Pinoresinol: A lignol of plant origin serving for defense in a caterpillar

Frank C. Schroeder, Marta L. del Campo, Jacqualine B. Grant, Douglas B. Weibel, Scott R. Smedley, Kelly L. Bolton, Jerrold Meinwald, and Thomas Eisner

This information is current as of June 2007.

Notes:

Pinoresinol: A lignol of plant origin serving for defense in a caterpillar

Frank C. Schroeder*, Marta L. del Campo†, Jacqualine B. Grant†, Douglas B. Weibel*, Scott R. Smedley‡, Kelly L. Bolton*, Jerrold Meinwald*§, and Thomas Eisner†

Departments of *Chemistry and Chemical Biology and †Neurobiology and Behavior, Cornell University, Ithaca, NY 14853; and ‡Department of Biology, Trinity College, Hartford, CT 06106

Pinoresinol, a lignan of wide distribution in plants, is found to occur as a minor component in the defensive secretion produced by glandular hairs of caterpillars of the cabbage butterfly, *Pieris rapae*. The compound or a derivative is appropriated by the larva from its normal food plant (the cabbage, *Brassica oleracea*). Pinoresinol was shown to be absent from the secretion if the larva was given a cabbage-free diet but present in the effluent if that diet was supplemented with pinoresinol. Pinoresinol is shown to be a feeding deterrent to ants (*Formica exsectoides*), indicating that it can complement the defensive action of the primary components of the secretion, a set of previously reported lipids called mayolenes. In the test with *F. exsectoides*, pinoresinol proved to be more potent than concomitantly tested mayolene-16.

acquired metabolite | chemical defense | insect antifeedant | Lepidoptera | sequestration

L ignans comprise a large class of secondary metabolites in vascular plants. Derived from the three phenyl-propanoid precursors, *p*-coumaryl alcohol (*1* in Fig. 1), coniferyl alcohol (*2* in Fig. 1) and sinapyl alcohol (*3* in Fig. 1), they fulfill important physiological functions in plants (1) and are of considerable pharmaceutical interest (2). For example, podophyllotoxin (*4* in Fig. 1) derivatives are used extensively in anticancer treatments and have been shown recently to possess antiviral properties as well (3). Pinoresinol (*5* in Fig. 1) is one of the structurally simplest lignans, being a dimer of coniferyl alcohol, and its frequent presence in woody or fibrous plants should come as no surprise [the Beilstein database (MDL Information Systems, San Leandro, CA) revealed 46 and 8 references, respectively, for the isolation of (+)-pinoresinol and (−)-pinoresinol from plants] (4). Virtually any plant capable of producing lignin can be presumed to have the enzymes necessary to link two units of coniferyl alcohol (*2* in Fig. 1) in a fashion leading to the bicyclic ring core of pinoresinol (*5* in Fig. 1).

The amount of pinoresinol produced by plants varies widely. Particularly high concentrations of pinoresinol have been found in young foliage, for example of *Forsythia* spp., as well as in the reproductive organs and seeds of many plants (5). The compound is therefore generally presumed to be a defensive agent, as is suggested also by its antihelminthic and antifungal activity (6–9). Animals are not known to produce pinoresinol or other dimeric lignols, nor have they been shown to acquire such compounds from plants. We here report the presence of pinoresinol in the defensive secretion of a caterpillar, the larva of *Pieris rapae*, the cabbage butterfly, one of the world's most familiar lepidopterans (10). We had earlier reported on the composition of this secretion, produced as droplets by glandular hairs on the back and flanks of the larva (11) (Fig. 2). We had noted the fluid to contain a series of structurally labile linolenic acid derivatives, the mayolenes (*6* in Fig. 3), which we demonstrated to be protective against ants (*Crematogaster lineolata*) (11). We have found pinoresinol itself to also be deterrent to ants (bioassay with *Formica exsectoides*), indicating that the compound could be part of the armamentarium of the larva. In fact,
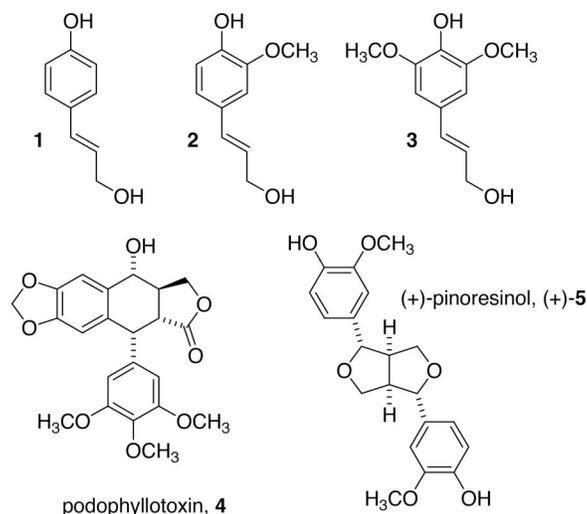

Fig. 1. Structures of the three phenyl-propanoid precursors of lignans and lignin, *p*-coumaryl alcohol (*1*), coniferyl alcohol (*2*), and sinapyl alcohol (*3*) and the dimeric lignans podophyllotoxin (*4*) and pinoresinol (*5*).

tested vis-à-vis *F. exsectoides*, pinoresinol proved to be significantly more potent than one of the mayolenes (mayolene-16), available to us as a synthetic sample. We also found evidence that pinoresinol is derived by *Pieris* from its larval food plant, *Brassica oleracea*. In fact, if the larvae are reared on an artificial cabbage-free diet, they can be shown to contain pinoresinol in their secretion only if that diet is supplemented with pinoresinol. Here, we present these results.

Results

Pinoresinol: Presence in the Secretion. ¹H-NMR-spectroscopic analyses of crude, unfractionated larval-secretion samples showed that the secretion consisted largely of the mayolenes described earlier (11). In addition, the spectra reproducibly showed several signals that appeared to belong to a less-abundant, previously unidentified component (Fig. 4*A*). This compound occurs at a concentration of only 3–5% in the secretion. To identify the structure of this compound by using NMR spectroscopic methods, it was necessary to collect secretions from a large number of larvae, which was accomplished by rinsing frozen larvae with dichloromethane at
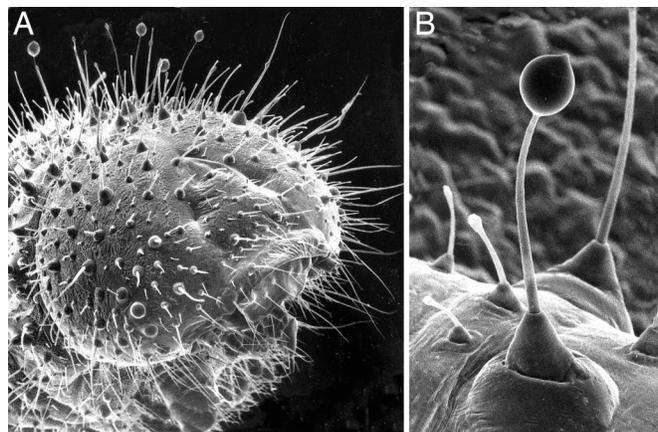
Fig. 2. Scanning electron micrographs of *P. rapae* larvae (early instar). (*A*) Frontal view showing several secretion-bearing glandular hairs. Some of the glandular hairs have lost their secretory droplets in the course of preparation of the larva for scanning electron microscopy. However, not all of the hairs projecting from the larva are glandular; most are nonglandular bristles. (*B*) Close-up view of a glandular hair.

−20°C. NMR-spectroscopic analyses showed that the resulting surface wash consisted largely of secretion in addition to smaller amounts of (probably cuticular) hydrocarbons. Combined surface-wash samples obtained from >5,000 larvae appeared to contain ≈100 μg of the unknown compound, which was deemed sufficient for further NMR-spectroscopic characterization via a standard set of two-dimensional spectra. In keeping with our recently described strategy to avoid chromatographic fractionation of natural product mixtures when possible (12), we decided not to pursue any attempts to isolate or purify the unknown compound before further NMR spectroscopic analysis, because chromatographic purification would almost certainly have led to some loss of material. Therefore, the unfractionated surface wash was used for the acquisition of the two-dimensional NMR spectra, including phase-sensitive double-quantum filtered correlation spectroscopy [(¹H,¹H)-dqf-COSY], (¹H,¹³C)-heteronuclear multiple-quantum correlation spectra, and magnitude-mode nongradient (¹H,¹³C)-heteronuclear multiple-bond correlation spectra. After NMR-spectroscopic analysis, the material was subjected to column chromatography on silica, and ≈50 μg of the compound was isolated, which was needed for MS analysis and acquisition of a reference one-dimensional ¹H-NMR spectrum. The spectroscopic data unambiguously define this minor compound in the secretion as pinoresinol (**5** in Fig. 1). The

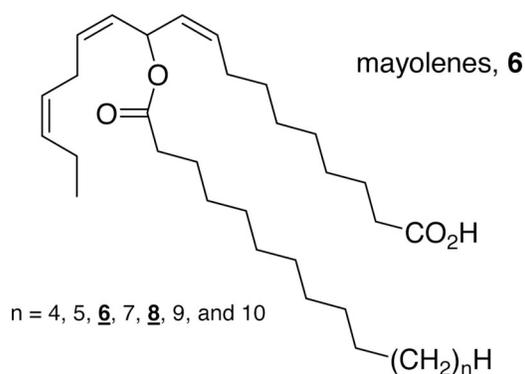mayolenes, **6**

n = 4, 5, **6**, 7, **8**, 9, and 10

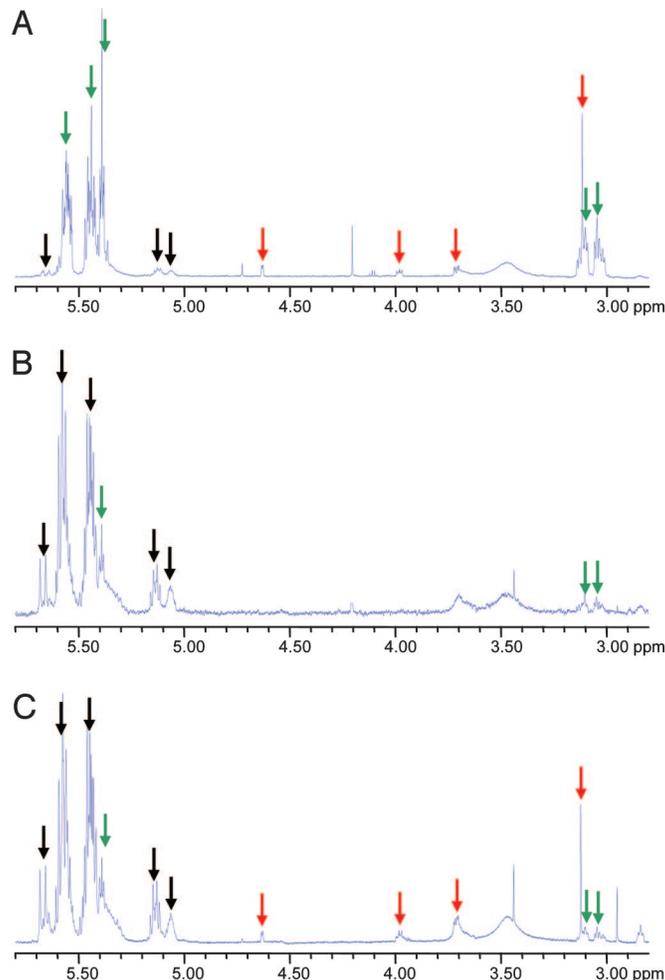**Fig. 3.** Structures of the mayolenes (*6*), the primary constituents of the *P. rapae* secretion.

Fig. 4. NMR-spectroscopic analysis. (*A*) Section of the ¹H-NMR spectrum of *P. rapae* secretion collected from cabbage-reared larvae, showing mayolenes (green arrows) and pinoresinol (red arrows) as well as smaller amounts of double-bond isomers and elimination products of the mayolenes (black arrows). (*B*) Equivalent section of the ¹H-NMR spectrum of *P. rapae* secretion collected from larvae reared on a cabbage-free diet, showing large amounts of double-bond isomers and elimination products of the mayolenes (black arrows) as well as smaller amounts of the mayolenes themselves (green arrows). Pinoresinol is absent. (*C*) Equivalent section of the ¹H-NMR spectrum of *P. rapae* secretion collected from larvae reared on a cabbage-free diet supplemented with pinoresinol.

caterpillar's secretion thus consists of a mixture of mayolenes (*6* in Fig. 3) and pinoresinol in a ratio of ≈96:4. For the determination of the absolute configuration of the pinoresinol, we used our recently introduced chiral silylation reagents, which showed the caterpillars' compound to be (−)-pinoresinol of 94% enantiomeric purity (13, 14).

Pinoresinol: Sequestration from the Cabbage Plant. Because it is a typical plant metabolite, *de novo* synthesis of pinoresinol by *P. rapae* seemed unlikely, suggesting its sequestration (or that of a simple derivative) from the cabbage plant. We therefore analyzed cabbage leaves for the presence of free pinoresinol and pinoresinol glycosides, as summarized in Fig. 5. ¹H-NMR-spectroscopic analyses of fractions obtained from column chromatography of the dichloromethane-soluble part of cabbage extracts showed no evidence for presence of pinoresinol. In addition, ¹H-NMR-spectroscopic analysis of the methanol-soluble part of the cabbage extract did not indicate the presence
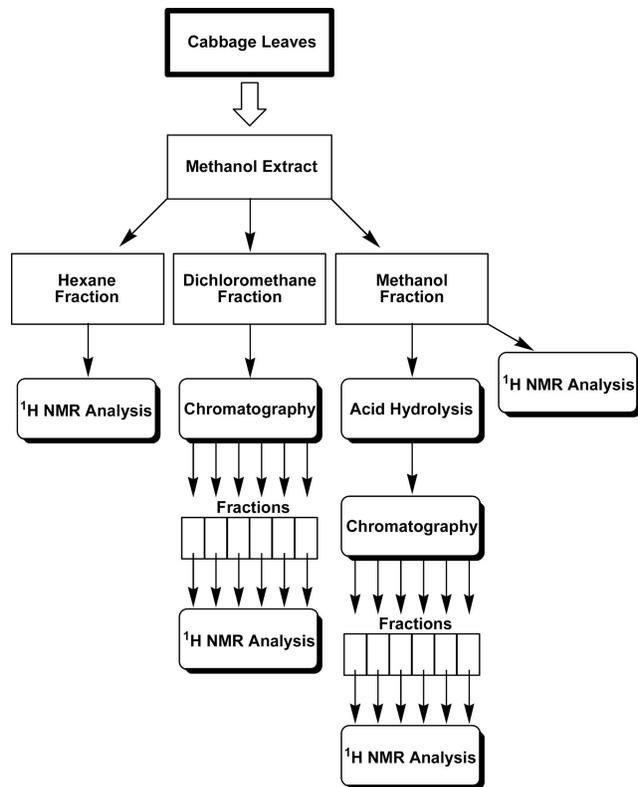
Fig. 5. Separation scheme for the detection of pinoresinol derivatives in the caterpillar's food plant, *B. oleracea*.
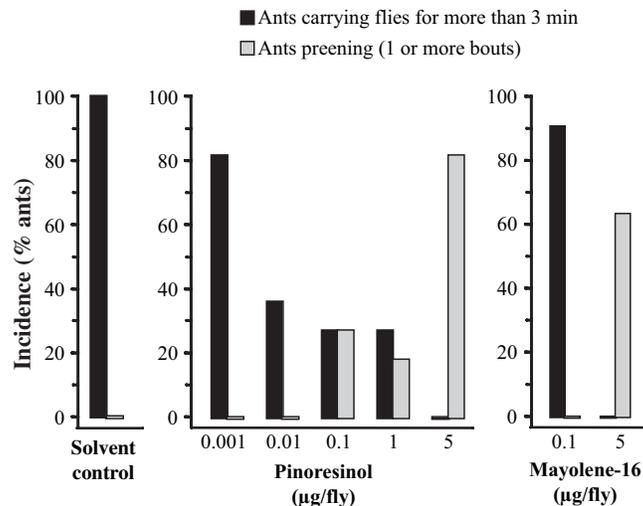

Fig. 6. Response of ants (*F. exsectoides*), individually presented with single fruit flies, plotted as a function of the dosage of pinoresinol or mayolene-16 applied topically (in 1 μl of methanolic solution) to the flies. Response is given as the fraction of ants per dosage category that carried the flies for more than 3 min or that responded by engaging in self-cleaning behavior, during the 10-min presentation of the fly. One microliter of methanol was the control. Sample size is $n = 11$ ants per category, including the control.

of any glycosides of pinoresinol. Thus, it was concluded that the *B. oleracea* variety we used did not produce significant quantities of free pinoresinol or of any low-molecular-weight pinoresinol glycosides.

We hypothesized that pinoresinol might still occur in cabbage leaves, but only in the form of high-molecular-weight glycosides, as part of the starchy components of a cabbage plant. In fact, careful acidic hydrolysis of an aqueous extract of cabbage leaves, followed by column chromatography, allowed us to isolate ≈0.1 mg of pinoresinol from the foliage of two entire cabbage plants along with comparable amounts of several structurally related lignans, including epi-pinoresinol. Comparable amounts of these lignans were also observed when samples of pure (+)-pinoresinol, isolated from *Forsythia* plants, were subjected to the conditions of the hydrolysis procedure, indicating that cabbage-derived pinoresinol might have partly isomerized or decomposed during acid hydrolysis. Therefore, the absolute configuration of cabbage-derived lignans was not determined.

Proof that dietary pinoresinol can, indeed, be incorporated into the secretion was provided by the demonstration (^{1}H-NMR spectroscopic analysis) that secretion samples from larvae fed on an artificial wheat-germ-based diet (15) lacked pinoresinol, whereas they contained (+)-pinoresinol when the diet was supplemented with the compound (Fig. 4 *B* and *C*). Both (+)- and (−)-pinoresinol occur in plants, and whereas sequestration from cabbage results in accumulation of almost pure (−)-pinoresinol in the larval secretion, our feeding experiments show that *P. rapae* is also capable of effectively sequestering the (+)-enantiomer.

Pinoresinol: Deterrency to Ants and Activity Relative to Mayolene-16. The ant used for assessment of the deterrency of these substrates was *F. exsectoides*, a species that we had used for

ascertainment of deterrency of natural products (16). We knew this ant to be aggressively insectivorous, even under cramped conditions. For present purposes, we offered individual worker ants single fruit flies (vestigial wing *Drosophila melanogaster*), treated by topical addition of either pinoresinol solution or mayolene-16 solution (and, for control purposes, of solvent alone) and kept track of how the ants dealt with these morsels during 10 min of presentation. We knew *F. exsectoides* to treat edible items thus offered by seizing them in the mandibles and then (sometimes after first spraying them with the formic acid-containing secretion from their poison sac) carrying them about.

Experience had taught us that the length of time that the ants kept the prey in their grip provides a measure of the acceptability of the item. We also knew that deterrent substances, when presented at higher dosages to *F. exsectoides* by way of fruit flies, could cause the ants to engage in conspicuous preening behavior (wiping of antennae with forelegs; drawing forelegs through the mouthparts) as a sequel, often, to no more than having briefly contacted or carried the fly. Preening behavior on the part of the ant can, therefore, provide a second criterion of food-item acceptability.

The results (Fig. 6) clearly showed the ants to be negatively affected by pinoresinol. Ants that received pinoresinol-treated flies showed a lesser disposition to carry these in their mouthparts for a period of >3 min than ants receiving the control flies ($P < 0.001$, $\chi^2 = 27.45$, df $= 5$, $n = 66$) and at the same time showed an increased tendency to undertake self-cleaning when the pinoresinol dosage was >1 μg per fly ($P < 0.001$, $\chi^2 = 29.69$, df $= 2$, $n = 66$) (Fig. 6). Mayolene-16 also proved to be a deterrent relative to control, but only at the dosage of 5 μg per fly (comparison of carrying persistence: $P < 0.001$, $\chi^2 = 11.0$, df $= 1$, $n = 22$). At the lower dosage of 0.1 μg per fly, the substance proved ineffective (comparison of carrying persistence: $P > 0.05$, $\chi^2 = 0.05$, df $= 1$, $n = 22$).

Discussion

The presence of pinoresinol in the *P. rapae* larval secretion, and the dietary origin of the compound in the larva, appear to be established, as is the antiinsectan potency of the compound. Ants

are known to attack *P. rapae* larvae under both field and laboratory conditions (17, 18), and there is little doubt that they figure worldwide among the enemies of this caterpillar. There is no reason, in fact, why *F. exsectoides* might not itself be an occasional threat to the larva. Interestingly, pinoresinol proved to be more active in the ant assays than mayolene-16, indicating that it could contribute to the defensive potency of the secretion in measure disproportionate to its concentration.

Secondary plant metabolites, of which pinoresinol is one example, are secondary in name only. Once regarded as waste products, they are now generally considered to be adaptive. The prevalent view is that many plant secondary metabolites play important roles as defensive agents, active more or less broadly against pathogens, helminths, and browsers (19). However, data on the actual effectiveness of specific plant metabolites are often hard to come by. Compounds may be assumed to be defensive, but actual proof of their repellency, deterrency, or toxicity to enemies is often lacking.

Interestingly, proof of the defensive role of a plant metabolite may be provided indirectly, by nature itself. Compounds used by plants in defense have sometimes evolved in parallel in insects, and it is through demonstration of the function of the compounds in insects, that light is cast on their role in plants. Thus, the production, for obvious defensive purposes, of acetylenic compounds by cantharid beetles (20), suggests that these compounds, as they occur, for instance, in asteraceous plants, serve similarly in defense (21). By the same token, the demonstration that cyclopentanoid monoterpenes serve defensively in insects strongly indicates that these compounds may play the same role when produced by plants (22–24).

Even more compelling is evidence provided by instances where insects make defensive use of metabolites that they appropriate from plants. In these cases, proof of function of the compounds in plants is more direct, because it is the plant products themselves that the insects put to use. The classic instance of such inferred function is provided by the monarch butterfly, which, by its defensive utilization of cardenolides from milkweeds, offers evidence that the compounds serve in defense in the milkweeds themselves (25).

Our findings with *Pieris* are interpretable in the light of such reasoning. Pinoresinol, given its proven antiinsectan potency, reflected in its antifeedant activity as well as in its action as a molting inhibitor in Hemiptera (26), can be envisioned to fulfill a defensive role in plants. However, this is not to say that the compound evolved strictly in response to selective pressures imposed by insects. Pinoresinol is a product of the dimerization of coniferyl alcohol, one of the basic building blocks of lignin, and, thus, its origin might well go back to preinsectan times, to when plants first hit upon the expedient of wood production. In *Pieris*, given our data, pinoresinol can be expected to play a protective role. Its appropriation by *P. rapae* can be viewed as being indicative of secondary evolutionary exploitation on the part of this lepidopteran, made possible by the animal's tolerance of the compound. Interestingly, neither free pinoresinol nor simple glycosides of pinoresinol could be detected in the caterpillar's food plant. Nonetheless, acid hydrolysis of the plant matter did yield pinoresinol, accompanied by smaller amounts of other lignans, suggesting that the caterpillar obtains the pinoresinol from ingested polymer-bound pinoresinol, most likely from starch-bound glycosides, or through partial hydrolysis of cabbage lignin. In either case, the caterpillar appears to sequester pinoresinol selectively from the mixture of plant-derived metabolites.

Other lignans, in all probability, share the defensive attributes of pinoresinol, thereby providing evidence for what is perhaps a principal adaptive justification for the compounds in plants. Given the ubiquitous presence of lignans in plants and the vastness of the insect presence on earth, it seems almost inevitable that other insects will be found that share *Pieris*' habit of putting acquired lignans to use.

Materials and Methods

Analytical Procedures. NMR Spectra were recorded at 25°C by using a Varian (Palo Alto, CA) INOVA (600 MHz proton, 151 MHz carbon) spectrometer with benzene-d_6 as the solvent. (^{1}H,^{1}H)-dqf-COSY spectra and phase-sensitive nuclear Overhauser effect spectroscopy spectra as well as heteronuclear multiple-bond and multiple-quantum correlation spectra were acquired by using the standard pulse sequences and phase cycling for coherence selection. For electrospray MS, a Hewlett-Packard (Houston, TX) 1090 II pump was linked to a Micromass (Cary, NC) Quattro I mass spectrometer operated in positive ion electrospray mode.

P. rapae. Larvae were taken from a colony maintained in confinement on either cabbage plants (*B. oleracea*) or an artificial wheat-germ-based diet (15) devoid of cabbage.

Pinoresinol. For reference purposes, a sample of (+)-pinoresinol (104.5 mg) was isolated from *Forsythia suspensa* by a previously described procedure (5).

Mayolene-16. The sample of this compound used in the assay with *F. exsectoides* was synthesized as described (27).

Collection of Larval Secretion. Collection of secretion from live larvae (mostly first and second instar) was effected by touching the tips of glass micropipettes to the secretory droplets and taking up the fluid by capillarity. Special care was taken to avoid contamination of the secretion samples with feces or plant material. Several such samples were separately collected and analyzed by ^{1}H-NMR spectroscopy.

Collection of Secretion by Rinsing of Whole Larvae. *P. rapae* caterpillars (five batches of ≈1,000 medium-sized, cabbage-reared larvae) were frozen at −20°C in flasks and submerged for 1 min in 30 ml of chilled (−20°C) dichloromethane. The use of frozen larvae and precooled solvent insured that the interior of the larvae was not extracted and resulted in very clean samples of secretion, containing only slightly larger amounts of cuticular lipids than the secretion samples collected with glass pipettes. The supernatant was taken up in pipettes, filtered through a small pad of Na_2SO_4, and concentrated by evaporation *in vacuo*, yielding 12.3 mg of an oily, slightly yellow residue.

Chemical Analysis of Larval Secretion. The sample of larval secretion obtained through rinsing of whole larvae was redissolved in 0.55 ml of benzene-d_6 and analyzed by NMR spectroscopy, which included acquisition of phase-sensitive (^{1}H,^{1}H)-dqf-COSY spectra, (^{1}H,^{1}H)-nuclear Overhauser spectroscopy spectra, (^{1}H,^{13}C)-heteronuclear multiple-quantum correlation spectra, and magnitude-mode non-gradient (^{1}H,^{13}C)-heteronuclear multiple-bond correlation spectra optimized for long-range ^{13}C-^{1}H-coupling constants of 4 Hz and 8 Hz. In addition, small samples of the residue were analyzed by using combinations of gas chromatography and electron-impact ionization MS as well as HPLC and electrospray-ionization MS. For additional NMR spectroscopic and MS analyses, the extract was subjected to column chromatography on silica with a 1:1 mixture of ethyl acetate and hexane as solvent, which produced 0.05 mg of pure pinoresinol. For determination of its absolute configuration, we used our recently described chiral silylation reagents (12, 13).

Pinoresinol: Presence in the Food Plant. Leaves of two *B. oleracea* plants were freeze-dried and ground, yielding 11.7 g of fine green powder. This material was extracted with three 200-ml portions of methanol, and the combined extracts were evaporated to

dryness *in vacuo*. The residue was placed in 300 ml of a water/methanol mixture (9:1), and the resulting suspension was extracted with two 300-ml portions of hexane. Subsequently, the water content of the methanol phase was increased to ≈30%, and the resulting solution was extracted with two 200-ml portions of dichloromethane. The combined dichloromethane extracts were evaporated, yielding 0.7 g of a yellowish residue. A 0.5-g sample of this material was subjected to column chromatography on silica, with a series of progressively more polar mixtures of dichloromethane and methanol as solvent, whereby the methanol content was increased stepwise from 5% to 90%. Fractions containing compounds with TLC retention factor (Rf) values similar to that of pinoresinol were combined and further fractionated through a second silica gel column, this time by using ethyl acetate with 50–0% hexane as solvent. Fractions thus obtained were characterized individually by ^{1}H-NMR spectroscopy. By using an authentic sample of pinoresinol, a detection limit of 5 μg of pinoresinol was established for this procedure.

Pinoresinol Glycosides. Leaves of two additional *B. oleracea* plants were dried, ground, and extracted as described above. The remaining aqueous extract was evaporated *in vacuo*, yielding 1.2 g of a yellowish solid residue, which was divided into two portions. A 0.2-g sample of this material was suspended in 5 ml of absolute methanol, and the resulting suspension was filtered and the filtrate evaporated *in vacuo*. The residue was dissolved in 0.6 ml of CD$_3$OD, followed by acquisition of ^{1}H-NMR and (^{1}H,^{1}H)-dqf-COSY spectra. The remaining 1.0 g of material was placed in a mixture of 10 ml of dioxane and 70 ml of 0.15 molar aqueous sulfuric acid (14). The mixture was stirred for 70 min at 65°C under argon and, after cooling to room temperature, was extracted with two 50-ml portions of a 1:1 mixture of ether and hexane. The combined organic extracts were washed with 20 ml of water and then dried over Na$_2$SO$_4$, filtered and evaporated *in vacuo*. The residue was subjected to column chromatography on silica by using ethyl acetate/hexane mixtures as described above. Fractions containing compounds with Rf values close to that of pinoresinol were characterized by ^{1}H-NMR spectroscopy. Of these fractions, those that appeared to contain lignans were characterized further by acquisition of (^{1}H,^{1}H)-dqf-COSY and nuclear Overhauser effect spectroscopy spectra.

Pinoresinol: Presence in the Secretion Linked to Presence in the Diet. Several samples of secretion were separately collected with micropipettes from larvae (mostly first and second instar), reared on an artificial wheat-germ-based diet (15), and analyzed by ^{1}H-NMR spectroscopy. One subgroup of these larvae had received a pinoresinol-enriched (0.1% of dry ingredients, added in ethanolic solution) version of this diet, whereas the others (controls) received the diet with added ethanol only.

Pinoresinol Deterrency to Ants and Activity Relative to Mayolene-16. The *F. exsectoides* ants used in the test with *D. melanogaster* fruit flies were from a colony that had become established in a large aquarium indoors after transference from their natural site. A test consisted of offering an individual fruit fly, treated by addition of test substance, to a single ant, confined in a Petri dish, and following the ensuing events for 10 min. The fruit flies, of a vestigial wing strain (and therefore more readily handled) were from a laboratory culture. Before addition of the test substance (pinoresinol or mayolene-16), the flies were immobilized by cooling. We tested pinoresinol at five dosages (0.001, 0.01, 0.1, 1.0, and 5.0 μg), applied to the fruit flies in methanolic solution (1 μl) with a micropipette. Mayolene-16 was comparably applied, but at dosages of 0.1 μg and 5 μg only. Control flies received 1 μl of methanol. After application of the test sample, flies were given a period of 10–30 min before being offered to the ants, to allow for evaporation of the methanol (the flies regained full mobility during this period). Eleven tests were performed per each dosage of the two test substances, as well as with the methanol controls, with the use of fresh ants and flies for each test. Tests were videotaped, allowing for accurate ascertainment of (*i*) the length of time the ants walked about with the fly in their grip, and (*ii*) whether at any time during the test the ants engaged in self-cleaning.

ECOLOGY

1. Gang DR, Kasahura H, Xia, Z-Q, Vander Mijnsbrugge K, Bauw G, Boerjan W, Van Montagu M, Davin LB, Lewis NG (1999) *J Biol Chem* 274:7516–7527.
2. Lewis NG, Davin LB (1999) in *Comprehensive Natural Product Chemistry*, eds Barton DHR, Nakanishi K, Meth-Cohn O (Elsevier, London), pp 639–712.
3. Ayres DC, Loike JD (1990) *Lignans: Chemical, Biological, and Clinical Properties* (Cambridge Univ Press, Cambridge, UK).
4. Tanaka R, Wada S-I, Kinouchi Y, Tokuda H, Matsunaga S (2004) *Planta Med* 70:877–880.
5. Kitagawa S, Hisada S, Nishibe S (1984) *Phytochemistry* 23:1635–1636.
6. García ES, Cabral MMO, Schaub GA, Gottlieb OR, Azambuja P (2000) *Phytochemistry* 55:611–616.
7. Cabral MMO, Azambuja P, Gottlieb OR, García ES (2000) *Fitoterapia* 71:1–9.
8. Carpinella MC, Ferrayoli CG, Palacios SM (2005) *J Agri Food Chem* 53:2922–2927.
9. Suga T, Ohta S, Munesada K, Kurokawa M, Shimizu M, Ohta E (1993) *Phytochemistry* 33:1395–1401.
10. Opler PA, Krizek GO (1984) *Butterflies East of the Great Plains* (Johns Hopkins Univ Press, Baltimore).
11. Smedley SR, Schroeder FC, Weibel DB, Meinwald J, Lafleur KA, Renwick JA, Rutowski R, Eisner T (2002) *Proc Natl Acad Sci USA* 99:6822–6827.
12. Taggi AE, Meinwald J, Eisner T, Schroeder FC (2004) *J Am Chem Soc* 126:10364–10369.
13. Schroeder FC, Weibel DB, Meinwald J (2004) *Org Lett* 6:3019–3022.
14. Weibel DB, Walker TR, Schroeder FC, Meinwald J (2000) *Org Lett* 2:2381–2383.
15. Webb SE, Shelton MS (1988) *NY Food Life Sci Bull* 122:1–6.
16. Carrel JE, Eisner T (1974) *Science* 183:755–757.
17. Jones RE (1987) *J Anim Ecol* 56:739–749.
18. Shiojiri K, Takabayashi J (2005) *Ecol Res* 20:695–700.
19. Rosenthal GA, Berenbaum MR (1992) *Herbivores: Their Interactions with Secondary Plant Metabolites* (Academic, San Diego).
20. Meinwald J, Chalmers AM, Eisner T (1968) *Science* 160:890–892.
21. Sörensen NA (1963) in *Chemical Plant Taxonomy*, ed Swain T (Academic, New York), pp 219–252.
22. Eisner T (1964) *Science* 146:1318–1320.
23. Meinwald J, Jones TH, Eisner T, Hicks K (1977) *Proc Natl Acad Sci USA* 74:2189–2193.
24. Eisner T, Eisner M, Aneshansley DJ, Wu C-L, Meinwald J (2000) *Chemoecology* 10:211–216.
25. Malcolm SB (1991) in *Herbivores, Their Interactions with Secondary Plant Metabolites*, eds Rosenthal GA, Berenbaum, MR (Academic, New York), pp 251–296.
26. Garcia ES, Azambuja P (2004) *Toxicon* 44:431–440.
27. Weibel DB, Shevy LE, Schroeder FC, Meinwald J (2002) *J Org Chem* 67:5896–5900.