 3.0	16.2 ± 2.3
myrcene	3.1 ± 2.2	5.0 ± 0.4	6.2 ± 0.6
β-phellandrene	0.3 ± 0.2	-	0.5 ± 0.2
limonene	17.8 ± 0.1	12.7 ± 2.2	15.4 ± 1.5
1,8-cineole	11.4 ± 1.1	6.3 ± 0.8	5.6 ± 0.9
trans-sabinene hydrate	0.9 ± 0.3	0.7 ± 0.2	0.3 ± 0.3
linalool	1.9 ± 0.2	2.5 ± 2.0	1.4 ± 0.9
β-elemene	0.7 ± 0.2	0.1 ± 0.1	0.7 ± 0.4
Total	100.0	100.0	100.0

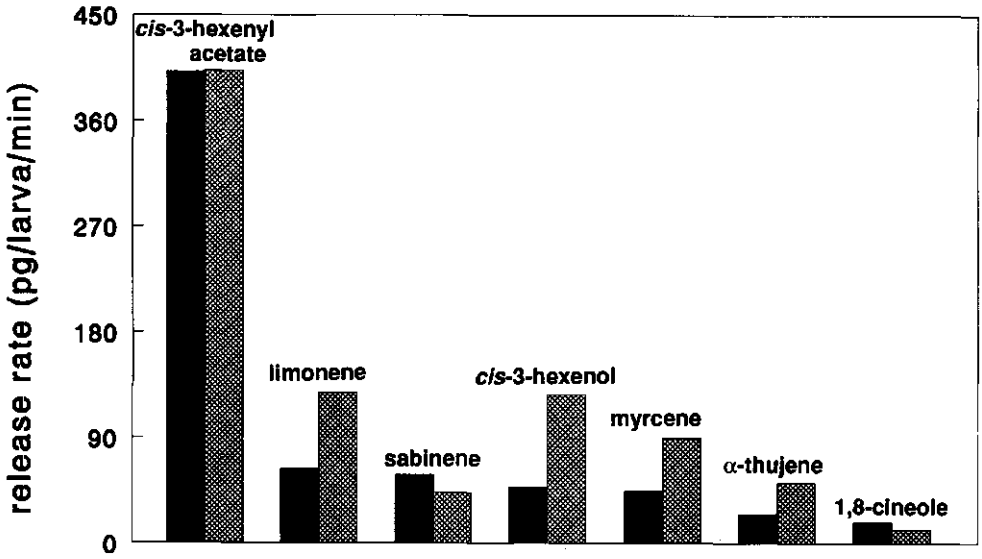


Figure 2. Amount of seven major headspace volatiles released by *B. oleracea* L. plants infested with either *P. brassicae* L. (solid bars) or *P. rapae* L. (hatched bars) first instar larvae. Net amounts released per caterpillar (*i.e.* corrected for the amount released by an average intact plant) over the 2.5 h collection period.

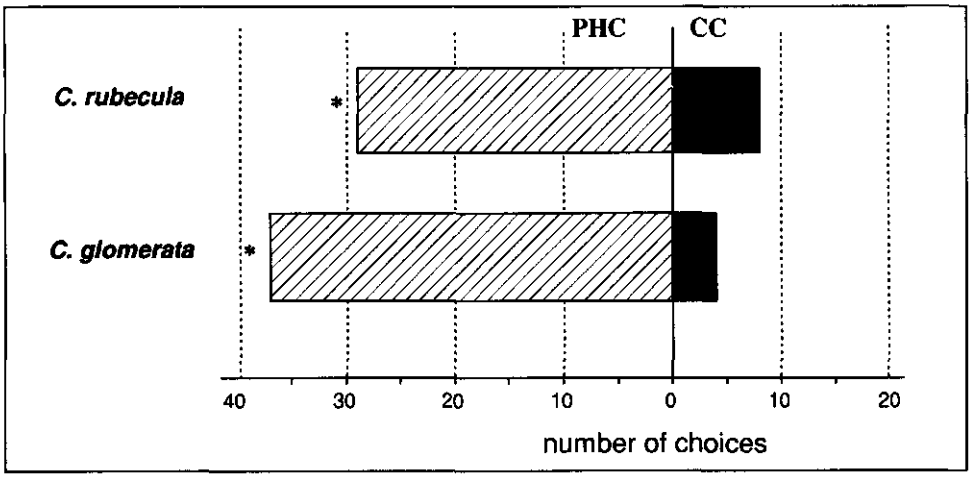


Figure 3. Distribution of choices for volatiles emitted by the plant-host complex (PHC) and the clean cabbage plant (CC) of *Cotesia glomerata* L. and *C. rubecula* Marshall Plant-host complexes consisted of cabbage, infested by the preferred host-species for the parasitoids, *Pieris brassicae* L. and *P. rapae* L. respectively. Significant preferences ($P < 0.05$) are indicated by *. N tested is 51 for *C. glomerata* L. and 54 for *C. rubecula* Marshall.

DISCUSSION

Production of volatiles per unit of fresh weight by intact cabbage plants followed a distinct seasonal trend, declining drastically in autumn, remaining very low in winter and increasing again in during spring. This phenomenon paralleled the severely reduced growth rates of the plants, indicating a role of the suboptimal physiological condition of the plants in causing the reduced emission of volatiles. Although few comparable studies are available, similar findings have been reported for other plant species (Sekiya *et al.*, 1977; Hatanaka *et al.*, 1987; Takabayashi *et al*, 1990). Our findings provided the criterion to select the summer period as most suitable for comparative studies of headspace volatiles collected from intact and herbivore-damaged plants.

Given the attention paid to *B. oleracea*-herbivore-natural enemy interactions (Nealis, 1986, 1990; Wiskerke and Vet 1991; Boland *et al.*, 1992; Kaiser and Cardé, 1992; Steinberg *et al.*, 1992; 1993) remarkably few data are available on the chemistry of volatile blends released by intact and herbivore damaged plants. The extensive qualitative study of Tollsten and Bergström (1988) on headspace volatiles collected from five intact (*i.e.* the main stem cut at ground level and put in water over 24-96 h) and mechanically damaged crucifer species did not include *B. oleracea* L., while they found many qualitative and indications of quantitative differences between the *Brassica* species.

Comparing headspace profiles of intact and infested plants reveals that two esters of cis-3-hexenol were found only from the cabbage-*P. brassicae* L. plant-host complex. *P. rapae* L. infested plants yielded qualitatively the same headspace composition as intact plants. Quantitative differences in contribution of three of the five major components (*cis*-3-hexenyl acetate, sabinene and 1,8-cineole) to headspaces of intact and both caterpillar infested plants were evident. Such comparisons are very scarce in the literature to date. A recent study by Takabayashi *et al.* (1991) demonstrated quantitative differences for three compounds between blends emitted by detached apple leaves infested by two different spider mite species. Our results confirm their conclusion that the plant is more important in affecting the composition of the volatile blend than the herbivore.

Behavioural evidence for the involvement of both plant and herbivore in producing a recognizable blend was found for *C. marginiventris* Cresson wasps orienting differently towards corn plants infested by either *Spodoptera frugiperda* J.E. Smith or *Trichoplusia ni* Hübner caterpillars (Sabelis and van de Baan, 1983; Dicke *et al.*, 1990; Turlings *et al.*, 1990b).

Cruciferous plants are phytochemically characterized by secondary plant substances called glucosinolates. These glucosinolates are the precursors of the volatile isothiocyanates. The Brussels sprouts plants we used contained at least two glucosinolates, glucobrassicin and sinigrin (van Loon *et al.*, 1992). Unexpectedly, isothiocyanates were absent in all headspace samples analyzed. It is unlikely that this is due to the choice of the adsorption material, because Tenax-TA gives good recoveries for isothiocyanates (Cole, 1980b). Knowledge of the release of isothiocyanates by *Brassica* species is mostly based on mechanically damaged plants (Cole, 1980a; Finch, 1978). Our data imply that injury by *P. brassicae* L. and *P. rapae* L. feeding does not induce release of these compounds.

C. glomerata L. is able to distinguish between intact plants and plants infested with larvae of *P. brassicae* L. or *P. rapae* L. (Wiskerke and Vet, 1991; Steinberg *et al.*, 1992; 1993). *C. glomerata* L., either naive or experienced, does not distinguish between plants infested with *P. brassicae* L. or with *P. rapae* L. (Wiskerke and Vet, 1991). This is in agreement with the generalistic nature of this species, which accepts several *Pieridae* species as host (Laing and Levin, 1982; Geervliet *et al.*, 1993). In a dual choice situation between intact and *P. rapae* L. infested plants, *C. rubecula* Marshall was shown to prefer plants infested with *P. rapae* L. (Kaiser and Cardé, 1992). Naive *C. rubecula* Marshall showed no discrimination between *P. brassicae* L. and *P. rapae* L. infested plants and both *Cotesia* species showed no discrimination between the two plant-host complexes after experience with either one of them (Geervliet, unpubl. results). Our chemical data show chemical differences which would allow discrimination by the two *Cotesia* species between intact plants and plants infested by their preferred hosts. It cannot be excluded, however, that there are synomones in the headspace with a concentration below the identification limit of the GC-MS (± 5 ng), nor that thermolabile components play a role. This lack of discrimination displayed by both parasitoids may not be surprising in view of the minor chemical differences between headspaces of both plant-host complexes. The host specificity of both *Cotesia* species found in the field (Rothschild *et al.*, 1977; Bradley, 1987; Geervliet unpubl. results) may be caused by contact cues rather than orientation cues. However, recently it has been shown that the species of plant studied may affect the ability of parasitoids to distinguish between two herbivore species feeding on the same plant (McCall *et al.*, 1993). Our current research is aimed to identify the minimal blend of components which is necessary for the searching behaviour of both *Cotesia* species.

REFERENCES

Boland, W., Feng, Z., Donath, J. and Gäbler, A. 1992. Are acyclic C_{11} and C_{16} homoterpenes plant volatiles indicating herbivory? *Naturwissenschaften* 79: 368-371.

Bradley, J. 1987. A study of the mortality factors in the *Apanteles glomeratus-Pieris* spp. system. Thesis of University of Newcastle upon Tyne.

Buttery, R.G., Xu, C. and Ling, L.C. 1985. Volatile components of wheat leaves (and stems): possible insect attractants. *J. Agric. Food Chem.* 33: 115-117.

Cole, R.A., 1980a. Volatile components produced during ontogeny of some cultivated crucifers. *J. Sci. Food Agric.* 31: 549-557.

Cole, R.A., 1980b. The use of porous polymers for the collection of plant volatiles. *J. Sci. Food Agric.* 31: 1242-1249.

David, W.A.L. and Gardiner, B.O.C. 1952. Laboratory breeding of *Pieris brassicae* L. and *Apanteles glomerata* L. *Proc. R. Entomol. Soc. Lond.* (A) 27: 54-56.

Dicke, M., 1988. Prey preference of phytoseiid mite *Typhlodromus pyri*: 1 response to volatile kairomones. *Exp. Appl. Acarol.* 4: 1-13.

Dicke, M., van Beek, T.A., Posthumus, M.A., Ben Dom, N., Bokhoven, van H. and de Groot, Ae. 1990. Isolation and identification of volatile kairomone that affects acarine predator prey interactions. Involvement of host plant in its production. *J. Chem. Ecol.* 16: 381-395.

Dicke, M. and Sabelis, M.W. 1988. How plants obtain predatory mites as body-guards. *Neth. J. Zool.* 38: 148-165.

Finch, S., 1978. Volatile plant chemicals and their effect on host plant finding by the cabbage root fly (*Delia brassicae*). *Entomol. Exp. Appl.* 24: 350-359.

Geervliet, J.B.F., van Aaken, R., Savelkoul, C., ter Smitte, S.M., Brodeur, J., Vet, L.E.M. and Dicke, M. 1993. Comparative approach to infochemical use by parasitoids for the case of *Cotesia glomerata* and *Cotesia rubecula*. *Proc. Exper. Appl. Entomol., N.E.V. Amsterdam* 4: 33-38.

Hatanaka, A., Kajiwara, T. and Sekiya, J. 1987. Biosynthetic pathway for C_6-aldehydes formation from linolenic acid in green leaves. *Chem. Phys. Lipids* 44: 341-361.

Kaiser, L. and Cardé, R.T. 1992. In-flight orientation to volatiles from the plant-host complex in *Cotesia rubecula* (Hym: Braconidae): increased sensitivity through olfactory experience. *Physiol. Entomol.* 17: 62-67.

Laing, J.E. and Levin, D.B. 1982. A review of the biology and a bibliography of *Apanteles glomeratus* (L.) (Hymenoptera: Braconidae). *Biocontrol News information* 3: 7-23.

McCall, P.J., Turlings, T.C.J., Lewis, W.J. and Tumlinson, J.H. 1993. Role of plant volatiles in host location by the specialist parasitoid *Microplitis croceipes* Cresson (Braconidae: Hymenoptera). *J. Insect Behav.* 6: 625-639.

Nealis, V.G., 1986. Reponses to host kairomones and foraging behaviour of the insect parasite *Cotesia rubecula* (Hymenoptera: Braconidae). *Can. J. Zool.* 64: 2393-2398.

Nealis, V.G., 1990. Factors affecting the rate of attack by *Cotesia rubecula* (Hymenoptera: Braconidae). *Ecol. Entomol.* 15: 163-168.

Richards, O.W., 1940. The biology of the small white butterfly (*Pieris rapae*), with special reference to factors controlling its abundance. *J. Animal Ecol.* 9: 243-298.

Rothschild, M., Valadon, G. and Mummery, R. 1977. Carotenoids of the pupae of the large white butterfly (*Pieris brassicae*) and the small white butterfly (*Pieris rapae*). *J. Zool. Lond.* 181: 323-339.

Sabelis, M.W. and van de Baan, H.E. 1983. Location of spider mite colonies by phytoseiid predators: demonstration of specific kairomones emitted by *Tetranychus urticae* and *Panonychus ulmi*. *Entomol. Exp. Appl.* 33: 304-314.

Saijo, R. and Takeo, T. 1975. Increase of *cis*-3-hexen-1-ol content in tea leaves following mechanical injury. *Phytochemistry* 14: 181-182.

Sekiya, J., Kajiwara, T. and Hatanaka, A. 1977. Seasonal changes in activity of the enzyme system producing *cis*-3-hexenol and *n*-hexanal from linolenic and linoleic acids in tea leaves. *Plant Cell Physiol.* 18: 283-286.

Sokal, R.R. and Rohlf, F.J. 1981. Biometry. The principles and practice of statistics in biological research. 2nd ed. W.H. Freeman and Comp., New York.

Steinberg, S., Dicke, M., Vet, L.E.M. and Wanningen, R. 1992. Response of the braconid parasitoid *Cotesia* (=*Apanteles*) *glomerata* to volatile infochemicals: effects of bioassay set-up, parasitoid age and experience and barometric flux. *Entomol. Exp. Appl.* 63: 163-175.

Steinberg, S., Dicke, M. and Vet, L.E.M. 1993. Relative importance of infochemicals from first and second trophic level in long-range host location by larval parasitoid *Cotesia glomerata*. *J. Chem. Ecol.* 19: 47-59.

Takabayashi, J., Dicke, M., Kemerink, J. and Veldhuizen, T. 1990. Environmental effects on production of a plant synomone that attracts predatory mites. *Symp. Biol. Hung.* 39: 541-542.

Takabayashi, J., Dicke, M. and Posthumus, M.A. 1991. Variation in composition of predator-attracting allelochemicals emitted by herbivore-infested plants: relative influence of plant and herbivore. *Chemoecology* 2: 1-6.

Takken, W., 1991. A new windtunnel for studies on host-seeking behaviour of mosquitoes. *Proc. Exper. Appl. Entomol. N.E.V. Amsterdam* 2: 171.

Tollsten, L. and Bergström, G. 1988. Headspace volatiles of whole plants and macerated plants parts of *Brassica* and *Sinapis*. *Phytochemistry* 27: 4013-4018.

Turlings, T.C.J., Tumlinson, J.H. and Lewis, W.J. 1990a. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250: 1251-1253.

Turlings, T.C.J., Scheepmaker, J.W.A., Vet, L.E.M., Tumlinson, J.H. and Lewis, W.J. 1990b. How contact foraging experiences affect preferences for host-related odors in the larval parasitoid *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae). *J. Chem. Ecol.* 16: 1577-1589.

Turlings, T.C.J., Wäckers, F.L., Vet, L.E.M., Lewis, W.J. and Tumlinson, J.H. 1993. Learning of host-finding cues by hymenopterous parasitoids, pp. 51-78, *in* D.R. Papaj and A.C. Lewis (eds.). Insect Learning. Ecological and Evolutionary Perspectives. Chapman and Hall, New York.

van Loon, J.J.A., Blaakmeer, A., Griepink, F.C., van Beek, T.A., Schoonhoven, L.M. and de Groot, Ae. 1992. Leaf surface compound from *Brassica oleracea* (Cruciferae) induces oviposition by *Pieris brassicae* (Lepidoptera: Pieridae). *Chemoecology* 3: 39-44.

van 't Riet, K. and Tramper, J. 1991. Basic Bioreactor Design. M. Dekker, Inc., New York.

Vet, L.E.M. and Dicke, M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37: 141-172.

Wallbank, B.E. and Wheatley, G.A. 1976. Volatile constituents from cauliflower and other crucifers. *Phytochemistry* 15: 763-766.

Whitman, D.W., 1988. Plant natural products as parasitoid cuing agents, pp 386-396, *in* H.G. Cuttler (ed.). Biologically Active Natural Products potential use in Agriculture. ACS Symp Ser 380. American Chemical Society, Washington D.C.

Whitman, D.W. and Eller, F.J. 1990. Parasitic wasps orient to green leaf volatiles. *Chemoecology* 1: 69-75.

Wiskerke, J.S.C. and Vet, L.E.M. 1991. Comparison of two *Cotesia* species foraging for solitarily and gregariously feeding *Pieris* host species. *Proc. Exper. and Appl. Entomol., N.E.V. Amsterdam* 2: 190-195.

GENERAL DISCUSSION AND CONCLUDING REMARKS

In this thesis the isolation and identification of infochemicals which are involved in *Cotesia-Pieris-Brassica* relationships and their eventual use in cabbage crop protection, are described.

In chapter 2, a strong oviposition stimulant for the large cabbage white butterfly present in the wax-layer of Brussels sprouts (*Brassica oleracea* L. var. *gemmifera* cv. Titurel) is identified as glucobrassicin. To prevent cabbage colonisation by *P. brassicae* L., the use of cabbage cultivars with a low level of glucobrassicin could be an option. However, glucosinolates and their hydrolysis products have been shown to provide also a chemical defence for crucifers. They are known to be toxic to bacteria, fungi and a number of insects feeding on other plants (Blakeman, 1973; Feeny, 1977; Louda and Mole, 1992). Thus the selection of cabbage cultivars with a low total glucosinolate content will not be a solution since the injury by micro organisms and other insects than the large cabbage white butterfly may become more serious than the possible protection against egg-laying by the large cabbage white itself.

In chapter 3, the identification of the supposed Host Marking Pheromone of the large cabbage white is described. Three oviposition deterrents (miriamide, miriamide 5-glucoside and 5-dehydroxy miriamide) were isolated from the eggs of the butterfly. In chapter 5, it becomes clear that the isolated compounds, described in chapter 3, are themselves not directly responsible for the observed oviposition deterrency of leaves or plants carrying eggs. This conclusion is based on the fact that the egg-borne deterrents could not be detected in cabbage leaves from which egg batches had been removed, one or two days after they had been deposited, while the leaves still showed oviposition deterrency. Evidence was obtained that cabbage leaves themselves react to deposited eggs by producing and/or secreting other compounds to the leaf surface which are deterrent and which differ from the isolated miriamides. This probably is the first example of a plant response to an insect product without prior injury being inflicted to the plant.
Although the isolated oviposition deterrents are not directly involved in the natural situation in which the oviposition behaviour of the butterfly is modified, they can be used in cabbage crop protection. When miriamide or 5-dehydroxy miriamide were used in dual choice bioassays, complete inhibition of oviposition on the treated leaves occurs in a large number of replicates at a dose of 2 µg/leaf and higher. Although no absolute protection may be obtained, cabbage crops could be protected when they are sprayed with these compounds.

They should preferably be used in combination with a push-pull, or stimulo-deterrent diversionary strategy (SDDS) (Pyke *et al.*, 1989; Miller, 1989; Pickett *et al.*, 1991) in which trap crop plants, containing high levels of the oviposition stimulating glucosinolates, are treated with an insecticide or a pathogenic biological control agent.

The structure-activity relationship of the three isolated miriamides and eight related synthesized compounds is described in chapter 4. Only minor loss of activity relative to the most active natural compound isolated (5-dehydroxy miriamide) was observed in six of the eight synthesized compounds. One of the synthesized structures was even more active than 5-dehydroxy miriamide.
In field experiments with pure synthesized miriamide, no oviposition deterrence or dispersal activity could be measured. Additional laboratory experiments showed that miriamide was unstable when exposed to direct daylight. So, more effort and research is necessary to investigate the photostability of the eight related synthesized compounds (and other compounds that have yet to be synthesized) in order to find structures which combine a high oviposition deterring activity with photostability.

In chapter 6, headspace composition, collected from intact cabbage plants and cabbage plants infested with either larvae of *P. brassicae* L. and *P. rapae* L. was determined. Major differences between intact and caterpillar damaged cabbage plants were revealed for hexyl acetate, *cis*-3-hexenyl acetate, myrcene, sabinene and 1,8-cineole. The minimal mixtures necessary for attracting two larval endoparasitoid species to the first instar larvae of both *Pieris* species have not yet been identified.
Identification of the infochemicals used by parasitoids to locate the larvae of *P. brassicae* L. and *P. rapae* L. may be used to select cabbage cultivars with an increased production of these chemicals after injury by herbivores in order to increase foraging efficiency of the natural enemies. The use of parasitoids in cabbage crop protection may yield only limited control of *Pieris* caterpillars, because the caterpillars are not directly killed but develop to the final larval instar before they die. However, a high parasitization level will reduce the second butterfly generation in the same year.

When doing bioassays with living materials like plants and insects it should be kept in mind that results obtained in the laboratory can be different from those obtained in the field. In order to prevent ourselves from wasting effort, time and money, it is desirable to repeat successful laboratory experiments outside in the field, when possible, at an early stage.

The problems we had in obtaining flight responses with the parasitoids in the windtunnel bioassays in the winter period are probably caused by the physiological state of the plants. The plant odour composition reflects the physiological state of the plant and changes with age and season. When the odour composition of the plant changes and the amount of volatiles decreases, it is wise to postpone this kind of research and to wait for the next summer period.

REFERENCES

Blakeman, J.P. 1973. The chemical environment of leaf surface with special reference to spore germination of pathogenic fungi. *Pestic. Sci.* 4:575-588.

Feeny, P. 1977. Defensive ecology of the Cruciferae. *Ann. Missouri Bot. Garden* 64:221-234.

Louda, S. and Mole, S. 1992. Glucosinolates: Chemistry and Ecology, pp. 124-164, *in* G.A. Rosenthal and M.R. Berenbaum (eds.). Herbivores: Their interactions with secondary plant metabolites. Academic press, New York.

Miller, J. R. 1989. Stimulo-deterrent diversionary cropping. The concept and suggested applications, p. 28, *in* proceedings of the symposium, Semiochemicals and Pest Control, Wageningen.

Pyke, B., Rice, M., Sabine, B. and Zalucki, M. 1987. The push-pull strategy - behavioural control of *Heliothis*. *The Australion Cotton Grower* 8:7-9.

Pickett, J.A., Wadhams, L.J. and Woodcock, C.M. 1991. New approaches to the development of semiochemicals for insect control, pp. 333-345, *in* Proc. Conf. Insect Chem. Ecol., Tábor, 1990. Academia Prague and SPB Acad. Publ., The Hague.

SUMMARY

In this thesis the isolation and identification of infochemicals which are involved in *Cotesia-Pieris-Brassica* relationships with the prospect of their eventual use in cabbage crop protection, are described. The study focuses on two topics: regulation of *Pieris* oviposition behaviour and host selection behaviour of parasitoids of *Pieris* larvae.

A general introduction about relationships between plants/insects and insects/insects, and more specifically the relationship between Crucifers and their associated insect herbivores as well as phytochemical information about Cruciferae is given in chapter 1.

In chapter 2 the isolation and identification of the oviposition stimulant for the large cabbage white butterfly, present in the leaf surface of Brussels sprout plants, is described. The oviposition stimulant could be identified as glucobrassicin (3-indolyl-methyl-glucosinolate), a secondary plant compound belonging to the glucosinolates which are characteristic for the genus *Brassica*.

The identification of oviposition deterrents from the eggs of the large cabbage white is described in chapter 3. Three compounds, responsible for the oviposition deterring activity of an egg wash when sprayed onto a cabbage leaf, were isolated and identified as *trans*-2-[3-(3,4,5-trihydroxy-phenylpropenoyl)-amino]-3,5-dihydroxy-benzoic acid (miriamide), *trans*-2-[3-(3,4-dihydroxyphenylpropenoyl)amino]-3,5-dihydroxybenzoic acid and *trans*-2-[3-(3,4-dihydroxy-5-β-glucopyranose-phenylpropenoyl)amino]-3,5-dihydroxybenzoic acid. The synthesis of the first two compounds is also described. The three previously unknown avenanthramide alkaloids (amides of derivatives of anthranilic and cinnamic acid) form a group of compounds that have not been reported from the animal kingdom before.

The structure-activity relationship of the isolated avenanthramide alkaloids (described in chapter 3) and eight related synthesized compounds, as oviposition deterrents for *P. brassicae* L., is studied in chapter 4. For ten of the tested compounds, the effective dosis at which an oviposition deterring index of 50 % (ED_{50}) occurred, has been calculated. At least three groups with different activity levels were found. Changes in the way both ring systems were connected had no influence on the deterrent activity, while modifications of groups linked to the anthranilic part of the molecule led to a reduction of activity compared to miriamide. Mono- and dihydroxy substituted cinnamic parts of the molecule increased its effectiveness. *trans*-(4-Hydroxyphenylpropenoyl)amino]-3,5-dihydroxybenzoic acid was found to be significantly more active than miriamide.

In chapter 5, the question whether ovipositing female butterflies, after landing on the upper surface of a cabbage leaf, can perceive the host marking pheromone (HMP) present on the eggs deposited on the lower side of the leaf is studied. The strongly oviposition deterring avenanthramide alkaloids could not be detected in leaf surface extracts from leaves from which egg batches had been removed. Thus the isolated avenanthramide alkaloids are not directly responsible for the HMP effect. Evidence is obtained that cabbage leaves themselves produce oviposition deterrents in response to oviposited egg batches, thus making the use of the term HMP disputable. Fractions containing potent oviposition deterrents were isolated from surface extract of leaves from which previously laid egg batches had been removed.

In chapter 6 headspace analysis of intact cabbage plants and cabbage plants infested with larvae of the small cabbage white and the large cabbage white is described. The volatile production of intact cabbage plants shows a seasonal fluctuation with the highest production rate in the summer period.
Major differences in the headspace profile of intact and caterpillar damaged plants were revealed for hexyl acetate, *cis*-3-hexenyl acetate, myrcene, sabinene and 1,8-cineole. No significant quantitative differences were found between the headspace of cabbage plants infested by one or the other caterpillar species. In a windtunnel bioassay (dual-choice), it was found that the solitary parasitoid of the small cabbage white and a gregarious parasitoid of the large cabbage white distinguish between intact cabbage plants and cabbage plants infested with their preferred hosts.

SAMENVATTING

In dit proefschrift wordt de isolatie en identificatie van aantal signaalstoffen beschreven die een rol spelen in interacties tussen spruitkool en adulten van het grote koolwitje (*Pieris brassicae* L.) en tussen twee parasitaire wespen en de rupsen van het grote en kleine koolwitje (*Pieris rapae* L.). Het onderzoek is gericht op twee onderwerpen: het eileggedrag van het grote koolwitje en de gastheer selectie van twee parasitaire sluipwespen.

In hoofdstuk 1 wordt een globaal overzicht gegeven van interacties tussen planten en insekten en tussen insekten onderling en een meer gericht overzicht van interacties tussen insekten en de familie van de Cruciferen. In het eerste overzicht wordt de waardplant selectie en de gastheer selectie voor de eileg van vlinders en sluipwespen beschreven.

In hoofdstuk 2 wordt de isolatie beschreven van de stof die verantwoordelijk is voor de waardplant selectie van het grote koolwitje. Deze stof is geïdentificeerd als glucobrassicine, een secundaire plantestof behorende tot de groep van glucosinolaten die de familie van de Cruciferen karakteriseren.

In hoofdstuk 3 wordt de isolatie en identificatie van het eilegremmend feromoon van het grote koolwitje beschreven. Drie stoffen, verantwoordelijk voor het eilegremmende effect van eispoelsel wanneer dit verneveld wordt op een koolblad, zijn geïsoleerd en geïdentificeerd als *trans*-2-[3-(3,4,5-trihydroxyfenylpropenoyl)-amino]-3,5-dihydroxybenzoëzuur (miriamide), *trans*-2-[3-(3,4-dihydroxyfenylpropenoyl)amino]-3,5-dihydroxybenzoëzuur en *trans*-2-[3-(3,4-dihydroxy-5-β-glucopyranose-fenylpropenoyl)amino]-3,5-dihydroxybenzoëzuur. Een synthese van de twee eerste verbindingen wordt ook in dit hoofdstuk beschreven. Deze drie, tot dusver onbekende verbindingen, behoren tot de groep van stoffen die avenanthramides (amides van anthranilzuur- en kaneelzuur-derivaten) genoemd worden, daar verwante verbindingen voor het eerst in het plantegeslacht *Avena* ontdekt zijn.

De structuuractiviteits relatie van de in hoofdstuk 3 geïsoleerde eilegremmende verbindingen en acht nauw verwante gesynthetiseerde derivaten wordt beschreven in hoofdstuk 4. Voor tien van de elf geteste verbindingen is de effectieve concentratie waarbij de ovipositie index 50 % bedraagt (ED_{50}) berekend. Er konden ten minste drie groepen met een significant verschillende effectiviteit onderscheiden worden. De onderzochte veranderingen in het verbindingstuk tussen de twee ringsystemen hebben geen invloed op de eilegremmende activiteit vergeleken met de activiteit van miriamide. Veranderingen in het anthranilzuur-deel leiden tot verlies aan activiteit.

Een mono- en dihydroxy gesubstitueerd kaneelzuur-deel leidt tot een hogere remming vergeleken met die veroorzaakt door miriamide. De meest actieve verbinding is *trans*-2-[3-(4-hydroxyfenylpropenoyl)amino]-3,5-dihydroxybenzoëzuur .

In hoofdstuk 5 wordt teruggekomen op het eilegremmend feromoon van het grote koolwitje. De vraag hoe een koolwitje na landing op de bovenkant van een koolblad de eilegremmende stoffen op al eerder gelegde eieren aan de onderkant van hetzelfde koolblad kan waarnemen wordt gedeeltelijk beantwoord. Translocatie van de eilegremmende miriamides afkomstig van de eieren afgezet aan de onderkant naar de waslaag aan de bovenkant van het koolblad kon niet worden aangetoond. Sterke aanwijzingen zijn gevonden dat het koolblad reageert op al gelegde eieren, waardoor er inductie optreedt van synthese en/of afgifte van andere dan de geïsoleerde verbindingen naar de waslaag van het blad. Waslaag-fracties van belegde koolbladeren met eilegremmende werking zijn geïsoleerd maar verdere opwerking tot één of meerdere zuivere stoffen is niet gelukt, vermoedelijk door instabiliteit van de betreffende verbindingen.

Resultaten van de headspace analyse van intacte koolplanten en koolplanten aangetast door rupsen van het grote en kleine koolwitje worden beschreven in hoofdstuk 6. De afgifte van vluchtige stoffen van intacte koolplanten, gemeten gedurende twee groeiseizoenen, is het hoogst in de zomer periode.
Belangrijke verschillen in de bijdrage aan de headspace samenstelling van intacte koolplanten en koolplanten aangetast door de rupsen van één van de twee vlinder soorten is gevonden voor hexylacetaat, *cis*-3-hexenylacetaat, myrceen, limoneen en 1,8-cineol. Er worden geen kwantitatieve verschillen gevonden tussen de headspace samenstelling van koolplanten aangetast door de twee rupsen soorten.
In een twee-keuze windtunnel experiment is gevonden dat een op de rupsen van het kleine koolwitje gespecialiseerde sluipwesp (*Cotesia rubecula* Marshall) op afstand duidelijk onderscheid maakt tussen de geur van een intacte koolplant en die van een koolplant aangetast door *P. rapae* L. rupsen. Een zelfde voorkeur voor de geur geproduceerd door koolplanten aangetast door rupsen van het grote koolwitje boven de geur van intacte planten, is ook gevonden voor een niet gespecialiseerde sluipwesp (*C. glomerata* L.).

CURRICULUM VITAE

Op 11 oktober 1964 ben ik, Anton Blaakmeer, geboren te Slootdorp. Na het behalen van het atheneum-diploma in 1984 aan de Rijksscholengemeenschap Wiringherlant te Wieringerwerf, ben ik in september van hetzelfde jaar begonnen aan de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit te Wageningen. Afstudeervakken heb ik verricht bij de vakgroep Organische Chemie (Dr. T.A. van Beek) en voor de vakgroep Plantenfysiologie op het I.T.A.L. (Dr. J.P.F.G. Helsper) te Wageningen. Het doctoraalexamen van de studierichting Moleculaire Wetenschappen, chemische oriëntatie, werd afgelegd in maart 1989. Van augustus 1989 tot en met december 1993 was ik als assistent in opleiding verbonden aan de vakgroep Organische Chemie van de Landbouwuniversiteit. Het in dit proefschrift beschreven onderzoek is uitgevoerd op de vakgroepen Organische Chemie en Entomologie, onder leiding van Dr. J.J.A. van Loon, Dr. T.A. van Beek, prof. Dr. L.M. Schoonhoven en prof. Dr. Ae. de Groot.

LIST OF PUBLICATIONS

T.A. van Beek and A. Blaakmeer. 1989. Determination of limonin in grapefruit juice and other citrus juices by high performance liquid chromatography. *J. of Chromatogr.* 464: 375-386.

H. Helsper and A. Blaakmeer. 1989. Biosynthesis of thiophenes in cell cultures of *Tagetes patula. Supplement to Plant Physiology* 4: 10.

J.J.A. van Loon, A. Blaakmeer, F.C. Griepink, T.A. van Beek, L.M. Schoonhoven and Ae. de Groot. 1992. Leaf surface compound from *Brassica oleracea* (Cruciferae) induces oviposition by *Pieris brassicae* (Lepidoptera: Pieridae). *Chemoecology* 3: 39-44.

A. Blaakmeer, A. Stork, A. van Veldhuizen, T.A. van Beek, Ae. de Groot, J.J.A. van Loon and L.M. Schoonhoven. Isolation, identification and synthesis of miriamides, new hostmarkers from eggs of *Pieris brassicae. J. Nat. Prod.* 57: 90-99.

A. Blaakmeer, D. Hagenbeek, T.A. van Beek, Ae. de Groot, J.J.A. van Loon and L.M. Schoonhoven. 1994. Plant response to eggs *vs.* host marking pheromone as factors inhibiting oviposition by *Pieris brassicae. J. Chem. Ecol.* 20: 1657-1665.

A. Blaakmeer, D. van der Wal, A. Stork, T.A. van Beek, Ae. de Groot and J.J.A. van Loon. Structure-activity relationship of isolated avenanthramide alkaloids and synthesized related compounds as oviposition deterrents for *Pieris brassicae* (Lepidoptera: Pieridae). *J. Nat. Prod.* Accepted for publication.

A. Blaakmeer, M.A. Posthumus, J.J.A. van Loon, T.A. van Beek, and Ae. de Groot. Comparative headspace analysis of cabbage plants damaged by two species of *Pieris* caterpillars: Consequences for in-flight host locationn by *Cotesia* parasitoids. *Entomol. Exp. Appl.* In press.