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CUTICULAR HYDROCARBONS AND NOVEL ALKENEDIOL DIACETATES FROM WHEAT STEM SAWFLY (*Cephus cinctus*): NATURAL OXIDATION TO PHEROMONE COMPONENTS[1]

ROBERT J. BARTELT,[2,]* ALLARD A. COSSÉ,[2] RICHARD J. PETROSKI,[2] and DAVID K. WEAVER[3]

[2]*USDA Agricultural Research Service*
National Center for Agricultural Utilization Research
Crop Bioprotection Research Unit
1815 N. University Street
Peoria, Illinois 61604

[3]*Montana State University*
Department of Entomology
P.O. Box 173020
Bozeman, Montana 59717-3020

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Abstract—The cuticular lipids of the wheat stem sawfly *Cephus cinctus* (Hymenoptera: Cephidae) were investigated as part of a chemical ecology project with this species. The major cuticular lipids were *n*-alkenes and *n*-alkanes. Alkenes were the most abundant and exhibited dramatic sexual dimorphism. (*Z*)-9-Tricosene accounted for about half of the total hydrocarbon in males but was nearly absent from females. The dominant alkenes in females were (*Z*)-9-pentacosene and (*Z*)-9-heptacosene. The alkane profiles were similar in both sexes, with *n*-tricosane being the most abundant, followed by *n*-pentacosane and *n*-heptacosane. In both sexes, there were minor amounts of alkanes and alkenes with other chain lengths and *n*-alkadienes of 29 and 31 carbons. In males, about one tenth of the surface lipids consisted of (*Z*)-9-alkene-1, ω-diol diacetates with 22-, 24-, and 26-carbon chains. The same compounds were also detected from females but in much smaller amounts. The structures of these novel diacetates were proven by synthesis. By analogy to methyl oleate, a well-studied food lipid, the alkenes and diacetates were expected to undergo slow oxidation in air to release specific aldehydes and other volatile products, and these were generally detected in volatiles collected from living sawflies.

* To whom correspondence should be addressed. E-mail: BartelRJ@NCAUR.USDA.gov

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Atmospheric oxidation of the diacetates was also demonstrated in the absence of sawflies. One product from the diacetates, 9-acetyloxynonanal, was shown in other research to be particularly active electrophysiologically and was also attractive in the field. Aldehydes from the alkenes also showed strong electrophysiological activity. The concept of volatile pheromones originating from heavy, unsaturated cuticular lipids is discussed.

Key Words—*Cephus cinctus*, wheat stem sawfly, pheromone, cuticular lipid, hydrocarbon, alkene, alkane, (*Z*)-9-docosene-1,22-diol diacetate, (*Z*)-9-tetracosene-1,24-diol diacetate, (*Z*)-9-hexacosene-1,26-diol diacetate, aldehyde, oxidation.

INTRODUCTION

The wheat stem sawfly *Cephus cinctus* Norton (Hymenoptera: Cephidae) remains an important pest of wheat in the northern Great Plains, and the chemical ecology of this species is now being investigated with the goal of finding new management tools. Aspects of this research have included the chemical identification, electrophysiological activity, and attractiveness of volatile compounds from the sawfly (Cossé et al., 2002). Compounds from sawfly volatiles collections that elicited strong electrophysiological responses included 9-acetyloxynonanal, nonanal, tetradecanal, phenylacetic acid, and other aldehydes and carboxylic acids. 9-Acetyloxynonanal was attractive to both sexes in preliminary field tests (Cossé et al., 2002).

In another sawfly species, *Pikonema alaskensis* (Rohwer) (Hymenoptera: Tenthredinidae), the volatile long-range pheromone also includes aldehyde components, and these originate in an unusual way, through the oxidation of essentially nonvolatile cuticular lipids (Bartelt and Jones, 1983). The cuticular lipids of *C. cinctus* were, therefore, studied to explore whether such a mechanism might operate in this species. The results suggest it does. This report includes a survey of the cuticular lipids of *C. cinctus*, the synthesis of three novel constituents to prove structure, and a discussion of the production of volatile pheromone components from unsaturated lipids by oxidation.

METHODS AND MATERIALS

Extracts and Collections of Volatile Compounds. Hexane extracts of whole, individual sawflies were prepared in Montana by one of us (D.K.W.) and sent to Peoria for analysis. The insects were field collected as prepupae and handled as described by Cossé et al. (2002). Individual adult sawflies were extracted for either 20 min or 24 hr in ca. 0.5 ml hexane, and the sex and age of each insect was recorded. Because mating occurs soon after emergence in the field, the chosen age

categories emphasized the younger insects: 1, 2, 3, 4, and 5 hr after emergence, 1–2 days old, and 5–6 days old. Some sawflies were also extracted before emergence; these were removed from the wheat stem stubs after they had eclosed from their pupae but before they had chewed through the plug in the stem. A total of 23 extracts of individuals were analyzed by GC-MS and compared. Later, additional hexane extracts were prepared in Peoria from insects received from Montana. These included 1 sec dips and 24 hr soaks of groups of 10–25 male or female sawflies.

The collection of volatiles from living sawflies was described by Cossé et al. (2002). The GC-MS data files for these previous volatiles collections were studied further for additional information.

Instrumentation. The details of the GC-MS system were given by Cossé et al. (2002). Briefly, the HP 5973 Mass Selective Detector was interfaced to an HP 6890 GC. Cuticular lipid samples were analyzed on a thin-film DB-1 GC column (15 m length, 0.25 mm ID, 0.1 μm film thickness; J & W Scientific, Folsom, California). The oven program started at 50°C for 1 min, increased at 10°C/min to 300°C, and remained at 300°C for 5 min. The inlet and transfer line were held at 300°C and 250°C, respectively. The usual MS scanning range was 40–550 amu (EI, 70 eV). The Wiley mass spectral library with 275,821 spectra (Wiley, 1995) was available on the MS data system. Quantitation of cuticular components was done by the external standard method on an HP 5890 Series II gas chromatograph equipped with flame ionization detector. High-performance liquid chromatography (HPLC) was used to separate geometrical isomers of alkenes and of one alkenediol diacetate. The system consisted of a Waters M6000 pump, Waters R401 differential refractometer detector, and $AgNO_3$-treated column. The $AgNO_3$ column was prepared from a 25-cm $\times$ 4.6-mm-ID silica column (Alltech, Adsorbosphere, 5 μm) by the method of Heath and Sonnet (1980). The elution solvent was 0.25% 1-hexene in hexane for alkene hydrocarbons and benzene for the alkenediol diacetate. HPLC on a silica column (Alltech, Econosphere Silica 5μ) was also used to separate the sawfly-derived alkenediol diacetates from the other cuticular lipids (10% ether in hexane). Infrared spectra of purified synthetic alkenediol diacetates were obtained as thin films on KBr plates using a Nicolet Impact 410 FT-IR spectrometer. The proton NMR spectrum was obtained at 400 MHz for a synthetic alkenediol diacetate on a Bruker Avance 400 instrument. The solvent was deuterobenzene, and the shifts were measured relative to TMS.

Dimethyl Disulfide Derivatives. Dimethyl disulfide (DMDS) adducts were prepared from the sawfly-derived olefinic lipids and the synthetic alkenediol diacetates so that locations of double bonds could be determined (Carlson et al., 1989). Briefly, the samples were evaporated to dryness under a stream of nitrogen, and DMDS and 5% iodine in ether were added (equal volumes, about 25 μl each). The samples were heated at 45°C for 1–2 hr, then diluted with hexane and worked up with aqueous sodium thiosulfate to destroy the iodine catalyst. The hexane layer, which contained the products, was dried over sodium sulfate, evaporated under

nitrogen, resuspended in about 10 μl of hexane, and analyzed by GC-MS. The MS scanning range for these was 40–650 amu.

Synthesis of Alkene-1, ω-Diol Diacetates. Three alkenediol diacetates (**8**, **9**, and **10**) were synthesized by one of us (R.J.P.), using standard methods, to confirm chemical identifications (Figure 1). The Wittig reaction was the key synthetic step, and most of the reactions in Figure 1 involved the creation of the required Wittig salts and aldehydes from commercially available starting materials and the addition or removal of protecting groups. The Wittig salts (**1–4**) were prepared from 9- or 11-carbon bromohydrins (Aldrich, Milwaukee, Wisconsin) by refluxing with triphenylphosphine in acetonitrile, followed by solvent removal and trituration with dry ether to remove impurities. For two of these (**1** and **2**), the hydroxyl group

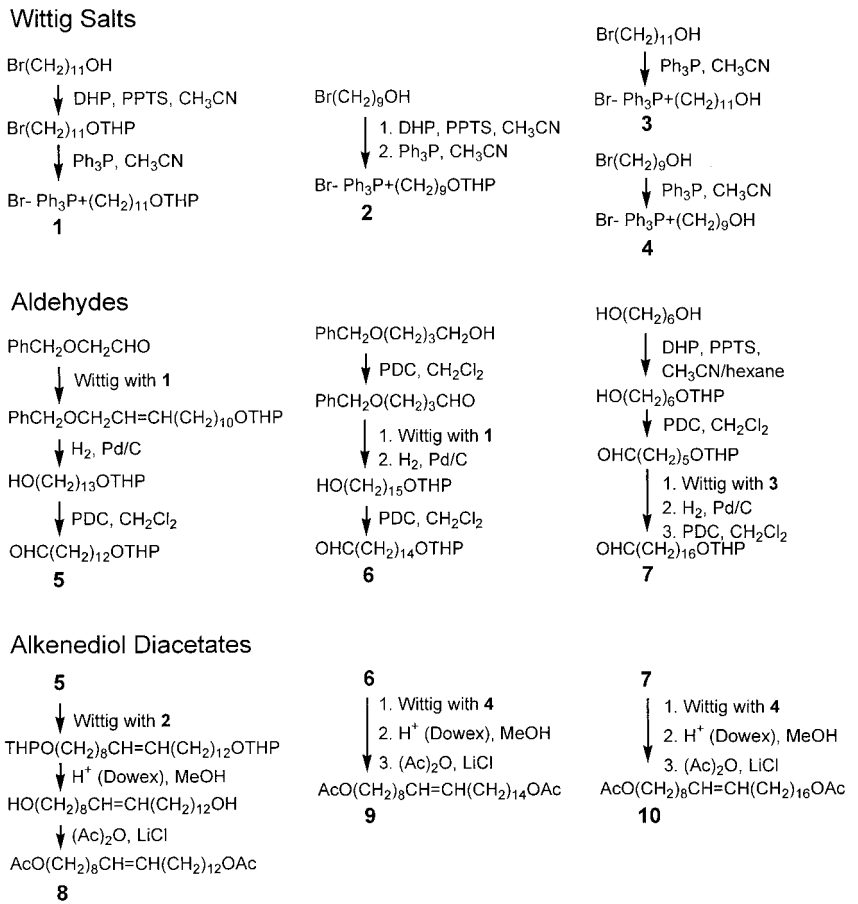

FIG. 1. Synthesis of the 9-alkene-1,ω-diol diacetates by Wittig routes (see text for abbreviations).

was protected as a tetrahydropyranyl (THP) ether before salt formation by reaction with 2,3-dihydropyran (DHP) in acetonitrile, using pyridinium *p*-toluene sulfonate (PPTS) as catalyst (Miyashita et al., 1977).

The carbon chains of the aldehydes (**5**–**7**) were all built up from smaller fragments with Wittig reactions (Sonnet, 1974). For **5** and **6**, two- and four-carbon benzyl-ether aldehydes were condensed with the 11-carbon THP Wittig salt. The four-carbon aldehyde was not commercially available but was made from its alcohol by oxidation with pyridinium dichromate (PDC) (Corey and Schmidt, 1979). Hydrogenation of these Wittig products over 10% palladium on carbon both removed the benzyl protecting group (but not the THP group) and reduced the double bond (Greene and Wuts, 1991). The free hydroxyls were subsequently oxidized to aldehydes with PDC. To make aldehyde **7**, the six-carbon diol was first converted to a mono-THP ether in a biphasic mixture of acetonitrile and hexane; the reactants were primarily in the more polar (acetonitrile) phase, but the mono ether migrated to the hexane phase, reducing the chance of further derivatization to the diether. The free hydroxyl was then oxidized to an aldehyde, and the aldehyde was condensed with hydroxy Wittig salt **3**. The double bond of the resulting 17-carbon product was hydrogenated over palladium on carbon, and the final aldehyde was formed by PDC oxidation of the alcohol.

The final Wittig condensations with the prepared aldehydes involved either the protected (THP) or hydroxy salts; both gave the expected products, although the yields in either case were only fair. The THP groups were removed in MeOH under acid catalysis (provided by Dowex 50W-X4 ion-exchange resin, which was later easily removed by filtration) (Beier and Mundy, 1979). Finally, the free alkenediols were acetylated with acetic anhydride, using lithium chloride as the Lewis acid catalyst (Sabitha et al., 1999).

The synthetic requirement was only to obtain enough material for chromatographic and spectral comparison with the natural material. Reactions were not optimized for yield, and in most cases, intermediates were used in subsequent reactions without purification. Overall yields were less than 1% from the starting materials, but the desired final products were distinct and readily recognizable in the reaction mixtures.

Reactions were monitored by mass spectrometry. Mass spectral and GC retention data for the syntheses in Figure 1 are summarized in Table 1. Mass spectra were not obtained for the Wittig salts **1**–**4**. The THP ether and diol intermediates for **9** and **10** were not analyzed by GC-MS but were converted directly to the diacetates before analysis. Synthetic **8**–**10** consisted of both *E* and *Z* isomers, which were separable by GC. Synthetic **10** was investigated further so that the proper configuration could be assigned to each GC peak. HPLC on the $AgNO_3$/silica column was used to separate the geometrical isomers, and infrared spectra were then obtained for these. A proton NMR spectrum was also acquired for the more abundant isomer.

TABLE 1. GC RETENTION TIMES AND MASS SPECTRAL DATA FOR INTERMEDIATES AND FINAL PRODUCTS IN SYNTHESIS OF 1,ω-ALKENEDIOL DIACETATES (SEE FIGURE 1)

Compound and (GC retention in min.)	Mass spectral fragments [m/z] and (relative abundance)
Intermediate for compound **1**	
$Br(CH_2)_{11}OTHP$ (15.92)	85 (100), 101 (8), 333 (2, M), 335 (2, M)
Intermediate for compound **2**	
$Br(CH_2)_9OTHP$ (13.98)	85 (100), 101 (10), 305 (3, M), 307 (3, M)
Intermediates for compound **5**	
$PhCH_2OCH_2CH{=}CH(CH_2)_{10}OTHP$ (21.57, 21.73)[a]	85 (100), 91 (77), 101 (13), 107 (9), 303 (4, M-85), 387 (0.1), 388 (0.1, M)
$HO(CH_2)_{13}OTHP$ (16.92)	41 (16), 43 (11), 55 (24), 56 (16), 57 (13), 67 (9), 69 (18), 81 (4), 83 (13), 84 (10), 85 (100), 95 (3), 97 (9), 101 (31), 111 (4), 125 (1), 215 (0.7, M-85), 299 (0.5), 300 (0.1, M)
$OHC(CH_2)_{12}OTHP$ (**5**) (16.30)	41 (17), 43 (12), 55 (21), 56 (18), 57 (14), 67 (10), 69 (9), 81 (7), 83 (8), 84 (12), 85 (100), 95 (10), 97 (6), 101 (22), 109 (6), 123 (2), 213 (0.7, M-85), 297 (0.4, M-1)
Intermediates for compound **6**	
$PhCH_2O(CH_2)_3CHO$ (7.75)	91 (100), 107 (33), 178 (0.6, M)
$PhCH_{2O}(CH_2)_3CH{=}CH(CH_2)_{10}OTHP$ (22.83, 23.00)[a]	85 (100), 91 (62), 101 (5), 107 (7), 331 (6, M-85), 415 (0.1), 416 (0.4, M)
$HO(CH_2)_{15}OTHP$ (18.64)	85 (100), 101 (34), 243 (0.9, M-85), 327 (0.4), 328 (0.1, M)
$OHC(CH_2)_{14}OTHP$ (**6**) (18.10)	85 (100), 101 (27), 241 (1, M-85), 325 (0.4), 326 (0.2, M)
Intermediates for compound **7**	
$HO(CH_2)_6OTHP$ (9.57)	85 (100), 101 (32), 117 (3, M-85), 201 (0.3), 202 (0.1, M)
$OHC(CH_2)_5OTHP$ (8.60)	85 (100), 101 (25), 115 (1, M-85), 199 (0.4), 200 (0.1, M)
$HO(CH_2)_{10}CH{=}CH(CH_2)_5OTHP$ (19.94, 20.02)[a]	85 (100), 101 (2), 252 (1, M-84-18), 270 (0.8, M-84)
$HO(CH_2)_{17}OTHP$ (20.22)	85 (100), 101 (36), 271 (1, M-85), 355 (0.3), 356 (0.1, M)
$OHC(CH_2)_{16}OTHP$ (**7**) (19.73)	85 (100), 101 (27), 269 (1, M-85)
Intermediates for compound **8**	
$THPO(CH_2)_8CH{=}CH(CH_2)_{12}OTHP$ (26.61, 26.73)[a]	85 (100), 101 (11), 340 (1, M-84-84), 423 (1, M-85)
$HO(CH_2)_8CH{=}CH(CH_2)_{12}OH$ (20.42, 20.50)[a]	41 (51), 55 (100), 57 (25), 67 (69), 69 (60), 81 (71), 82 (94), 95 (63), 96 (69), 109 (32), 123 (16), 135 (10), 149 (4), 304 (1, M-18-18), 322 (3, M-18), 340 (1, M)

TABLE 1. CONTINUED

Compound and (GC retention in min.)	Mass spectral fragments [*m/z*] and (relative abundance)
$AcO(CH_2)_8CH{=}CH(CH_2)_{12}OAc$ (**8**) (22.08, 22.15)[a]	41 (34), 43 (100), 55 (81), 57 (15), 61 (21), 67 (71), 69 (52), 81 (75), 82 (74), 95 (67), 96 (66), 109 (37), 110 (30), 121 (25), 123 (20), 135 (19), 137 (11), 149 (8), 151 (5), 152 (5), 304 (4, M-60-60), 321 (3, M-60-43), 364 (3, M-60) 424 (4, M)
Compound **9**	
$AcO(CH_2)_8CH{=}CH(CH_2)_{14}OAc$ (23.43, 23.51)[a]	41 (34), 43 (100), 55 (85), 57 (22), 61 (24), 67 (65), 69 (59), 81 (74), 82 (70), 95 (64), 96 (63), 109 (36), 110 (29), 121 (22), 123 (21), 135 (17), 137 (12), 149 (7), 151 (5), 152 (6), 332 (4, M-60-60), 349 (3, M-60-43), 392 (4, M-60) 452 (5, M)
Compound **10**	
$AcO(CH_2)_8CH{=}CH(CH_2)_{16}OAc$ (24.75, 24.81)[a]	41 (33), 43 (100), 55 (85), 57 (22), 61 (25), 67 (65), 69 (61), 81 (74), 82 (72), 95 (67), 96 (64), 109 (36), 110 (30), 121 (20), 123 (22), 135 (16), 137 (11), 149 (7), 151 (5), 152 (5), 360 (4, M-60-60), 377 (2, M-60-43), 420 (4, M-60) 480 (6, M)

[a] *Z* and *E* isomers with nearly identical mass spectra. The spectral data correspond to the earlier, larger GC peak (*Z* isomer) in each case.

Other Standards. Samples of 8-acetyloxyoctanal and 10-acetyloxydecanal were prepared for comparison of GC retention times and mass spectra with components in sawfly volatile collections. The commercially obtained 8- and 10-carbon diols were converted to mono-THP ethers as described above, oxidized with PDC to mono-THP aldehydes, then deprotected and acetylated. The 13-, 15-, and 17-carbon aldehyde acetates were obtained by ozonolysis (see Cossé et al., 2002) of alkenediol diacetate samples. The saturated aldehydes of 6–14 and 16 carbons were either commercially available (6–14 carbons, Aldrich) or were prepared by ozonolysis of appropriate alkene hydrocarbons. Octanoic, nonanoic, decanoic, and phenylacetic acids were obtained commercially (Aldrich). Synthetic *E* and *Z* alkenes and various other alcohols, hydrocarbons, and 2*E*-alkenals were on hand from other research.

Laboratory Oxidation of Alkenediol Diacetates. Preliminary study of light-induced, abiotic oxidation of alkenediol diacetates was performed using a Suntest CPS solar simulator (Atlas, Gainesville, Florida), with the system set up as described by McGuire et al. (2000). Purified (HPLC) samples containing about 500 ng of synthetic **10** or natural male alkenediol diacetates were evaporated to dryness on the inside of quartz cuvettes. Cuvettes were capped and sealed with Teflon tape and placed in the solar simulator. The outside of the treated cuvette was in direct contact with the cooled (15°C) instrument surface. Samples were exposed for 2 hr

to light irradiation similar in intensity and wavelength composition (300–1100 nm) to sunlight. As a control, a sample of synthetic **10** was set up identically but without the light. All samples were resuspended in hexane after 2 hr and then analyzed by GC-MS.

RESULTS AND DISCUSSION

Cuticular Hydrocarbons. The cuticular hydrocarbons were the major compounds in the sawfly hexane extracts (Figure 2, Table 2). Both sexes had large amounts of *n*-alkanes and *n*-alkenes, primarily with odd numbers of carbons. The *n*-alkane profiles were similar between sexes, with tricosane being most abundant and with smaller amounts of 25-, 27-, 29-, and 31-carbon alkanes. There were traces of the intervening even-chain compounds and also the 21- and 33-carbon alkanes. No branched alkanes were detected.

The *n*-alkenes were the most abundant class of hydrocarbons, and their profiles differed dramatically between sexes. In males, the 23-carbon alkene accounted for about half of the total hydrocarbon. In females, this alkene was far less

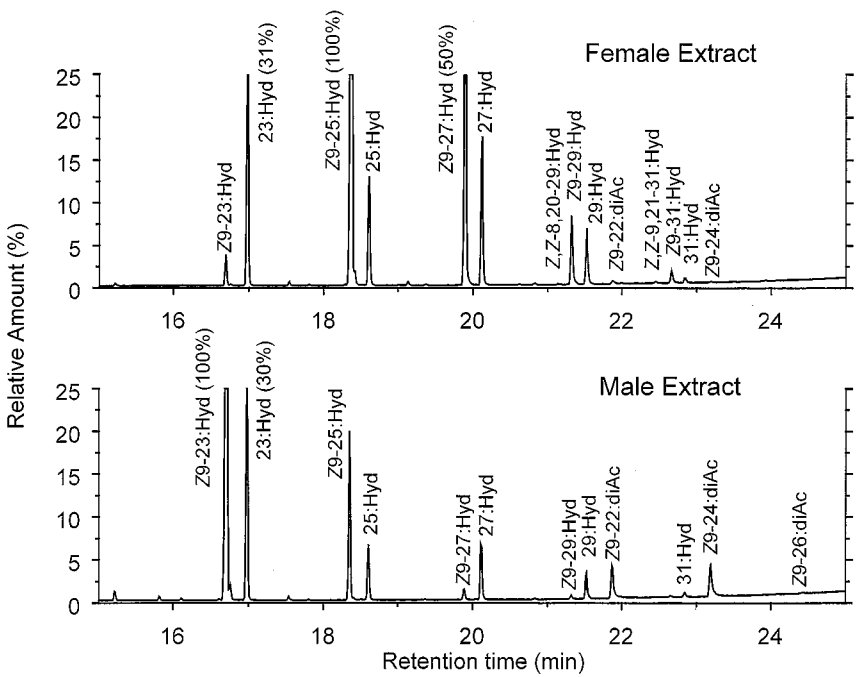

FIG. 2. Gas chromatograms of extracts from female and male *C. cinctus*. Selected hydrocarbons (:Hyd) and diacetates (:diAc) are labeled. Relative areas of off-scale peaks are shown in parentheses.

TABLE 2. HYDROCARBONS EXTRACTED FROM 2-TO 5-DAY-OLD MALE AND FEMALE *C. cinctus* CUTICLE

Hydrocarbon	Relative amount (% of total)		MS data: molecular ion of hydrocarbons (and key ions of DMDS adducts)
	Females	Males	
n-Alkanes (by numbers of carbons)			
21	0.31	0.57	296
22	0.13	0.29	310
23	13.9	17.5	324
24	0.24	0.23	338
25	8.6	6.1	352
26	0.32	0.20	366
27	11.1	5.9	380
28	0.20	0.14	394
29	3.5	3.4	408
30	0.038	0.074	422
31	0.52	1.2	436
33	0.10	0.36	464
n-Alkenes (by double bond descriptions and numbers of carbons)			
(*Z*)-9-21	0.016	0.24	294, (173, 215, 388)
(*Z*)-9-22	0.027	0.48	308, (173, 229, 402)
(*Z*)-9-23, [(*Z*)-7-23][a]	4.4	48.0	322, (173, 243, 416, [145, 271][a])
(*Z*)-9-24	0.53	0.68	336, (173, 257, 430)
(*Z*)-9-25	34.9	13.4	350, (173, 271, 444)
(*Z*)-9-26	0.50	0.072	364, (173, 285, 458)
(*Z*)-9-27	16.6	0.92	378, (173, 299, 472)
(*Z*)-9-28	0.15	ND[b]	392, (173, 313, 486)
(*Z*)-9-29	3.1	0.17	406, (173, 327, 500)
(*Z*)-9-30	0.049	ND	420, (173, 341, 514)
(*Z*)-9-31	0.67	0.071	434, (173, 355, 528)
n-Alkadienes (by double bond descriptions and numbers of carbons)			
(*Z*,*Z*)-8,20-29	0.034	0.020	404, (159, 173, 325, 339, 498, 592)
(*Z*,*Z*)-9,21-31	0.042	0.015	432, (173, 187, 339, 353, 526, 620)

[a] Present in trace amount (<1% of major isomer).
[b] ND denotes not detected.

abundant, and the 25- and 27-carbon alkenes were dominant instead. There were smaller amounts of alkenes with 21, 29, and 31 carbons in both sexes and also the intervening even-chain compounds. In both sexes, the double bonds were almost exclusively in the nine position for all chain lengths, based on mass spectra of the DMDS derivatives. The 23-carbon alkenes from both sexes did have trace amounts of 7-alkene (<1% as much as the 9-isomer). The observed molecular ions (alkene molecular weights plus 94) and the key fragment ions (from cleavage between the carbons with the thiomethyl groups) are given in Table 2. By comparison to *E* and *Z* synthetic standards (underivatized) on the $AgNO_3$ HPLC

column, all of the sawfly alkenes had the *Z* configuration. No branched alkenes were detected.

In females, hydrocarbon mass spectra indicated the presence of small amounts of alkadienes of 29 and 31 carbons (molecular ions at $m/z = 404$ and 432, respectively). The mass spectra of the DMDS derivatives were interpreted according to Carlson et al. (1989). Fragments listed in Table 2 represent the molecular ion (addition of two equivalents of DMDS), the loss of 94 (one DMDS) as a neutral molecule, and subsequent cleavage at the remaining DMDS site as with the alkenes (yielding four ions, representing the saturated and unsaturated side of each double bond). These spectra determined the double bonds to be in the 9 and 21 positions from one end of the chain for both dienes. (However, rules of nomenclature require that the 29-carbon compound be named as 8,20-nonacosadiene so that the locant numbers are as small as possible). GC retention times were consistent with straight chains and *Z* configurations at the double bonds (Bartelt et al., 1986). These dienes were also detected from males, but in smaller amounts.

The total amount of hydrocarbon per sawfly was about 15 μg, with males and females having very similar quantities. Extraction for 20 min or 24 hr gave comparable profiles and amounts, although the longer soak also extracted fatty acids (primarily palmitic and oleic) and sterols from the insects. Even a 1 sec rinse gave essentially the same profiles, indicating that the compounds were present on the immediate surface. The lipid profiles and amounts were consistent in major features for all ages between 1 hr and 5 days. In contrast, adults removed from wheat stubs before natural emergence had the typical amount of alkane but only about 10% of the usual amount of alkene. Apparently, biosynthesis of alkenes is intense around the time of emergence. Finally, younger females (several hours to 1 day old) had larger amounts of the alkadienes than older ones, with the 29-carbon diene amounting to as much as 0.5% of the total hydrocarbon rather than less than 0.05%. This difference was not seen in males. The female in Figure 2 was less than 1 day old at the time of extraction.

Diacetate Lipids. Initial comparison of chromatograms revealed two compounds in male extracts (approximately 1 μg of each per male) that were far less abundant in females (Figure 2, retention times of 21.9 and 23.2 min). The mass spectrum of the first of these indicated a molecular weight of 424 (Figure 3), which was not consistent with a hydrocarbon. (A fully saturated hydrocarbon of 30 carbons would have a molecular weight of 422; thus oxygen was probably present in the sawfly compound to account for the higher molecular weight). Several subtle features in the mass spectrum gave important information about the structure. The first was a fragment at m/z 61 (19% of base peak), which is characteristic for an acetate group (McLafferty, 1980). Examination of library spectra of acetates indicated that the m/z 61 peak is most intense when the acetate group is at or near the end of the carbon chain and is distant from other functional groups or branches. Second, the spectrum of the sawfly compound lacked the characteristic peaks that

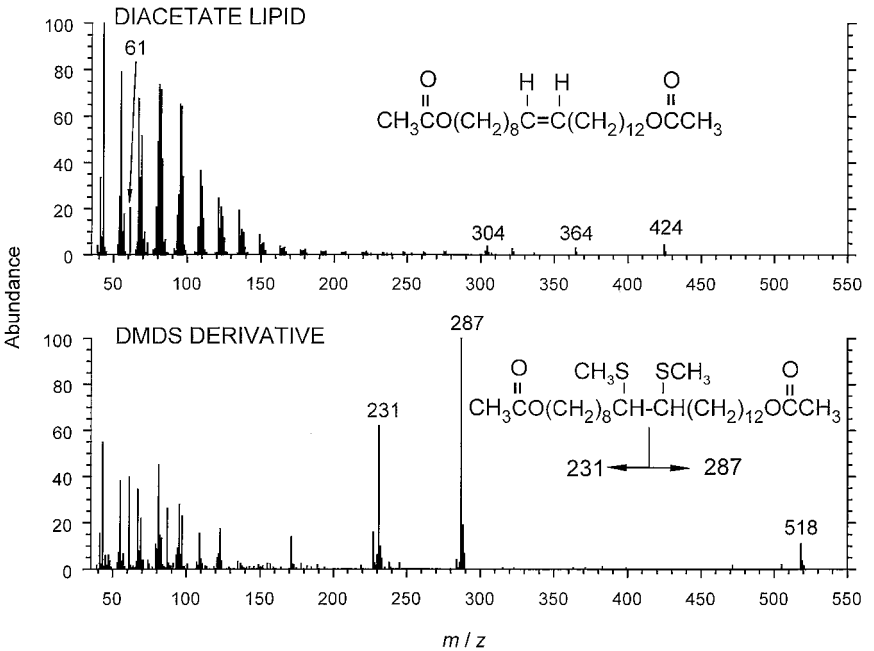

FIG. 3. EI-mass spectrum of 22-carbon diacetate lipid from male *C. cinctus* (top) and EI-mass spectrum of the DMDS derivative of this lipid, showing origins of major fragments (bottom).

would be expected if the acetates were secondary; for example, an acetate on the second carbon of a chain leads to a prominant spectral peak at *m*/*z* 87 due to chain cleavage adjacent to the acetate. Finally, there were fragments at *m*/*z* 364 and *m*/*z* 304 that corresponded to two consecutive losses of 60 from the molecular ion (believed to represent the loss of two acetic acid molecules). Taken together, this evidence suggested a lipid chain of 22 carbons with an acetate group on each end. However, the molecular weight for a saturated chain would be 426 instead of 424; thus a double bond was probably present in the chain. The DMDS derivative formed readily (molecular ion at *m*/*z* 518 = 424 + 94), and the fragment ions (*m*/*z* 231 and 287) were consistent with a double bond being present in the 9 position of the original lipid (Figure 3).

The data for the second lipid were analogous, with molecular weight of 452 and key fragments at *m*/*z* 61, 332, and 392. The DMDS adduct gave key MS ions at *m*/*z* 231, 315, and 546, consistent with a 24-carbon diacetate, again with the double bond in the 9 position. Closer inspection of the gas chromatograms revealed a third, still later-eluting member of this series, about 10% as abundant as the first two. Its mass spectrum included ions at *m*/*z* 61, 360, 420, and 480, and the MS

ions of its DMDS derivative included 231, 343, and 574. These data indicated a 26-carbon diacetate, once more with the double bond in the 9 position. There was no evidence for other double-bond positional isomers. Close examination of female-derived extracts and their DMDS derivatives by GC-MS revealed that the same three compounds were present but at much lower levels (about 10% of the amounts in males, see Figure 2).

Synthetic Alkenediol Diacetates. Synthetic alkenediol diacetates were prepared to confirm the proposed structures and to establish double-bond configuration of the natural material. The mass spectral data (Table 1) supported that the synthetic reactions proceeded as shown in Figure 1 because the synthetic intermediates and products had the expected molecular weights and reasonable fragmentation patterns. For example, THP ethers were readily recognized by their mass spectra (Table 1) because of the base peak at m/z 85 and a moderately intense ion at m/z 101, due to cleavage on either side of the ether oxygen. The molecular ion and an M-1 ion were usually seen for the THP ethers, but the intensity for both was low (ordinarily $<1\%$). The M-85 peak was similarly small but was still reliably present and served as further confirmation of molecular weight. Example details for one THP ether alcohol and one THP ether aldehyde are given under the intermediates for compound **5** in Table 1; for other THP ethers, only key ions are listed. Analogously to THP ethers, benzyl ethers had spectral peaks at m/z 91 and 107. The THP ether bromides (intermediates for **1** and **2**) had characteristic spectra because of the bromine isotopes. For the di-THP diether precursor of **8**, the molecular ion was not seen, but the M-85 fragment was present, and there was another ion corresponding to two losses of a deprotonated THP group (m/z 340). After deprotection, the free diol exhibited a small molecular ion and ions corresponding to one and two neutral losses of water. Features of the mass spectra of alkenediol diacetates were discussed above.

Patterns in GC retention times supported the mass spectral interpretations and synthetic expectations. For example, the Wittig reactions always gave two products (the *E* and *Z* isomers) that were separated by about 0.1 min but had nearly identical mass spectra. Catalytic hydrogenation always collapsed the E/Z peak doublet into a single peak of still higher GC retention time. Also, oxidation of a THP ether alcohol to the corresponding THP ether aldehyde consistently decreased the GC retention time (Table 1).

By GC of synthetic **8–10**, the earlier-eluting isomer was always the more abundant one; this was expected to be the *Z* isomer because the Wittig reaction conditions favor production of *Z* double bonds (Sonnet, 1974). Compound **10** was analyzed further by AgNO3 HPLC to confirm this. The more abundant isomer eluted later than the less abundant one (24–26 ml vs. 20–21 ml, based on GC analysis of HPLC fractions), which also supported that the more abundant isomer was *Z* (Heath and Sonnet, 1980). The IR spectra further corroborated this conclusion. The more abundant isomer was a liquid at room temperature and had key IR

absorbances (cm^{-1}) indicative of an olefin (3003, w, =C–H), hydrocarbon chain (2925 and 2854, both s, $-CH_2-$), and acetate group (1741, s, C=O; 1365, m, 1240, s, and 1037, m, other acetate-related peaks), but the spectrum did not indicate presence of an *E* double bond. On the other hand, the less abundant isomer was a solid at room temperature and had corresponding absorbances at 2918, 2850, 1732, 1369, 1247, and 1041; it also had the absorbance (962, m) that confirmed the *E* double bond. The proton NMR spectrum of the more abundant isomer supported the structure for synthetic **10** as drawn in Figure 1, but it gave no further information about double-bond configuration because the olefinic protons had the same shift and the coupling constant could not be observed: δ 5.56 (2 H, m, =C$\underline{H}$–), 4.04 (4 H, m, O–C$\underline{H}_2$–), 2.17 (4 H, m, =CH–C$\underline{H}_2$), 1.74 (6 H, s, C$\underline{H}_3$(C=O)O), 1.2–1.6 (40 H, other –C$\underline{H}_2$–, m).

Comparison of Natural and Synthetic Alkenediol Diacetates. Based on same-day comparisons of GC retention times, each natural alkenediol diacetate matched exactly the *Z* isomer of the corresponding synthetic compound for all three chain lengths. They also matched with respect to mass spectra and with respect to GC rentention times and mass spectra of the DMDS derivatives.

Compounds in Nature Related to Diacetates. This is believed to be the first report of natural alkenediol diacetates, but related compounds are known. Beeswax contains saturated 1,ω-diols of 24–28 carbons and esters of these, but the acid moieties have 16–24 carbons instead of just two (Tulloch, 1971). Free 1,ω-alkanediols of 22, 24, and 26 carbons have been isolated from various plants (Chatterjee and Chakraborty, 1967; Austin et al., 1969; Rizvi and Sultana, 1972; Amparo-Lopez et al., 1976; Hatam et al., 1990). 1,16-Hexadecanediol diacetate has been reported in the defensive secretion of the gulf fritillary butterfly (M. Blum, personal communication). Recently Oliver et al. (2000) and Doss et al. (2000) isolated 3-hydroxypropanoyl esters of (*Z*)-9-docosene-1,22-diol and (*Z*)-9-tetracosene-1,24-diol from bruchid weevils. The compounds are potent plant growth regulators, and callus tissue develops on legume pods exposed to these compounds during oviposition by female beetles.

Oxidation of Cuticular Alkenes. "Spontaneous" oxidation of unsaturated lipids has been studied extensively, especially in relation to rancidity of food oils, and much is known about the chemical mechanisms involved (reviewed by Frankel, 1998). Oxidation is complicated and involves cascades of free-radical reactions. It can be light induced (photooxidation) or simply occur by exposure to molecular oxygen under ambient conditions (autoxidation) if triggered by trace catalysts or reactive species such as free radicals. Once initiated, the reactions tend to be self-sustaining. Both mechanisms generally lead to the same volatile products, but proportions can differ. The major volatile products are usually aldehydes.

One food-related lipid, methyl oleate, provides a reasonable predictive model for the nonenzymatic oxidation of the alkenes and cuticular alkenediol diacetates of *C. cinctus*. Methyl oleate is like the sawfly compounds in having just one *Z* double

bond that is distant from other functional groups or chain ends. The mechanism for oxidation of methyl oleate to volatile products (Frankel, 1998) suggests a specific series of compounds that would result from the oxidation of particular alkenes, and occurrence of these compounds in the volatile collections from sawflies would support that such a mechanism is operating.

The oxidation scheme and expected products from (*Z*)-9-tricosene, the only abundant alkene in *C. cinctus* males, are summarized in Figure 4. The initial reaction is the formation of allylic hydroperoxides (four positional isomers). The hydroperoxides decompose either thermally or catalytically (e.g., by metal ions) into alkoxyl radicals, and these can then react in a number of ways, including cleavage into volatile products. Scission on either side of the radical group (indicated by "A" or "B" in Figure 4), yields a specific free aldehyde plus an intermediate that reacts further to give a characteristic hydrocarbon, alcohol, or other aldehyde. This general scheme is shown at the bottom of Figure 4, and the expected products from each of the hydroperoxide isomers are summarized at the upper right. By analogy to methyl oleate (Frankel, 1998), nonanal and tetradecanal should be the most abundant products.

The compounds actually detected in volatile collections are shown in bold print in Figure 4; relatively large and intermediate quantities are indicated by ** and *, respectively. The compounds were identified by mass spectrum and GC retention time, relative to authentic standards. The more abundant products are also indicated in the total ion chromatogram in Figure 5, which represents a volatile collection from male sawflies.

Nonanal and tetradecanal were, in fact, the most abundant of the expected oxidation products from males. Octanal and decanal were clearly present but were less abundant. Amounts were still smaller for the other compounds noted in bold in Figure 4. Some of the products expected but not found (heptanal, heptane, heptanol, octane) are relatively volatile and would have occurred too near the solvent front of the GC run to yield sharp peaks and be easily detected. Others, such as 2-undecenal, were probably present, but small amounts and coelution with more abundant compounds prevented positive identification. The heavier (15- and 16-carbon) 2-alkenals were not detected. Their lower volatilities would have reduced the amounts collected. Furthermore, these compounds are especially prone to further oxidation, and remaining in the oxidizing lipid mixture instead of evaporating out would have enhanced their degradation.

Female sawflies have very little 23-carbon alkene but have large amounts of the 25- and 27-carbon alkenes. Oxidation of these would be analogous to tricosene. All of the scission products with 11 or fewer carbons (Figure 4) would be expected from any of the 9-alkenes, but those of 12 or more carbons expected from 9-tricosene would be replaced by homologs with two or four additional carbons if the parent alkene is 9-pentacosene or 9-heptacosene. Hence, there was relatively little tetradecanal from females, but hexadecanal was abundant instead (Cossé

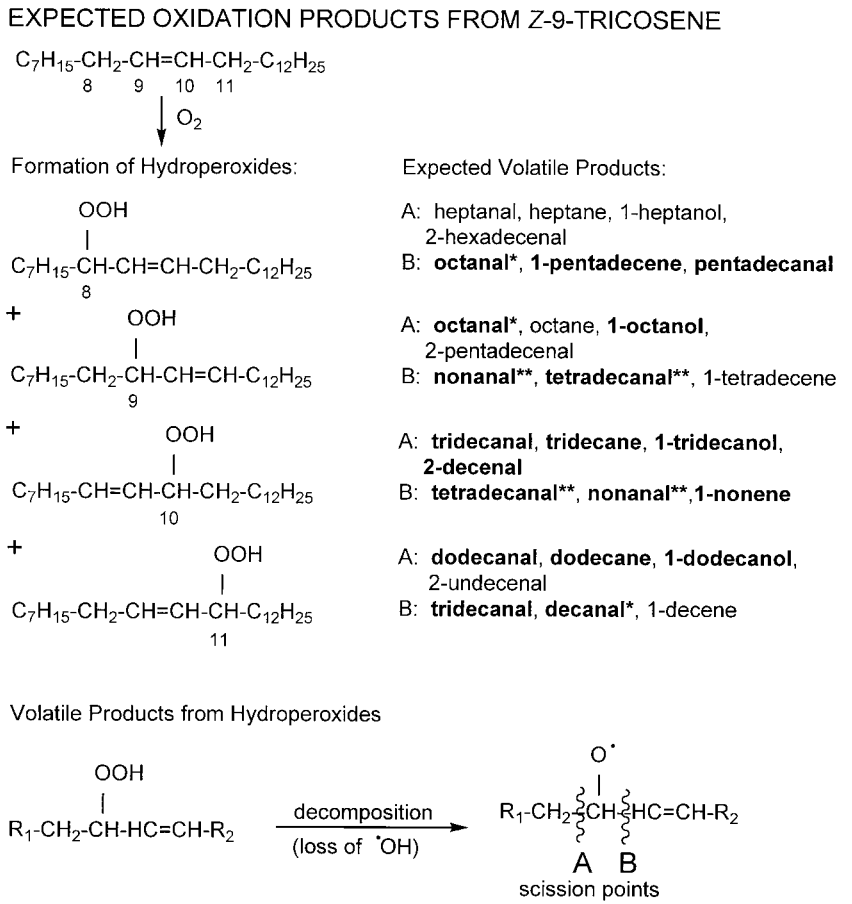

FIG. 4. Expected products from the autoxidation of (*Z*)-9-tricosene, based on the process for methyl oleate (Frankel, 1998). Compounds in bold print were detected in volatile collections from male sawflies. High and intermediate relative abundance denoted by ** and *, respectively.

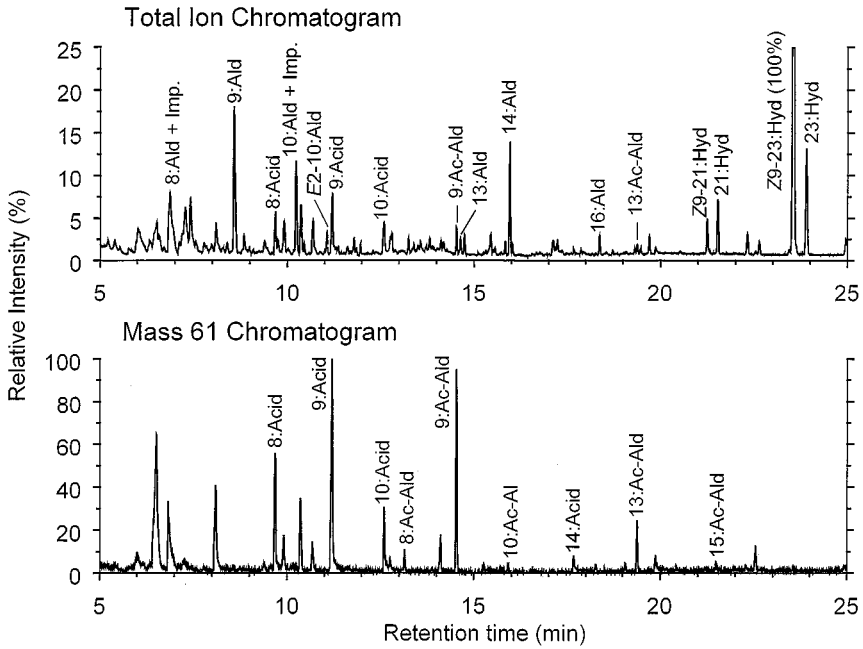

FIG. 5. GC-MS chromatograms of a volatile collection from male *C. cinctus*. The upper trace is total ion chromatogram. The lower trace is for mass 61 only, which shows the acetyloxyaldehydes (and also the carboxylic acids) very clearly. Peaks are labeled as aldehydes (:Ald), hydrocarbons (:Hyd), carboxylic acids (:Acid), or acetyloxyaldehydes (:Ac-Ald). Nonbiological impurities are denoted by "Imp."

et al., 2002). The 18-carbon aldehyde was observed in the collections of volatiles but in smaller amounts. Its lower volatility probably reduced its abundance in the collections.

The observed product patterns support the formation of the volatiles by a mechanism like that described for methyl oleate. Further oxidation of these aldehydes would yield carboxylic acids, and those of 8, 9, and 10 carbons were found consistently in the volatile collections from sawflies (Cossé et al., 2002) (Figure 5), although these may have other, as yet unexplained sources as well. The emission of phenylacetic acid from sawflies is not, however, accounted for by the oxidation mechanism.

The amounts of oxidation products collected from the sawflies were minute relative to the amounts of alkene present. The most abundant aldehydes from males were nonanal and tetradecanal, but typical collected amounts were only 50 and 15 ng/day per male, respectively, while males have about 7 μg of the parent 9-tricosene. Monounsaturated compounds such as methyl oleate oxidize

only 1–2% as rapidly as the polyunsaturated analogs (Frankel, 1998); thus, the minor yields of volatile products from alkenes is not surprising. Nevertheless, the levels of volatiles observed are still sufficiently high to be detectable to sawfly antennae. Intriguingly, even though the intact alkenes were not found to elicit electrophysiological responses (Cossé et al., 2002), the male–female dimorphism in alkenes could still lead to behavioral recognition of sexes at a distance by way of the aldehyde (or other) oxidation products.

Dienes. The alkadienes, which are more abundant in females, would release additional, unique oxidation products. These would include dodecanedial and unsaturated aldehydes of 20, 21, and 22 carbons with a double bond in the 12 position. None of these compounds were detected, but the amount of diene starting material that was present was extremely small.

Oxidation of Alkene-1,ω-diol Diacetates. By analogy to the alkenes, the alkenediol diacetates of 22, 24, and 26 carbons would all be expected to produce 9-acetyloxynonanal, and each diester would also generate a unique, heavier homolog from the other end of the lipid chain (Figure 6).

The mass spectral data files for collections of sawfly volatiles were examined for these compounds by extracting single-mass chromatograms for *m*/*z* 61 (for

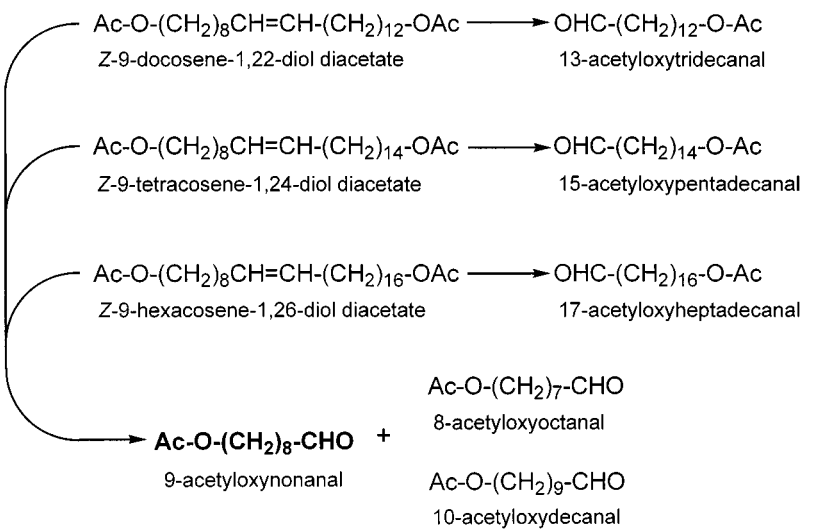

FIG. 6. Acetyloxyaldehydes expected from the oxidative cleavage of the alkenediol diacetates of *C. cinctus*. The major product would be 9-acetyloxynonanal because it can be derived from all three parent lipids, and acetyloxyaldehydes with 13-, 15-, and 17-carbon chains would result from the other ends of the parent lipid chains. Analogs of these acetyloxyaldehydes having one additional or one fewer carbon would also be expected (see text), but only those related to 9-acetyloxynonanal were likely to be detectable.

example, Figure 5). This approach revealed compounds containing the primary acetate group without interference from many other functional classes. (Simple aldehydes, alcohols, and hydrocarbons do not exhibit the *m*/*z* 61 fragment. Organic acids do show up, however, because there is always an isotope peak or protonated fragment corresponding to the major ion at *m*/*z* 60). 13-Acetyloxytridecanal was readily found in samples in which 9-acetyloxynonanal was prominent (about 20% as abundant as the major product, Figure 5). The 15-carbon homolog was sometimes detectable but was even less abundant, probably because of lower volatility. 17-Acetyloxyheptadecanal was never found, presumably because of still lower volatility and the much smaller amount of precursor. All four of these aldehyde acetates (of 9, 13, 15, and 17 carbons) were detected when the crude hexane extract of sawflies was ozonized, and the 13-carbon homolog clearly stimulated antennae (Cossé et al., 2002).

8-Acetyloxyoctanal and 10-acetyloxydecanal were also detected in volatiles collections but not from the lipid ozonolysis experiment. These compounds would be expected if the alkenediol diacetates oxidized according to the scheme for the alkenes and methyl oleate (but not from ozonolysis). Thus the pattern of aldehyde acetates observed in volatiles collections further supported the idea that they were formed by "weathering" of cuticular lipids. Interestingly, in tests with the synthetic standards, 8-acetyloxyoctanal and 10-acetyloxydecanal were clearly active by GC-EAD. Analogs with one additional and one fewer carbon would also be expected for the acetyloxyaldehydes with 13-, 15-, and 17-carbon chains, but these would have been present at extremely low levels and were not detected.

Under laboratory conditions, the amount of 9-acetyloxynonanal emitted from male sawflies was small (1–10 ng per sawfly per day) (Cossé et al., 2002) relative to the amounts of precursor alkenediol diacetates present in males (about 2 μg), but it was roughly proportional to the amounts of aldehydes apparently derived from 9-tricosene, further supporting a similar origin. In addition, the relatively greater production of 9-acetyloxynonanal from male sawflies than females (Cossé et al., 2002) was consistent with the greater amounts of the alkenediol diacetates present in males.

These qualitative and quantitative relationships strongly suggest that the alkenediol diacetates are the precursors to 9-acetyloxynonanal, a key compound in the sawfly volatiles collections that elicited intense antennal activity and had demonstrable activity in field tests (Cossé et al., 2002), and furthermore, that passive oxidation of the cuticular lipids could account for the amounts obtained in our volatiles collections from living sawflies.

Laboratory Oxidation of Alkenediol Diacetates. The synthetic alkenediol diacetate **10** was free of detectable acetyloxyaldehydes prior to artificial sunlight exposure. However, after 2 hr of irradiation, and as expected from our chemical model (Figure 6), 8-acetyloxyoctanal, 9-acetyloxynonanal, 16-acetyloxyhexadecanal, and 17-acetyloxyheptadecanal could be readily detected and were 0.09%, 0.4%,

0.3%, and 0.9% as abundant as the recovered starting material **10** (calculated from peak areas in the total ion chromatogram). 10-Acetyloxydecanal and 18-acetyloxyoctadecanal were not detected. An HPLC preparation of natural alkenediol diacetates, which contained **8** and **9** in an 8:1 ratio, was also free of detectable acetyloxyaldehydes before the light treatment. After 2 hr of light exposure, the results were analogous to synthetic **10:** 8-acetyloxyoctanal, 9-acetyloxynonanal, 12-acetyloxydodecanal, 13-acetyloxytridecanal, and 15-acetyloxypentadecanal were detected and were, respectively, 0.1%, 0.6%, 0.2%, 0.8%, and 0.2% as abundant as starting compound **8**. The experiment with synthetic **10** without exposure to light gave results similar to when the light was used, but the amounts of the acetyloxyaldehydes were lower (the acetyloxyaldehydes with 8-, 9-, 16-, and 17-carbon chains were 0.03%, 0.2%, 0.1%, and 0.4% as abundant as the starting material, respectively).

Although this experiment was preliminary, it clearly demonstrated that the biologically active acetyloxyaldehydes can be produced from the alkenediol diacetates in the absence of living sawflies. Furthermore, the amounts of acetyloxyaldehydes produced, relative to the amounts of parent alkenediol diacetates, were remarkably similar to those encountered with living sawflies (e.g., low nanogram quantities of acetyloxyaldehydes emitted per sawfly per day versus low microgram quantities of alkenediol diacetate present on the cuticle). Based on the GC-EAD studies of Cossé et al. (2002), the amounts of acetyloxyaldehydes produced by this process are large enough to be biologically relevant.

Behavioral and Environmental Enhancement of Oxidation Reactions. Certain features of sawfly biology would favor the oxidation of cuticular lipids. The insects are most active when the weather is warm (microclimate as hot as 35°C) and sunny (D. K. Weaver, unpublished). The oxidation reactions are accelerated by both heat and sunlight (Frankel, 1998, and our preliminary experiment). Sawfly grooming behavior (D. K. Weaver, unpublished) could distribute pheromonal precursors over the cuticle in a way that enhances oxidation rates. Thus it is possible that the reactions may be biologically (behaviorally) controlled even if there is no direct enzymatic mediation. The abiotic oxidation of unsaturates is complicated to study, especially in the early stages of the process, because it can be triggered or influenced by so many factors. These include the presence of trace amounts of metal ions, ketones, hydroperoxides, and other reaction-initiating substances, the action of ultraviolet light, and the effect of temperature (Frankel, 1998). On the other hand, involvement of enzymes has not been ruled out, and resolving this issue will be essential to understanding this pheromone system.

Pheromones Produced by Oxidation. The oxidation of relatively nonvolatile cuticular lipids is known to release volatiles with pheromonal activity in other hymenopterans. For example, females of the yellowheaded spruce sawfly, *P. alaskensis*, possess a series of (*Z*,*Z*)-9,19-alkadienes of 28–37 carbons that is absent in males (Bartelt et al., 1982). All of these dienes have a common oxidation

product, (*Z*)-10-nonadecenal, that is highly attractive to males in the field (Bartelt and Jones, 1983). Similarly, in the parasitic wasp, *Macrocentrus grandii* Goidanich (Braconidae), the females have a series of (*Z*,*Z*)-9,13-alkadienes of 27–41 carbons that the males lack (Swedenborg and Jones, 1992). With these dienes, the common oxidation product is (*Z*)-4-tridecanal, and as with *P. alaskensis*, the aldehyde is clearly attractive to males in the field. In laboratory wind-tunnel bioassays, as little as 17 pg of this aldehyde elicited dramatic upwind orientation.

C. cinctus differs in two important ways from the above examples. First and most obviously, the males are the major source of the key compounds (e.g., the diacetate lipids and the derived aldehyde acetates) instead of the females. Second, *both* sexes do have measurable amounts of the compounds, unlike the usual pattern with insect sex pheromones. Although the pheromone research on *C. cinctus* is in its early stages, the pheromone biology of the species already appears to be quite atypical and may provide a useful model for comparison in the future.

The existence of oxidation-derived pheromones in insects has not been frequently explored. The distinctive alkenediol diacetates of *C. cinctus* and their chemical relationship to the acetyloxyaldehydes through oxidation made this connection between nonvolatile precursors and volatile pheromones very clear, but as demonstrated with the alkenes, very typical compounds can also release volatiles that could be every bit as important biologically as the more esoteric ones. The reported examples of biologically active oxidation products are from the Hymenoptera, but it seems likely that the phenomenon could occur far more widely.

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