Journal of Chemical Ecology, Vol. 23, No. 7, 1997

GROWTH INHIBITORY, INSECTICIDAL, AND FEEDING DETERRENT EFFECTS OF (12Z,15Z)-1-ACETOXY-2-HYDROXY-4-OXO-HENEICOSA-12,15-DIENE, A COMPOUND FROM AVOCADO FRUIT, TO *Spodoptera exigua*

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(Received October 31, 1996; accepted March 11, 1997)

Abstract—We isolated and identified (12Z,15Z)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (persin) from avocado as a compound toxic to *Spodoptera exigua* larvae. Persin was obtained from oil extracted from the specialized idioblast cells of avocado fruit. When tested for activity using neonates in no-choice artificial diet bioassays, persin at concentrations of 200 μg/g inhibited growth; larvae gained only ~50% of the weight attained by control insects after seven days. At concentrations of 400 μg/g or above, larval growth was reduced by >70% as measured by weight gain, and significant mortality occurred. In choice tests, persin at concentrations of 400 μg/g or higher deterred feeding, with significantly more larvae being found on control diet than on persin-treated diet. Possible applications of persin as a natural insecticide are discussed.

Key Words—*Spodoptera exigua*, *Persea americana*, avocado, idioblast cells, persin, (12Z,15Z)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene, Lepidoptera.

INTRODUCTION

Public health and environmental safety concerns have contributed to a reduction in the use of synthetic insecticides in commercial agriculture (Trumble, 1990).

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0098-0331/97/0700-1819$12.50/0

Consequently, more target-selective and biodegradable compounds are needed to replace the environmentally persistent chemicals with broad-spectrum toxicity that are being phased out (Alkofahi et al., 1989). Alternative sources of potentially suitable insecticides include phytochemicals that may have evolved for plant defense against herbivores (Duke, 1990). One possible source of such chemicals is the unusual idioblast oil cells that occur in the leaves and other organs of dicotyledonous plant species in numerous families (Postek and Tucker, 1983; Baas and Gregory, 1985). These oil cells usually differ from their neighboring mesophyll cells both in morphology and composition. For example, Maron and Fahn (1979) reported that oil cells in *Laurus nobilis* L. are larger, have lower starch contents, and differ in their plastid organization from mesophyll cells. Other reports (Mariani et al., 1989) have shown the presence of insecticidal sesquiterpene lactones in oil cells of *Liriodendron tulipifera* L.

An early study indicated that idioblast oil cells compose approximately 2% of the tissue volume of avocado fruit (*Persea americana* Mill.) (Cummings and Schroeder, 1942). More recently, Platt and Thomson (1992) described avocado idioblast cells in detail and developed a method for their extraction in multigram quantities from ripe fruit. They also reported that these cells are present in the leaf, root, and seed as well as the flesh of fruit. Preliminary analyses indicated the presence of alkaloids, sesquiterpene hydroperoxides, and possibly other terpenes.

Subsequent studies showed that the oil from avocado idioblast cells is biologically active. Kobiler et al. (1993) demonstrated the activity of two compounds against the fungus, *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. More recently, Rodriguez-Saona and Trumble (1996) showed that crude oil extracted from idioblast cells was not only toxic to early [LC_{50} (95% Fiducial Limits (FL) = 0.16% (0.15–0.16%)] and late instars [LC_{50} (95% FL) = 0.51% (0.44–0.61%)] of the generalist herbivore *Spodoptera exigua* (Hübner), but also deterred feeding.

The objectives of the study reported here were: (1) to identify the compound present in the idioblast cell oil most insecticidal to *S. exigua*, and (2) to test the effects of this compound on early-instar *S. exigua* growth, mortality, and feeding behavior.

METHODS AND MATERIALS

Extraction of Oil. Ripe avocado, *P. americana*, fruit were obtained from trees of the Haas cultivar grown at the University of California's South Coast Research and Extension Center, Santa Ana, California. The idioblast cells were separated and the oil was extracted as previously described (Rodriguez-Saona and Trumble, 1996). One kilogram of ripe fruit yielded an average of 7.3 ± 1.5 g of crude oil.

Insects. S. exigua larvae were reared on an artificial diet modified from Patana (1969), at 28 ± 2°C and a photoperiod of 14L:10D. The colony was originally collected from Orange County, California, in 1982 and had new genetic material added periodically, including within 12 months prior to the study. Cohorts of unfed neonates were used within 12 hr of eclosion. Incubators used in bioassays were maintained at 28 ± 2°C, a relative humidity of ~75%, and a photoperiod of 14L:10D with fluorescent lighting.

Isolation and Identification of Active Compounds. NMR spectra were taken on $CDCl_3$ solutions, using a GE 300 instrument (General Electric, Fremont, California), at 300 MHz for protons, and 75.48 MHz for ^{13}C. Exact mass chemical ionization mass spectra (NH_3) were recorded with a VG 7070 EHF high resolution mass spectrometer (VG Instrument Co., Beverly, Massachusetts).

A portion of the crude oil (4.5 g) was fractionated by flash chromatography on silica gel (230–400 mesh; 5 cm ID × 25 cm) (Aldrich Chemical, St. Louis, Missouri), eluting sequentially with 2 liters each of toluene–ethyl ether–acetic acid at 70:30:1 (v/v/v), 50:50:1, and 10:90:1. The material remaining on the column was then stripped off with ethanol (2 liters). Fractions were checked by thin-layer chromatography (TLC) on silica plates developed with toluene–ethyl ether–acetic acid (70:30:1 v/v/v). Spots on developed plates were visualized using UV (254 nm) followed by spraying with H_2SO_4 and charring with a hot air blower. Subfractions were combined to yield eight fractions that were concentrated under reduced pressure, followed by exposure to ~0.5 mm Hg vacuum to remove traces of solvent. The concentrated fractions were weighed, then diluted with acetone to a final volume of 10 ml, and refrigerated at 4°C until needed.

A portion (~15%) of the most polar active fraction (fraction 3, 0.22 g total weight) was further purified by HPLC, using a 2.25- × 25-cm Econosil silica column (Alltech, Deerfield, Illinois, 10 μm particle size) eluted with 15% acetone in hexane (4 ml/min), monitoring the effluent with a Dynamax RI-2 refractive index detector (Rainin Instrument Co., Emeryville, California). Five fractions were taken, of which the major peak, constituting the active material, eluted at about 32 min. The fractions were concentrated, then taken up in 1.5 ml acetone to maintain the same dilution as was used in the original fraction. Aliquots of the active compound were removed for spectral analysis. Chemical ionization exact mass (direct insertion probe, NH_3 reagent gas): Calcd. for $C_{23}H_{44}NO_4$ ($M+NH_4$): 398.3270; Found: 398.3281. ^1H NMR: 5.45–5.27 (m, 4H), 4.37–4.27 (m, 1H), 4.15–4.02 (m, 2H), 2.78 (br t, 2H, J = 5.6 Hz), 2.63 (d, 2H, J = 6.0 Hz), 2.46 (t, 2H, J = 7.4 Hz), 2.12 (s, 3H), 2.05 (br quartet, 4H, J = 6.6 Hz), 1.4–1.22 (m, ~16H), 0.89 (t, 3H, J = 7 Hz). ^{13}C NMR: 210.8, 171.2, 130.2, 130.0, 128.1, 127.9, 67.2, 66.0, 45.1, 43.6, 31.5, 29.6, 29.3, 29.3, 29.1, 27.2, 27.2, 25.6, 23.5, 22.6, 20.8, 14.1. The ^{13}C and ^1H NMR spectra matched those reported for the known compound

(12Z,15Z)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (persin) previously isolated from avocado (with the exception that the original report neglected to report the quartet at 2.05, corresponding to the four allylic protons; Prusky et al., 1982).

Bioassays. Bioactivity of the fractions was tested against early-instar *S. exigua* in an artificial diet bioassay, using rates equivalent to 2% crude idioblast cell oil in diet. This rate (20 mg/g) was selected because previous tests using early instars showed high mortality and a significant reduction of larval development at this concentration (Rodriguez-Saona and Trumble, 1996). Treated diets were prepared by transferring 750 μl of acetone solutions of fractions (equivalent to 0.34 g crude oil) into 50-ml polypropylene centrifuge tubes (Fisher, Pittsburgh, Pennsylvania), evaporating the acetone, adding 2 ml of 0.1% Tween-80 solution (Fisher), homogenizing with an ultrasonic homogenizer (Cole-Parmer, Chicago, Illinois), and finally adding prepared diet to produce a final weight of 15 g. The mixture was vortexed for 3 min. Control diet was prepared by mixing 2 ml of Tween solution and 13 g of artificial diet to produce a final weight of 15 g. Control and treated diets were poured into 24-well (15.9 mm diameter and 15.9 mm depth) bioassay trays (C-D International Inc., Pitman, New Jersey). One neonate larva was added per well, and the trays were placed in an incubator under the previously described conditions. Twenty-four neonates were tested for each fraction and the control. Mortality and larval weight were recorded after seven days.

The effect of the purified compound (fraction 3-3) on larval growth and mortality of *S. exigua* was tested. Treated and control diet were prepared as previously described. Eight concentrations were examined: 0 (control), 200, 300, 400, 500, 600, 700, and 800 μg/g. Twenty-four neonates were used per treatment. Bioassays were conducted under standard incubator conditions (see above), and were replicated four times for each treatment (i.e., a total of 96 larvae were tested with each treatment). After seven days, larvae were weighed, and the EC (effective concentration) values were calculated by subtracting the mean larval weight for each treatment from the mean weight of the controls and dividing by the mean weight of the controls. The EC_{50} value (the concentration required to inhibit growth by 50% relative to controls) was estimated by interpolation. Larval mortality was recorded after seven days.

Choice Tests. The ability of early-instar *S. exigua* to discriminate between treated and control diets was assessed in choice tests (as described in detail by Rodriguez-Saona and Trumble, 1996). Briefly, five neonates were placed inside an arena constructed from a 30-ml plastic cup lined with ~1 cm of 4% agar (w/v). Each cup had holes cut in opposite sides where two 1.5-ml polypropylene microcentrifuge tubes were placed. One of the tubes contained the control diet alone and the other contained the treated diet. The tubes were completely filled with the diet. To determine if low concentrations of the active compound

could elicit a larval response, one concentration below the EC_{50} was tested (100 μg/g). In addition, one concentration near the EC_{50} (200 μg/g) and two concentrations above the EC_{50} (400 and 600 μg/g) were tested. Treated and control diet were prepared as previously described. The arenas were held in an incubator under standard conditions (see above). The positions of the larvae were recorded at the beginning of the photophase and again 12 hr later for four days. Each arena was treated as a replicate, and each treatment had a total of 50 replicates.

Data Analysis. In no-choice tests, inhibition of larval growth was related to dose by an exponential function of the general form $Y = 100\ [1 - \exp(-kx)]$ (Smirle and Wei, 1996), where Y is the growth inhibition index expressed as a percent, x is dose, and k is a proportionality constant. Mortality data were analyzed using probit analysis (Finney, 1971). Other comparisons were done using Minitab (1991). Choice test data were analyzed by calculating the number of larvae present in the control diet minus the number of larvae present in the treated diet divided by the total larvae at each observation. We thus tested the hypothesis that the difference was significantly different from zero, using t tests (after Horton, 1995) [the test statistic was approximately t because of the large sample size ($N = 50$)] calculated with Super Anova (1989).

RESULTS

Isolation and Identification of Active Compounds. The first fractionation of the crude oil yielded two fractions (fractions 1 and 3), which produced high levels of larval mortality ($\geq 50\%$) (Figure 1a) and significantly reduced larval weight (Figure 1b, $P < 0.05$) compared to the other fractions. Larvae fed diet mixed with fractions 1 and 3 weighed an average of 4.43 $\pm$ 1.63 (SE) and 5.36 $\pm$ 1.96 (SE) mg, respectively, while control larvae weighed an average of 64.57 $\pm$ 7.38 (SE) mg. In contrast, diet treated with fractions 4 and 5 resulted in significant increases in weight versus the control diet (Figure 1b, $P < 0.05$).

Further efforts were focused on the most insecticidal fraction (fraction 3). This fraction consisted primarily of one compound, as determined by TLC. A portion was further purified by normal-phase HPLC, yielding the major compound in pure form (fraction 3-3). The compound degraded upon attempted coupled GC-MS analysis. However, exact mass measurement using a direct insertion probe and chemical ionization yielded a molecular formula of $C_{23}H_{40}O_4$, corresponding to four sites of unsaturation. The ^{13}C NMR spectrum showed two carbonyl peaks at 210.8 (ketone) and 171.2 ppm (ester), and four peaks (130.2, 130.0, 128.1, 127.9), each with a single attached proton, corresponding to carbons of two nonconjugated 1,2-disubstituted double bonds. These six peaks accounted for all four sites of unsaturation. Two further peaks shifted downfield

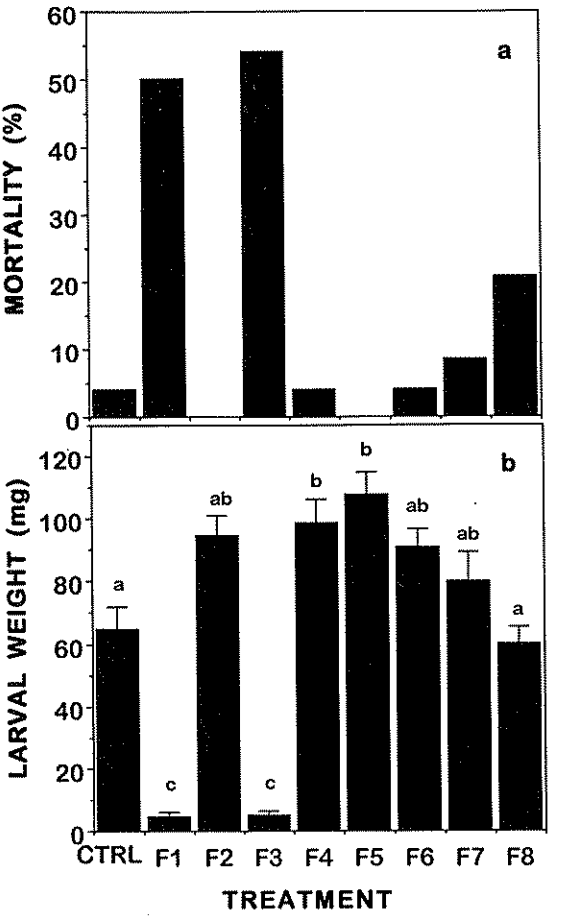
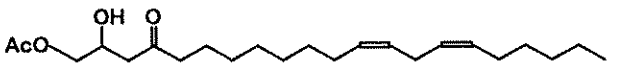


FIG. 1. Percent mortality (a) and weight (b) of *S. exigua* larvae fed control diet and diet containing fractions from avocado idioblast cell oil, from flash chromatographic fractionation. Bars at each data point indicate the standard error. Different letters indicate statistical differences between treatments (Tukey's Pairwise Comparisons, $P < 0.05$).

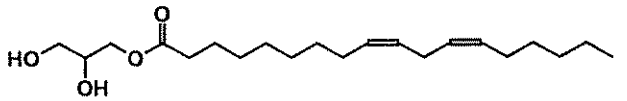
(67.2 ppm, two attached protons; 66.0 ppm, one attached proton) were assigned as methylene and methine carbons bonded to an oxygen, accounting for the two remaining noncarbonyl oxygen atoms. One of these could be attributed to the previously identified ester function, while the other could only belong to an alcohol, since there were no other carbon atoms with a chemical shift indicative of an attached oxygen. There were two methyl groups (20.8 and 14.1 ppm); all other carbons were methylenes.

Further structural details were supplied by the ^1H spectrum. A two-proton triplet (2.46 ppm) and a two-proton doublet (2.63 ppm) corresponded to methylenes on either side of a ketone, with the former being adjacent to another methylene, and the latter, shifted slightly further downfield, being adjacent to a methine. The methine proton was located as a complex multiplet centered at 4.32 ppm, with the downfield shift suggesting an attached oxygen atom. Furthermore, the complexity of the coupling pattern suggested coupling to at least two more protons on an adjacent carbon. These data were corroborated by a complex two-proton multiplet centered at 4.08, indicative of a diastereotopic methylene with an attached oxygen atom. A three-proton singlet (2.12 ppm) was assigned as the methyl portion of an acetate ester. Thus, the "head" end of the molecule was assigned the partial structure shown in the first six atoms of persin (Figure 2, I), with the only uncertainty being the relative positions of the acetate and hydroxyl functions at positions 1 and 2.

The remainder of the structure was assigned as follows. A three-proton triplet (0.89 ppm) indicated a methyl group terminating an alkyl chain. A multiplet consisting of four similar olefinic protons (5.45–5.27 ppm), a broadened quartet consisting of four allylic protons (2.05 ppm), and a broadened triplet (2.78 ppm) consisting of two bisallylic protons suggested a 1,4-diene system isolated in an alkyl chain, typical of an unsaturated fatty acid-type moiety. The only other NMR signal was the large methylene envelope, corresponding to 16 protons on eight methylene units. Assembling all of the above information, the structure was tentatively assigned as a 1-acetoxy-2-hydroxy-4-oxo-heneicosadiene, with only the position and geometry of the 1,4-diene system and the relative positions of the acetoxy and hydroxy groups being in question. A search



(12Z,15Z)-1-Acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (I)



Monoglyceride of linoleic acid (II)

FIG. 2. Structural relationship between the biologically active compound, (12Z, 15Z)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene) (persin) (I), and the monoglyceride of linoleic acid (II).

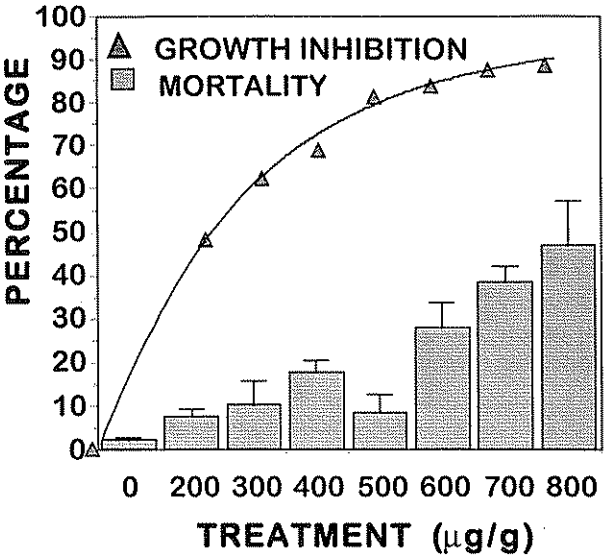


FIG. 4. Percent growth inhibition and mortality of *S. exigua* larvae fed diet containing different concentrations of persin. The equation for growth inhibition is: $Y = 100\ [1 - \exp(-0.0037X)]$. Bars at each data point indicate the standard error.

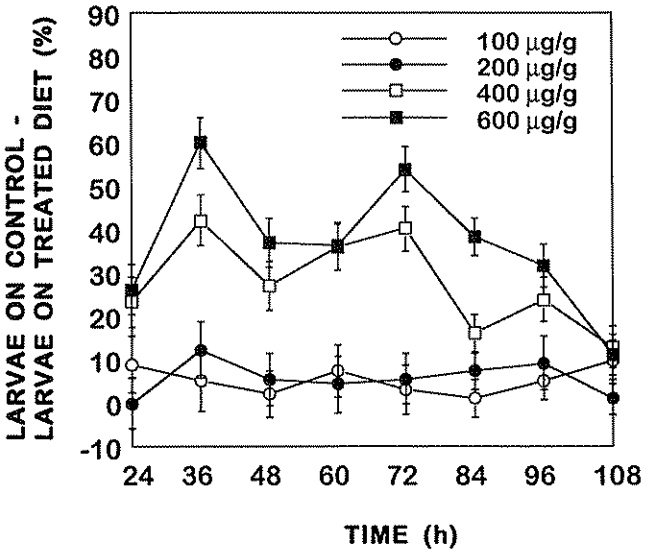


FIG. 5. Feeding deterrency of persin to *S. exigua* larvae in choice tests (first observation recorded 24 hr after initiation of the experiment). Bars at each data point indicate the standard error.

DISCUSSION

Persin, (12Z, 15Z)-1-acetoxy-2-hydroxy-4-oxo-henecoisa-12,15-diene, was isolated by bioassay-driven fractionation of avocado fruit idioblast cell oil and determined to be both toxic and a feeding deterrent to larvae of *S. exigua*. This compound has been previously isolated, and it apparently has a wide spectrum of biological activity. It was first isolated from avocado leaves and reported to inhibit growth of silkworm larvae, *Bombyx mori* L. (Chang et al., 1975; Murakoshi et al., 1976). Prusky et al. (1982) subsequently isolated persin from the peel of unripe avocado fruit and found that it inhibited in vitro vegetative growth of the avocado fungal pathogen *C. gloeosporioides*. More recently, Kobiler et al. (1993) reported the presence of persin in specialized oil cells of the avocado mesocarp.

At doses of 200 μg/g, persin inhibited growth of fourth-instar *B. mori* by approximately 40% over six days (Murakoshi et al., 1976). We found similar activity at 200 μg/g against early instars of the generalist herbivore *S. exigua*, where growth was inhibited by approximately 50% in seven days. We had anticipated differences between these species because the specialist feeder *B. mori*, with a long history of cultivation exclusively on mulberry, has been protected from most natural or synthetic chemicals. Conversely, *S. exigua* is a generalist herbivore that is constantly exposed to a variety of plant natural products, and it is the frequent target of pesticide applications (Brewer et al., 1990). Apparently, the metabolic enzymes providing substantial resistance for *S. exigua* larvae to pesticides or secondary plant compounds such as the linear furanocoumarins (Brewer et al., 1995) are not as effective against persin.

Although *S. exigua* shows a similar susceptibility to persin as *B. mori*, both insects are affected by concentrations much lower than the levels found in the pericarp and mesocarp of avocado fruit. Kobiler et al. (1993) reported concentrations in avocado fruit pericarp of different ages (starting from 75 days after fruit set until full maturity nearly 170 days later) ranging from 760 to 2280 μg/g of fresh weight. Concentrations in the mesocarp ranged from 1500 to 5800 μg/g. *S. exigua* is not listed as an avocado pest (McKenzie, 1935); this could be explained, at least in part, by the presence of persin in most tissues of the plant.

Our study showed that persin also modifies larval behavior of *S. exigua* by acting as a feeding deterrent at sublethal concentrations. Deterrency was observed on diet treated with persin at concentrations of 400 μg/g or higher during the first 72 hr, after which deterrency declined (Figure 5). Three factors may contribute to this observed decline in activity. First, persin is degraded by exposure to light (Carman and Duffield, 1995). Second, larger larvae may be more capable of tolerating the treated diet. Third, larvae may have habituated. Because only the two highest rates of persin produced avoidance, it is uncertain if these data

represent a threshold effect or a dose–response effect. In a comparison of diets with 400 μg/g and 600 μg/g (Figure 5), significantly more avoidance at the high rate was documented for three of eight observations, suggesting that a dose–response may be occurring.

Choice tests show that early instars of *S. exigua* fed persin at a concentration of 200 μg/g may not avoid treated diet (no behavioral effects, Figure 5), but no-choice tests show physiological effects; larval weights decreased compared to controls (Figure 4), even though larvae suffered low mortality (< 10%). Thus, persin detrimentally affects *S. exigua* larval growth at concentrations in diet of 200 μg/g or higher, but has deterrent effects only at concentrations of 400 μg/g or higher.

The LC_{50} for early-instar *S. exigua* is 936 μg/g persin in diet after seven days, which can be misleading because physiological effects were observed at a much lower concentration (200 μg/g, Figure 4), indicating that persin has strong growth inhibitory effects at a sublethal concentration. In comparison, Prabhaker et al. (1986), in similar diet-incorporation bioassays, found that seed extracts from the neem tree, *Azadirachta indica* A. Juss, at 200 μg/g in artificial diet killed 53% of neonate *S. exigua* larvae in eight days. Similarly, Moar and Trumble (1987) reported an LC_{50} for neem of 130 μg/g to early-instar *S. exigua*. Although the biological activity of persin is in the same range as the published LC_{50}s for neem, the LC_{50} for persin is substantially higher, suggesting that the primary value of this compound may be as a model for more stable and toxic synthetic compounds.

Our results indicate that persin, or more stable analogs, may have potential for insect control. However, persin does have substantial biological activity in mammals. Seawright et al. (1995) reported the experimental use of persin for treatment of mammalian cancers and that persin inhibits or prevents lactation. In particular, persin is a close structural mimic of the monoglyceride of linoleic acid (Figure 2, II), even in the position and the stereochemistry of the two double bonds in the chain. Seawright et al. (1995) speculate that the physiological action of persin in mammals may be due to interference, perhaps irreversibly, with glyceride biosynthesis. Nonetheless, the comparatively high concentrations in avocados have not been reported as a human health concern, even though humans ingest fresh and processed avocados, and avocado oil is widely used in cosmetics (Anonymous, 1980). One possible explanation for this lack of reported toxicity may be due to the nature of the idioblast cells; these cells have a tough cell wall that is probably not degraded by human digestion, so that the cells may pass through the digestive system intact.

Furthermore, substantial information will be required on field rates, mammalian toxicity, photostability, and phytotoxicity before the compound can be considered for commercialization. Should the extraction of persin from relatively expensive avocado fruit prove commercially impractical, total synthesis of the

compound is a possibility (Bull and Carman, 1994; MacLeod and Schäffeler, 1995), and it may also be possible to develop more photostable, more selective, or more potent analogs.

The isolation and identification of the bioactive compounds in fraction 1 (Figure 1) is in progress. This fraction is complex, and ongoing studies have indicated biological activity in at least three subfractions. The identities and biological activities of the purified compounds will be reported in due course.

Acknowledgments—We thank Kristina White, Gregory Kund, Mitchel White, Stephanie Young, and Claudia Trübenbach for assistance in the laboratory. We also thank Carlos Coviella, William Carson, and Drs. Stuart Reitz, J. Daniel Hare, and P. Kirk Visscher for their critical reviews of the manuscript. We acknowledge Dr. Robert Beaver for statistical advice, Drs. Richard Kondrat and Ron New (UC Riverside Analytical Facility) for mass spectra, and the collaborations of Drs. Kathryn Platt and William Thomson. This work was supported by the California Celery Research Advisory Board and the California Tomato Commission.

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