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ISOLATION, IDENTIFICATION, AND SYNTHESIS OF MIRIAMIDES, NEW HOSTMARKERS FROM EGGS OF *PIERIS BRASSICAE*

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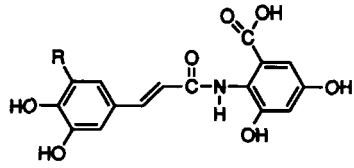
ABSTRACT.—The large white butterfly, *Pieris brassicae* L., a herbivorous pest of crucifers, produces egg-associated chemical markers that inhibit its oviposition. The identification of the marker compounds is reported herein. Separation by means of reversed-phase hplc demonstrated the presence of three active substances, which were identified as *trans*-2-[3-(3,4,5-trihydroxyphenylpropenoyl)-amino]-3,5-dihydroxybenzoic 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 the 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 colonization 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.

Females of several herbivorous insect species are known to deposit a marking substance on or near the eggs (1–3). 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 (HMPs), formerly often labeled as oviposition-deterring pheromones (ODPs), attract much attention. Egg-associated substances also affect related herbivorous species and natural enemies of the herbivores (4–7). Oviposition deterring activity has also been found in feces from larvae (8–10). A more detailed analysis of the ecological role of an HMP requires its chemical identification. Thus far, only a few attempts to identify egg-associated HMPs have been successful (11–13), with a notable example concerning the cherry fruit fly, *Rhagoletis cerasi* (11).

Within the Lepidoptera, several potential uses of HMPs have been reported (14,15). The large white butterfly, *Pieris brassicae* L., a specialized herbivore of cabbage (*Brassica oleracea*) and other cruciferous plants, has been studied in detail (14,16,17). Oviposition by *P. brassicae* is inhibited when a potential host plant carries conspecific eggs or is sprayed in a methanolic egg wash (14,16). 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 (18). Based on a two-choice bioassay, the inhibition of oviposition by egg-associated compounds was quantified (17). Herein the identification and synthesis of HMPs isolated from eggs of *P. brassicae* is presented.

RESULTS AND DISCUSSION

Eggs of *P. brassicae*, freshly laid on cabbage leaves, *Brassica oleracea* L. var. *gemmifera*



1 R=OH
2 R=O-β-Glucopyranosyl
3 R=H

cv. Titurel, were collected. Out of 20 hplc fractions, four (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 eims 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 atom was expected to be present in the form of a primary or secondary aromatic amine or an amide. No alkaloidal reaction with Dragendorff reagent was given by **1**. The high uv absorbance (λ max 353 nm) suggested a highly conjugated system. The ^{1}H-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 (δ 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).

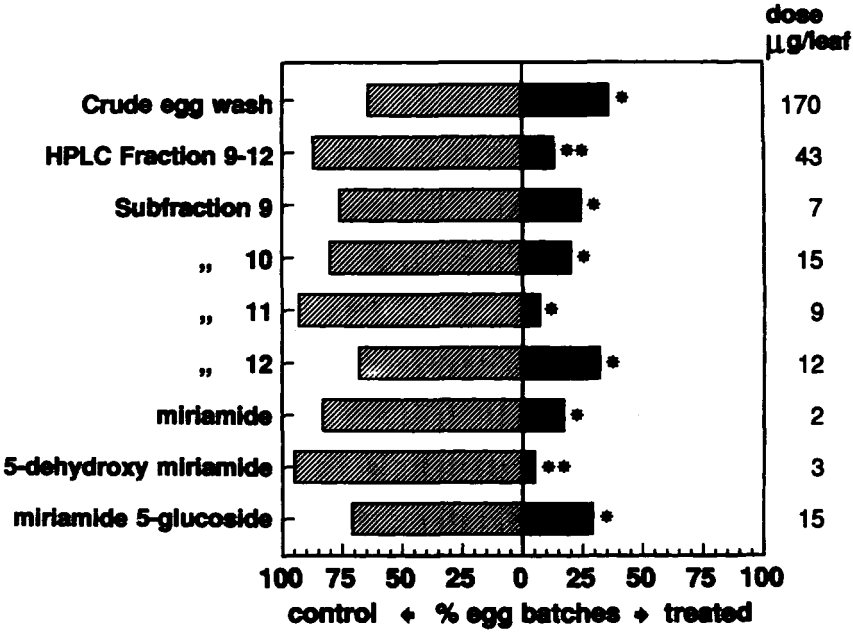


FIGURE 1. Oviposition preferences displayed by *Pieris brassicae* female butterflies in a dual-choice situation. Asterisks (*) indicate that treated leaves were significantly less preferred according to Wilcoxon's matched pair signed rank test (two tailed; 23), under the null hypothesis that the total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

TABLE 1. ^{1}H-Nmr Data of the Three Miriamides **1**, **2**, and **3**.[a]

Position	**1**	**2**	**3**
Benzoic acid part			
H-4	6.57, d, J=2.7	6.60, d, J=2.9	6.60, d, J=2.7
H-6	7.02, d, J=2.7	7.03, d, J=2.9	7.03, d, J=2.7
Cinnamic acid part			
H-2	6.62, s	6.83, d, J=1.7	7.07, d, J=1.9
H-5			6.78, d, J=8.3
H-6	6.62, s	7.11, d, J=1.7	6.98, dd, J=1.9, 8.3
H-7	7.45, d, J=15.5	7.52, d, J=15.5	7.54, d, J=15.5
H-8	6.35, d, J=15.5	6.63, d, J=15.5	6.59, d, J=15.5
Sugar part			
H-1		4.82, d, J=7.1	
H-2, H-3, H-4, and H-5		3.30–3.55, br m	
H-6		3.97, dd, J=2.1, 11.9	
H-6′		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).

The ^{13}C-nmr spectrum of **1** (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. A COLOC nmr experiment used for ^{1}H-^{13}C couplings, in this case optimized for

TABLE 2. ^{13}C-Nmr Data of **1**+D_2SO_4, **2**, and **3** (Synthetic Product).[a]

Position	**1**	**1**+D_2SO_4	**2**	**3**
Benzoic acid part				
C-1	124.9	126.0	124.9	127.9
C-2	120.8	115.4	120.9	121.0
C-3	153.2	154.8	153.3	153.1
C-4	110.8 +	114.0	110.4	110.9
C-5	157.0	158.6	157.2	157.0
C-6	110.8 +	114.0	110.9	110.9
C-7	170.8	167.6	170.9	170.9
Cinnamic acid part				
C-1	127.0	128.7	127.3	128.0
C-2	108.6 +	108.5	111.9	115.3
C-3	147.1	146.9	147.5	146.7
C-4	137.3	138.1	139.2	149.2
C-5	147.1	146.9	147.5	122.8
C-6	108.6 +	108.5	110.9	116.5
C-7	144.7 +	147.4	144.3	144.5
C-8	117.7 +	114.0	118.6	117.4
C-9	168.1	169.9	168.0	168.0
Sugar part				
C-1			104.4	
C-2			77.7	
C-3			78.5	
C-4			71.5	
C-5			75.0	
C-6			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.

4 and 8 Hz, respectively (Figure 2), showed one part of the molecule to consist of a substituted cinnamic acid, in which two equivalent protons were at positions 2 and 6 relative to the double bond. In the other region, two protons were coupled with six carbon atoms, one of which belonged to a carboxylic acid (δ 170.8). This information, combined with that from the ^{1}H-nmr spectra, indicated a substituted benzoic acid moiety with the two protons at positions 2 and 4 relative to the carboxylic acid group. The last structural features that had to be solved were 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 CD_3OD, gave only small chemical shift changes for 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 benzoic and cinnamic portions are linked via an amide bond. The trivial name miriamide is proposed for **1**, in honor of Miriam Rothschild, who was the first to notice the oviposition-deterrent activity of compounds associated with the eggs of *P. brassicae*.

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 spectrum (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

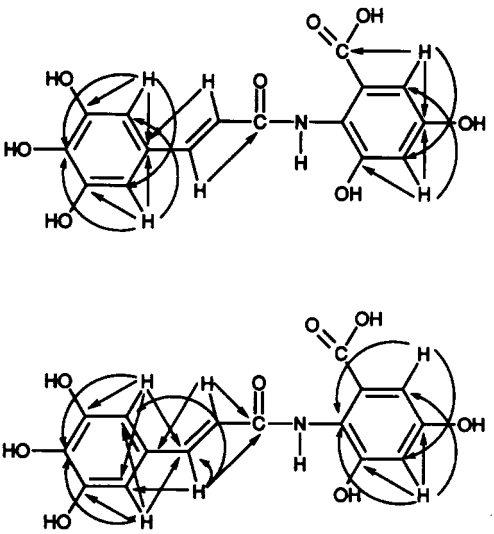


FIGURE 2. Long-range ^{1}H-^{13}C-nmr connectivities observed in **1** optimized for 4 Hz couplings (above) and 8 Hz couplings (below).

from those in **1**, while the other 13 carbons possessed chemical shifts almost identical to those 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 glucose. In contrast, hydrolysis with an α-glucosidase gave only starting material. Thus, a β-glucopyranose had to be attached to the ring next to a proton in the cinnamic portion. 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**].

Fraction 12 contained 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 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 positions 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-deoxymiriamide) [**3**], a structure wherein the hydroxy group at C-5 in the cinnamic acid part was replaced by a proton.

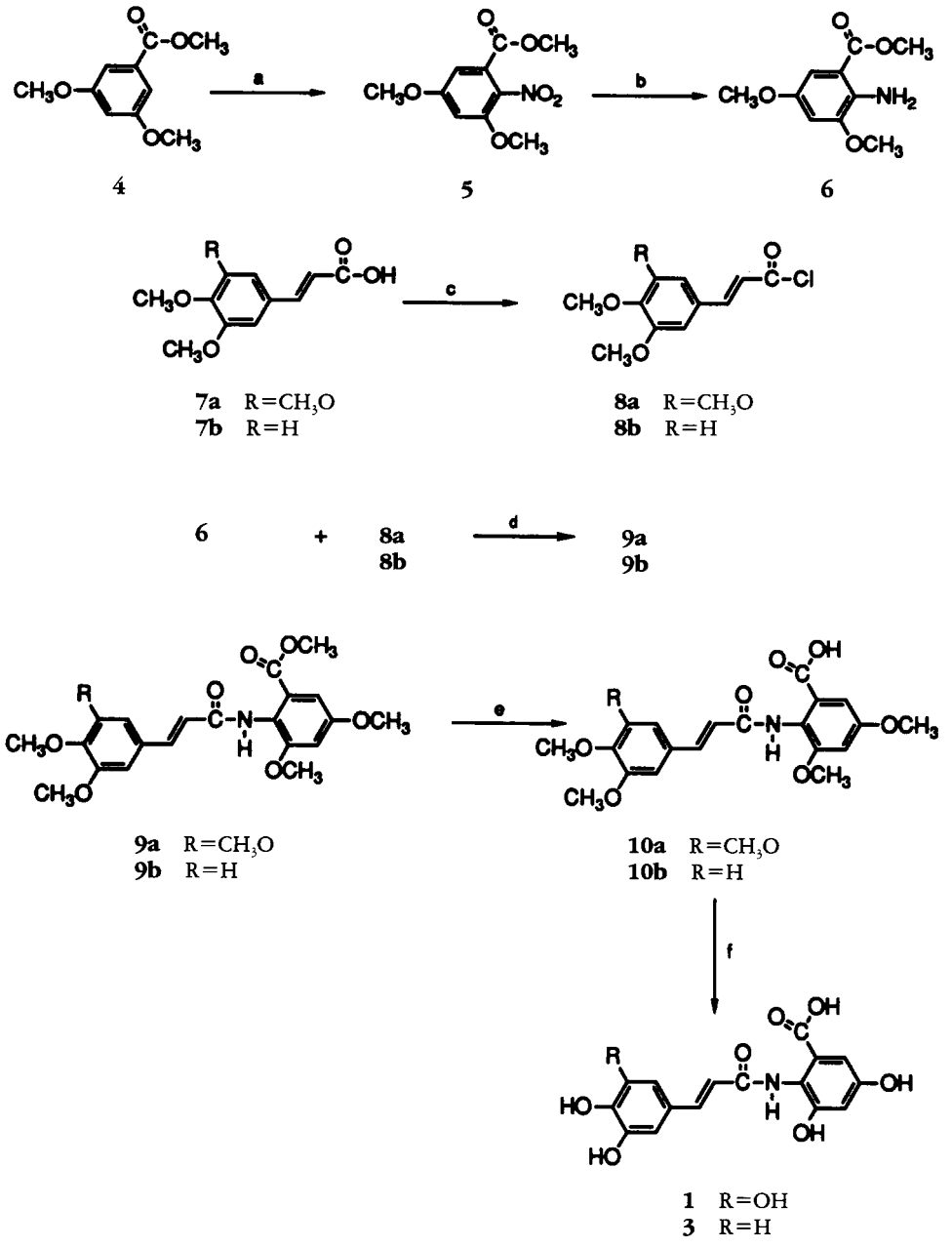
Structural assignments of miriamide [**1**] and 5-deoxymiriamide [**3**] were confirmed by synthesis (Scheme 1). Compound **1** was prepared starting from methyl-3,5-dimethoxybenzoate [**4**] and *trans*-(3,4,5-trimethoxy)cinnamic acid [**7a**]. Nitration of **4** gave methyl-2-nitro-3,5-dimethoxybenzoate [**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**]. Hydrolysis of the methyl ester **9a** with KOH resulted in *trans*-2-[3-(3,4,5-trimethoxyphenylpropenoyl)amino]-3,5-dimethoxybenzoic acid [**10a**] and demethylation of this acid with BBr_3 gave miriamide [**1**]. The synthesis of 5-deoxymiriamide **3** was accomplished in the same way starting from *trans*-(3,4-dimethoxy)-cinnamic acid [**7b**] and methyl-3,5-dimethoxy-benzoate [**4**].

Structures of **1–3** are, to the best of our knowledge, unknown in the animal kingdom. Structurally related compounds have been documented from plants and were found in *Avena* coleoptiles (19).

When miriamide [**1**] was applied to cabbage leaves at doses of 2.2 μg/leaf and higher (Figure 3), complete inhibition of oviposition on the treated leaf occurred in a large number of replicates. In this dose range the average percentage deterrence was 80%, which means that the control leaf receives ten times as many egg batches as the treated leaf in this relatively crowded bioassay set-up. It is also clear (Figures 1 and 3) that **2** is less active than the other two miriamides.

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* 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 three miriamides. Behan and Schoonhoven (20) already suggested that the accessory glands contained an inactive or less active form of the pheromone. It is likely that after secretion of miriamide 5-



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.

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 (6). Interestingly, the HMPs 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* and *Pieris napi* L. egg washes indicated the presence of the miriamides in these eggs as well. Dual-choice bioassays

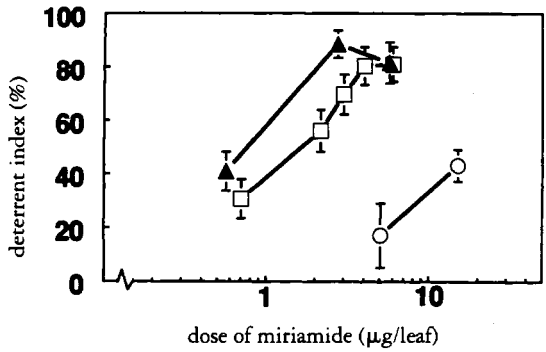


FIGURE 3. Oviposition deterrent index in the dual-choice oviposition assay as a function of the doses of the three pure miriamides. Deterrent index was calculated as: (C−T)×100/(C+T), in which C is the number of egg batches on control leaf and T the number of egg batches on the treated leaf. Means ± standard error of the mean (SEM) of 6–12 replicates are shown. Triangles: 5-deoxymiriamide [**3**]; rectangles: miriamide [**1**]; circles: miriamide 5-glucoside [**2**].

with *P. rapae* 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. (both 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 (13). The latter compounds have previously been documented as semiochemicals from hymenopteran insects (21). 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 behavior of *Pieris brassicae* was altered (18). 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 behavior in the initial phase of plant colonization 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.

EXPERIMENTAL

GENERAL EXPERIMENTAL METHODS.—Hplc: Pumps (model 302 and 303) manometric module 802C, Dynamic mixer 811 and uv-detector 116 (all 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 (flow-rate 3 ml/min) and a Microsorb RP C18 250×4 mm (flow-rate 1 ml/min), both 5-μm particle size and 100 Å pore size (Rainin Instrument Co.).

Nmr: All ^{1}H-nmr spectra were recorded at 200 MHz (Bruker AC-E 200) in CD_3OD $CDCl_3$ or DMSO-d_6, 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-ion fast-atom-bombardment mass spectrum (fabms) ($[M+H]^+$, $[M+H+glycerol]^+$) and a negative-ion fabms ($[M-H]^-$, $[M-H+glycerol]^-$) of **1** using a Finnigan MAT 95 mass spectrometer, $[M-H]^-$, *m/z* 346.0572 (calcd 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* var. *gemmifera* cv. Titurel plants were reared in a greenhouse (20–30°, 50–80% RH, 16l:8d) in standard potting soil. Illumination consisted of daylight supplemented by high-pressure sodium/mercury vapor lamps hanging 0.75 m above pot level. A voucher specimen, *van Setten 1073*, has been deposited at the Herbarium Vadense (WAG), Wageningen, Netherlands.

INSECTS.—*Pieris brassicae* adults were obtained from a laboratory colony maintained on *Brassica oleracea*. 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 (22). Voucher specimen 378,421 has been deposited at the collection of the Department of Entomology, Wageningen Agricultural University.

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°. 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 leaf and one treated leaf was placed in diagonal opposite corners, alternated between replicates, to minimize positional effects. Females could oviposit on the leaves during 5 h (8 AM–1 PM) periods. Preference of the butterflies was measured by comparing the number of egg batches on the treated leaves with the controls. A replicate 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 (23).

EXTRACTION AND ISOLATION.—About 150,000 eggs (30 g) were washed during five periods of five minutes each with pure MeOH. The MeOH egg washes were evaporated to dryness and dissolved in a small volume of pure MeOH. This yellow crude egg extract was separated into 20 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 flow-rate was 3.0 ml/min. A fraction was collected every 2 min.

Fractions 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. The flow-rate 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 1 h at 37°.

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°). The temperature was maintained between 8° and 15°. 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 H_2O (100 ml). After crystallization from MeOH, methyl-2-nitro-3,5-dimethoxybenozate [**5**] (10.6 g, 88%) was isolated. ^1H 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.* (24). After reacting for 120 h, the amine was isolated (89% yield). ^1H-nmr spectrum ($CDCl_3$, 200 MHz) δ 5.72 (NH_2, s), 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)cinnamoyl chloride* [**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)cinnamoyl chloride [**8a**] (7.05 g, 92%). ^1H-nmr spectrum ($CDCl_3$, 200 MHz) δ 6.58 (d, *J*=15.4 Hz, H-8), 6.83 (s, H-2/H-6), 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° 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°. ^{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°.

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 (8 ml, 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°. After stirring for 2 h at 0°, 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-d_6, 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=14.7 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.

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