eration of the residue pattern in lake trout.

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Registry No. Toxicant B, 51775-36-1; toxaphene, 8001-35-2; 2,2,5-*endo*-6-*exo*-8,9,9,10-octachlorobornane, 58002-18-9; 2,2,5-*endo*-6-*exo*-8,8,9,10-octachlorobornane, 58002-19-0.

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Macrolide Aggregation Pheromones in *Oryzaephilus surinamensis* and *Oryzaephilus mercator* (Coleoptera: Cucujidae)

Aldona M. Pierce,* Harold D. Pierce, Jr., Allan C. Oehlschlager, and John H. Borden

Oryzaephilus surinamensis (L.), the sawtoothed grain beetle, and *O. mercator* (Fauvel), the merchant grain beetle, utilize male-produced, marcolide aggregation pheromones. These compounds were isolated from the Porapak Q-captured volatiles obtained from adult beetles feeding on rolled oats. The pheromones were identified as (*Z*)-3-dodecen-11-olide (I) and (*Z,Z*)-3,6-dodecadien-11-olide (II) for *O. mercator* and (*Z,Z*)-3,6-dodecadien-11-olide (II), (*Z,Z*)-3,6-dodecadienolide (III), and (*Z,Z*)-5,8-tetradecadien-13-olide (IV) for *O. surinamensis*. Structures of isolated I, II, III, and IV were confirmed by comparison with synthesized materials. Laboratory bioassays with *O. surinamensis* indicated that IV synergized the response to a mixture of II and III, while I and II were not synergistic for *O. mercator*.

INTRODUCTION

The sawtoothed grain beetle, *Oryzaephilus surinamensis* (L.), and the merchant grain beetle, *O. mercator* (Fauvel), are stored-product pests of worldwide distribution. A recent survey of stored-product insects across the United States (Mueller, 1982) ranked *O. surinamensis* first in importance as a pest of stored products and processed food and second in importance as a problem on raw grain. Being less tolerant of low ambient temperatures (Howe, 1956), *O. mercator* has become firmly established as a household pest in North America, especially on cereal products (Loschiavo and Sabourin, 1982) and on processed food in general (Mueller, 1982). Moreover, *O. mercator* frequently has been misidentified as *O. surinamensis* due to the morphological similarity of the two species (Loschiavo and Sabourin, 1982).

Identification of pheromones and food attractants for *Oryzaephilus* species could contribute to the development

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of integrated control programs (Levinson and Levinson, 1979; Burkholder, 1981) for these economically important pests. We previously have shown that *O. surinamensis* and *O. mercator* were attracted to beetle-produced volatiles as well as to volatiles from rolled oats and brewer's yeast (Pierce et al., 1981). Some attractive volatiles from rolled oats recently have been chemically identified for *O. surinamensis* (Mikolajczak et al., 1984). Research in our laboratory into the identificaton of attractive semiochemicals for another cucujid, the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens), has led to the identification of a new class of male-produced aggregation pheromones, the macrolide lactones (Wong et al., 1983). Preliminary field tests have shown that small, disposable traps baited with synthetic macrolide pheromones were very effective in recapturing released *C. ferrugineus* (Lindgren et al., 1985), indicating that these pheromones could be used in semiochemical-based, pest monitoring systems.

For both *Oryzaephilus* species we report herein the identification of male-produced, macrolide lactones which serve as aggregation pheromones.

MATERIALS AND METHODS

Experimental Insects. *O. mercator* and *O. surinamensis* were reared on large-flake rolled oats and brewer's yeast (95:5, wt/wt) (Pierce et al., 1981). In order to obtain populations exclusively of either sex, males and females of both species were separated as adults (Halstead, 1963.)

Pheromone Collection. Beetle-produced volatiles for preparative collections were obtained by aerating adults of mixed sex and age on a food source in all glass systems (Pierce et al., 1981) at 23–24 °C in darkness. Typically, 100,000 beetles (1–3 months posteclosion) were maintained on 1.5 kg rolled oats in a vertically oriented chamber (15.5 cm i.d. × 27 cm). Charcoal-filtered, humidified air was drawn by aspiration (1.9 L/min) through the culture and then through a trap filled with Porapak Q (50-80 mesh, Applied Sciences Division, Milton Roy Laboratory Group, State College, PA 16801). Every 7 days the rolled oat medium was changed, and the aeration was continued for a total of 28 days. An aeration was done in the same manner but without beetles to check for volatiles derived from the rolled oats. Volatiles were recovered by extraction of the Porapak Q with purified pentane in a Soxhlet extractor for 24 h and concentrated to ca. 5 mL by distilling the pentane through a Dufton column.

Instrumental Methods. A Hewlett-Packard 5830A gas chromatograph equipped with a 18835B capillary inlet system and a flame-ionization detector (FID) was employed for analyses by gas chromatography (GC). Samples were analyzed on a 48-50 m × 0.5 mm i.d. open-tubular glass column coated with SUPEROX 4 (Alltech Associates, Deerfield, IL 60015). The temperature program for analytical GC was 70 °C for 2 min and then 4 °C/min to 180 °C, holding for 30 min. A Varian 1200 gas chromatograph equipped with a 10:1 effluent splitter, FID, and thermal gradient collector (Brownlee and Silverstein, 1968) was used for isothermal (170 °C) micropreparative separation of Porapak Q-trapped volatiles. The column was a 3 m × 3.17 mm o.d. stainless steel tube packed with 10% SP-1000 on Supelcoport (100/120 mesh) (Supelco, Bellafonte, PA 16823). Before micropreparative separation, an aliquot of the pentane extract was concentrated to 30–50 μL by evaporation under a gentle stream of N_2 at −10 °C. For both chromatographs, helium was the carrier gas, and the injection port and detector temperatures were 260 and 270 °C, respectively. The candidate pheromones were quantified by GC with methyl myristate as an internal standard.

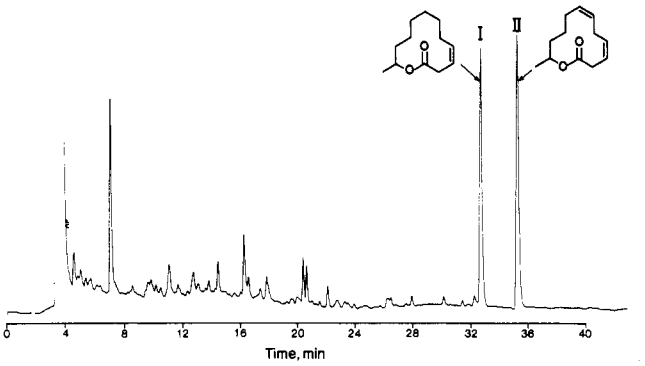

Figure 1. Gas chromatogram of Porapak Q-captured volatiles from mixed-sex *O. mercator* feeding on rolled oats, with structures of macrolides I and II.

Mass spectra were determined on a Hewlett-Packard 5895A GC/MS/DS fitted with a 30 m × 0.25 mm i.d. fused silica column coated with Carbowax 20M (J&W Scientific, Rancho Cordova, CA 95670) with helium as the carrier gas.

Nuclear magnetic resonance spectra (NMR) were recorded with a Bruker WM400 spectrometer. Samples separated by micropreparative GC were rinsed from collection tubes into NMR tubes with $CDCl_3$ (minimum isotopic purity 99.96 atom % D, Merck Sharp & Dohme, Canada Ltd., Kirkland, PQ H9R 4P7) containing 0.005% tetramethylsilane.

Bioassay Procedures. A two-choice, pitfall olfactometer (Pierce et al., 1981) was utilized to test suspect attractants. Two glass vials were suspended from holes in the bottom of a plastic petri dish. Filter paper disks treated with a 10 μL aliquot of either an experimental stimulus in purified pentane or purified pentane as a control were put into the bottom of the appropriate vials. Twelve beetles were released into each dish, and the dish lids were replaced. Bioassays for each test solution were replicated 12 times by using fresh beetles in each replicate. After 2 h in darkness, the numbers of beetles in experimental and control vials were recorded. The raw data were analyzed with a *t* test for correlated, paired data. Results were expressed as the mean percent response of the total number of beetles per treatment.

Bioassays were conducted at 23 °C and 50–60% relative humidity from 1600–2000 h by using test beetles 5–12 weeks posteclosion. To ensure a uniform state of preconditioning for *O. mercator*, each replicate of 12 beetles was held in a 60-mL glass vial without food for 18 h at 23 °C in darkness prior to a bioassay. To obtain maximum responsiveness from *O. surinamensis*, up to 1500 beetles were preconditioned without food for 48 h at 23 °C in darkness in a 6-L Erlenmeyer flask through which charcoal-filtered, humidified air was drawn at 1.9 L/min. Since response of *O. surinamensis* in the two-choice olfactometer was extremely sensitive to population density (Pierce et al., 1983), test beetles were maintained at a reduced population density of 1000 beetles per kg medium at least one week before bioassays commenced.

RESULTS AND DISCUSSION

***O. mercator* Volatiles.** A gas chromatogram of Porapak Q-trapped volatiles from mixed-sex *O. mercator* feeding on rolled oats is shown in Figure 1. In addition to volatiles from the rolled oats, there were two predominant compounds, I and II. Aeration of separated sexes determined that I and II were male-produced. Compounds I [(*Z*)-3-dodecen-11-olide] and II [(*Z,Z*)-3,6-dodecadien-11-olide] are unsaturated macrolide lactones (Figure 1).

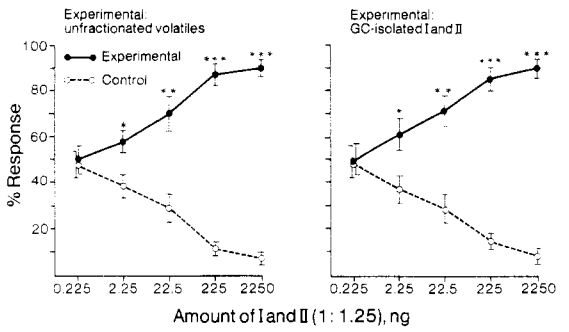

Figure 2. Response ($\bar{X}$ ± SE) by mixed-sex *O. mercator* in two-choice, pitfall bioassay to the unfractionated volatiles from mixed-sex *O. mercator* feeding on rolled oats and to GC-isolated I and II. Significant response to experimental stimulus indicated by the following: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. n = 12 replicates.

Their structures were established by comparison of mass and NMR spectra of the GC-isolated compounds to spectra of I and II isolated from *C. ferrugineus* (Wong et al., 1983). The structures of I and II have been confirmed by synthesis (Oehlschlager et al., 1983; Millar and Oehlschlager, 1984).

In order to establish if these macrolides were pheromones, GC-isolated I and II were recombined in the natural ratio (1:1.25) and tested in the pitfall olfactometer over a 10 000-fold dose range. Comparison of the attraction of mixed-sex *O. mercator* to the two-part mixture with the attraction shown to the unfractionated extract indicated that most, if not all, of the activity of the unfractionated extract was accounted for by the presence of I and II (Figure 2).

Natural I and II were bioassayed individually and as a 1:1 mixture over a 10 000-fold dose range with either male or female *O. mercator* as test beetles (Figure 3). (Response by mixed-sex adults to the 1:1 mixture was no different than response to the 1:1.25 mixture.) Males and females responded differentially to I and II. For macrolide I, males had a lower threshold for positive response (1 ng) and demonstrated a greater attraction at higher experimental doses, compared to the response shown by females. For macrolide II, females had a lower threshold for positive response (1 ng) compared to that of males, although both sexes were strongly attracted to higher doses of II. Although both I and II are apparently aggregation pheromones for *O. mercator*, the maximum response by either sex to the 1:1 mixture of I and II over the total dose range suggests that the two-part mixture would be the most effective bait in a field trap. Since mixed-sex *O. mercator* were increasingly attracted to natural I, II, and the 1:1 mixture over a 100 000-fold dose range (Figure 4), these pheromones might be effective as lures in field traps.

Male *C. ferrugineus* produce nearly optically pure (*S*)-I as a synergistic aggregation pheromone; synthetic (*R*)-I was completely inactive when bioassayed in the two-choice olfactometer (Wong et al., 1983). The chiralities of *O. mercator* I and II have not yet been determined, and at the amounts bioassayed I and II were not synergistic. The metasternal gland secretion of the cerambycid beetle *Phoracantha synonyma* (Newman) contains macrolide lactones (Moore and Brown, 1976), one of which, (*Z*)-5-dodecen-11-olide, differs from I only in the position of the double bond. The macrolides produced by *P. synonyma* have not been implicated as pheromones.

***O. surinamensis* Volatiles.** Figure 5 shows a gas chromatogram of Porapak Q-captured volatiles from mixed-sex *O. surinamensis* feeding on rolled oats. There

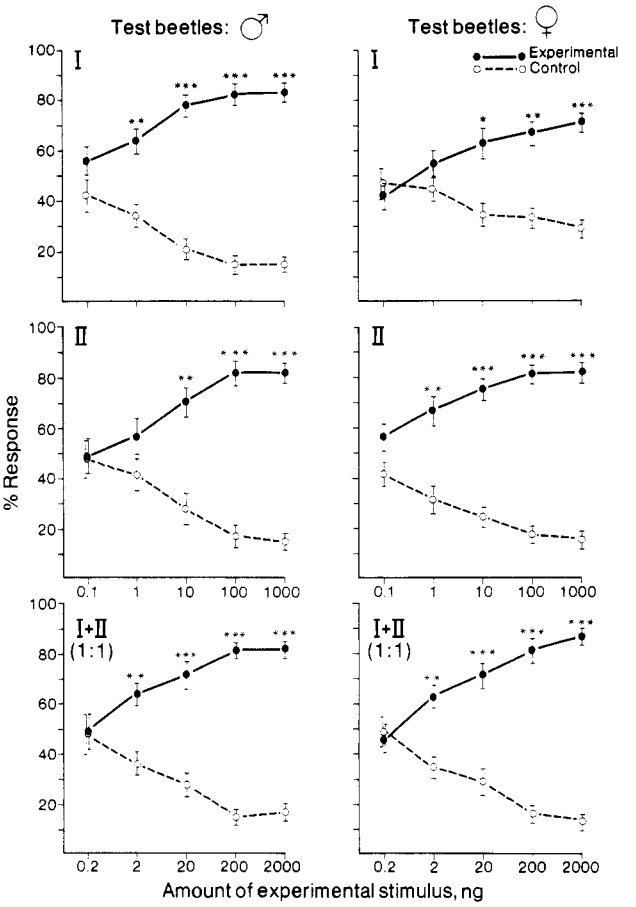

Figure 3. Response ($\bar{X}$ ± SE) by male or female *O. mercator* in two-choice, pitfall bioassay to macrolides I and II isolated from Porapak Q-captured volatiles of mixed-sex *O. mercator* feeding on rolled oats. Significant response to experimental stimulus indicated by the following: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. n = 12 replicates.

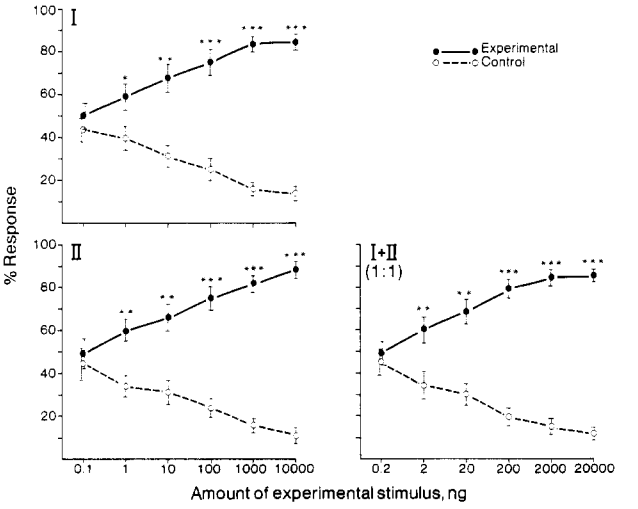

Figure 4. Response ($\bar{X}$ ± SE) by mixed-sex *O. mercator* in two-choice, pitfall bioassay to macrolides I and II isolated from Porapak Q-captured volatiles of mixed-sex *O. mercator* feeding on rolled oats. Significant response to experimental stimulus indicated by the following: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. n = 12 replicates.

were three predominant compounds, II, III, and IV, which aeration of separated sexes indicated were male-produced. The mass and NMR spectra of II from *O. surinamensis* were identical with those of II from *O. mercator*. Thus, (*Z,Z*)-3,6-dodecadien-11-olide (chirality unknown) is pro-

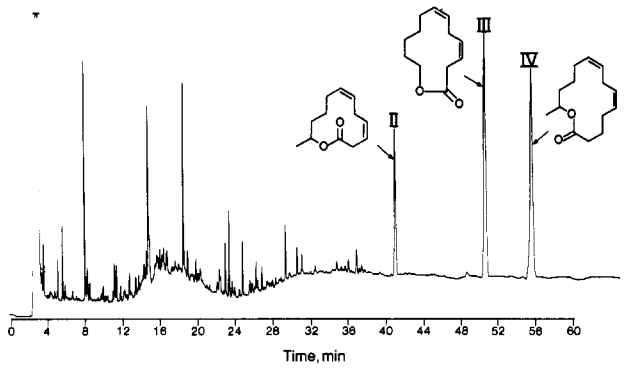

Figure 5. Gas chromatogram of Porapak Q-captured volatiles from mixed-sex *O. surinamensis* feeding on rolled oats, with structures of macrolides II, III, and IV.

Table I. NMR Spectral Data of Natural III Isolated from Porapak Q-Captured Volatiles of Mixed-Sex *O. surinamensis* Feeding on Rolled Oats

proton	chemical shift, $\delta^{a,b}$	coupling constants, Hz
2	3.09 (d)	$J_{2,3}$ = 7.75
3	ca. 5.48 (m)	$J_{3,4}$ = 10.9, $J_{3,5}$ = 1.9
4	ca. 5.54 (m)	
5	2.91 (dd)	$J_{4,5} = J_{5,6}$ = ca. 7
6	ca. 5.54 (m)	$J_{6,7}$ = 11
7	ca. 5.48 (m)	
8	2.05 (q)	$J_{7,8}$ = 7.5
9	1.34 (m)	$J_{8,9}$ = 7.5
10	1.46 (m)	$J_{9,10}$ = 5.0
11	1.71 (m)	$J_{10,11}$ = 6.6
12	4.11 (t)	$J_{11,12}$ = 5.0

[a] In ppm downfield from tetramethylsilane. [b] d = doublet, m = multiplet, q = quartet, t = triplet.

duced by both male *O. mercator* and *O. surinamensis*.

The mass spectrum of compound III exhibited M^+ at m/z 194 and was quite similar to that of II (Wong et al., 1983), but lacked a fragment ion at m/z 179 ($M^+ - CH_3$) which was present in the mass spectra of I and II. Thus, III probably bore no side chain methyl group. The results of the analyses of the decoupled NMR spectra obtained from GC-isolated III are given in Table I. The protons α to the carbonyl absorbed as a doublet at δ 3.09 were coupled (J = 7.75 Hz) to one of the olefinic protons (C-3 H) in the multiplet centered at δ 5.48. Decoupling of the bis allylic protons at δ 2.91 permitted assignment of the olefinic resonance at δ 5.54 to the hydrogens attached to C-4 and C-6 as well as measurement of the vinyl coupling constants. The observed couplings (Table I) indicated that both double bonds were of Z geometry. The remaining allylic protons (δ 2.05) were found to be coupled to an adjacent methylene (δ 1.34) and to one of the olefinic hydrogens (C-7 H) in the multiplet at δ 5.48. The chemical shift of the triplet at δ 4.11 suggested that the hydrogens were bound to the carbon bearing the ester oxygen. Irradiation of the multiplet at δ 1.71 collapsed this triplet to a singlet. On the basis of these mass and NMR spectral data, III was formulated as (*Z,Z*)-3,6-dodecadienolide. The proposed structure for III has been recently confirmed by synthesis (Millar and Oehlschlager, 1984).

The mass spectrum of the longest eluting compound (IV) showed M^+ at m/z 222 with characteristic ions at m/z 207 ($M^+ - CH_3$) and m/z 204 ($M^+ - H_2O$) and for m/z <140 was similar to those of II and III. The mass spectral data suggested that IV was a bis(methylene) homologue of II. Key features of the NMR spectrum of natural IV were a methyl doublet at δ 1.24 (J = 6 Hz) and complex olefinic absorptions at δ 5.29 (1 H) and 5.43 (3 H). Absence of absorptions below δ 6.00 indicated the double bonds were not conjugated. The carbinyl proton absorbed at δ 5.03 and was coupled to a methyl group and an adjacent methylene ($J_{13,14}$ = 9.5 Hz and $J_{13,14}$ = 3.5 Hz). In II and III, the Z double bonds are located on the $\omega - 6$ and $\omega - 9$ carbons. Based upon this pattern of double bond position and geometry, IV was formulated as (*Z,Z*)-5,8-tetradecadien-13-olide. The proposed structure of IV has also been confirmed by synthesis (Millar and Oehlschlager, 1984).

Table II. Response by *O. surinamensis* to Macrolides II, III, and IV Isolated from Porapak Q-Captured Volatiles of Mixed-Sex *O. surinamensis* Feeding on Rolled Oats

		% response ($\bar{X}$ + SE)[d]		
	experimental stimulus, ng	experimental stimulus	pentane control	
II	1[a]	51.0 ± 6.1	38.5 ± 5.9	NS
	10	65.5 ± 6.8	26.9 ± 5.4	*
	100	66.4 ± 4.4	25.0 ± 4.1	**
III	1	45.1 ± 6.5	36.6 ± 5.1	NS
	10	63.6 ± 5.3	33.1 ± 4.4	*
	100	61.3 ± 4.5	25.4 ± 4.8	*
IV	3	42.0 ± 4.6	46.1 ± 5.1	NS
	30	44.1 ± 6.1	48.8 ± 5.6	NS
	300	51.4 ± 5.7	32.6 ± 4.9	NS
II + III	1 + 1	62.9 ± 6.1	27.9 ± 5.2	*
	10 + 10	65.5 ± 4.5	25.2 ± 3.7	**
	100 + 100	70.3 ± 3.5	21.7 ± 2.9	***
II + IV	1 + 3	57.4 ± 5.1	34.1 ± 4.7	NS
	10 + 30	65.0 ± 4.8	30.0 ± 4.1	*
	100 + 300	72.0 ± 3.6	22.0 ± 3.1	***
III + IV	1 + 3	57.4 ± 5.6	32.6 ± 4.9	NS
	10 + 30	66.0 ± 5.9	30.8 ± 4.6	*
	100 + 300	69.5 ± 3.6	24.2 ± 3.4	**
II + III + IV	1 + 1 + 3	67.4 ± 4.0	22.9 ± 3.2	**
	10 + 10 + 30	78.2 ± 3.4	19.3 ± 3.3	***
	100 + 100 + 300	82.7 ± 3.3	13.7 ± 3.1	***
	100 + 100 + 300[b]	75.8 ± 4.1	15.2 ± 2.7	***
	100 + 100 + 300[c]	85.4 ± 2.9	12.5 ± 2.5	***

[a] Test beetles, mixed-sex. [b] Test beetles, males. [c] Test beetles, females. [d] Significant response to experimental stimulus indicated by the following: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significant. n = 12 replicates.

The natural products, II, III, and IV, were bioassayed individually and in various mixtures (Table II). Substantial amounts of II or III elicited a low aggregation response from mixed-sex *O. surinamensis*; the positive response was enhanced somewhat when II and III were combined in a 1:1 mixture. Macrolide IV elicited no significant response alone, but slightly enhanced the response to II or III when combined with either compound. The 1:1:3 mixture of II, III, and IV (approximately the natural ratio produced by the bioassay beetles) evoked the strongest aggregation response from mixed-sex, male, or female *O. surnamensis* over the experimental dose range. Macrolides II, III, and IV are concluded to be aggregation pheromones, with IV synergizing the response to the combined mixture of II and III. While a minimum amount of IV was necessary for consistent, positive response, progressively increasing the proportion of IV in the three-part mixture from 1:1:1 to 1:1:10 did not affect the aggregation response of mixed-sex *O. surinamensis* (data not shown).

Male *Cryptolestes turcicus* (Grouvelle) produced IV (chirality unknown) as a synergistic aggregation pheromone (Millar, 1983). Macrolide IV has been identified also in the metasternal gland secretion of *P. synonyma* (Moore and Brown, 1976).

Chirality. The response of *Oryzaephilus* species to synthetic racemic and chiral macrolide pheromones will be reported elsewhere.

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Registry No. I, 97372-89-9; II, 86583-51-9; III, 90134-39-7; IV, 86583-52-0.

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Lipoxygenase-2 Isozyme Is Responsible for Generation of *n*-Hexanal in Soybean Homogenate

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The process of development of *n*-hexanal from soybean homogenate was investigated by using *Glycine max* var. Suzuyutaka (wild type) and the following lipoxygenase (L) deficient mutant seeds (L null); L-1 null, L-2 null, L-3 null, and L-1, -3 null. *n*-Hexanal was determined during the incubation of the homogenates of these seeds at 25 °C. The level of *n*-hexanal was the lowest in the L-2 null homogenate and the highest in the L-1, -3 null homogenate. After the addition of linoleic acid to the homogenates, the level of *n*-hexanal increased remarkably in the homogenates from the seeds except for L-2 null. *n*-Hexanal was scarcely generated in the L-2 null homogenate. These results suggest that L-2 isozyme is responsible for *n*-hexanal formation by using free linoleic acid as the substrate. When the soybean extract prepared from these seeds was incubated at 70 °C, *n*-hexanal formation was the lowest in the L-2 null soybean extract.

INTRODUCTION

The consumption of soy proteins as food ingredients is being encouraged, because soy protein is not only an economical resource, but also has high qualities of nutrition and functionality. However, characteristic grassy beany and green flavors build a barrier for wide utilization of soy protein. Major contributors to these flavors are the volatile carbonyl compounds which are enzymatically derived from hydroperoxides of unsaturated fatty acids (Rackis et al., 1979). Lipoxygenase (EC 1.13.11.12) catalyzes the hydroperoxidation of linoleic acid and other polyunsaturated lipids that contain a *cis,cis*-1,4-pentadiene moiety.

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Soybean contains three types of lipoxygenase isozymes (lipoxygenase-1 (L-1), L-2, and L-3) which exhibit different kinetic behaviors (Galliard and Chan, 1980; Axelrod et al., 1981). Therefore, each isozyme might be expected to take part in the development of grassy beany and green flavors through a different mode of action. However, at present it is not clear how the individual isozymes participate in the generation of the flavors. Recently, soybean seeds that lack lipoxygenase isozymes were found as the result of screening a variety of seeds to get soybean with low level of the objectionable flavors (Hildebrand and Hymowitz, 1981; Hildebrand and Kito, 1984; Kitamura et al., 1983; Kitamura, 1984).

In the present study, in order to elucidate the mechanism of the development of grassy beany and green flavor from soybean, we investigated which lipoxygenase isozyme(s) was predominantly responsible for the formation of *n*-hexanal, one of major elements of soybean flavor, by using a wild type and the isozyme deficient mutants of soybeans such as L-1, L-2, L-3, and L-1, -3.

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