Full Papers

Composition and Electrophysiological Activity of Constituents Identified in Male Wing Gland Secretion of the Bumblebee Parasite *Aphomia sociella*

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Male wing gland secretion and volatiles emanating from calling males were investigated in the bumble bee wax moth, *Aphomia. sociella,* using comprehensive two-dimensional gas chromatography−mass spectrometry (GCxGC-TOF-MS), gas chromatography−infrared spectroscopy (GC-FTIR), enantioselective gas chromatography, electroantennography (EAG), gas chromatography with electroantennographic detection (GC-EAD), and NMR. GC-EAD analysis of the male wing gland secretion revealed seven active areas, corresponding to 1-hexanol (**1**), 2-phenylethanol (**2**), [(*R*),(*Z*)]-nona-2,6-dien-4-olide (**3**), [(*S*),(*Z*)]-nona-6-en-4-olide (**4**), mellein (**5**), phytone (**6**), and a mixture of C_{18} fatty acids (**7**). Solid-phase microextraction (SPME) confirmed the presence of 2-phenylethanol, nona-2,6-dien-4-olide, nona-6-en-4-olide, mellein, and phytone in volatiles emanating from a calling male. Though the abundance of these compounds slightly differed in SPME and gland secretion analysis, nona-2,6-dien-4-olide and mellein dominated in both samples, followed by 2-phenylethanol, nona-6-en-4-olide, and phytone. The strong antennal responses elicited by components of the secretion suggest that one or more of these compounds constitute the sex pheromone. Both sexes perceived male wing gland secretion, with females being significantly more sensitive compared to males.

Bumblebees are traditionally reared for agricultural use as pollinators in greenhouses. Due to the recent massive decline in domesticated honeybee populations, bumblebees also represent an alternative for pollination in open fields. Similar to honeybees, bumblebee colonies suffer diseases and parasite attacks.

Bumble bee wax moth, *Aphomia sociella* L., is a parasite of wild social bees, wasps, and especially bumblebees. The larvae destroy the host nest by consuming the brood comb and the offspring. Unlike most Lepidoptera, reproductive behavior of *A. sociella* and other members of the subfamily Galleriinae (family Pyralidae) is initiated by males and includes both ultrasonic and pheromonal signals.[1−5] Sex pheromone is produced in male-specific glands located at the forewing base.[6] Males release the pheromone during calling in scotophase. The calling behavior is characterized by stationary wing-fanning associated with ultrasound production and pheromone release.[7] In nature, males call presumably in the vicinity of bumblebee nests. Similar to other wax moths, female close-range approaching behavior to calling males observed in the laboratory is nontypical. They may directly contact the male, wing-fan, and/or circle in close proximity, or remain stationary until the male initiates a courtship.[3,8,9] In *A. sociella,* the female dependence on chemical and acoustic signals has not been clarified. The dependence of mating partners on chemical and acoustic communication varies in different species of Galleriinae. Female *Galleria mellonela* approach the pheromone source in the absence of acoustic stimulation; however, the searching behavior is more efficient when sound is present.[10] Deaf *Achroia grisella* are unable to respond to a calling male in spite of the fact that pheromone is released.[1] Lures impregnated with gland extract were ineffective attractants for *Eldana saccharina* in the absence of sound.[11] In closely related *A. gullaris,* racemic (Z)- nona-2,6-dien-4-olide (major pheromone component) was ineffective to attract females,[12] and although the individual enantiomers have been synthesized,[13] their semiochemical importance is still unknown. In a study on gland extracts and volatiles released by calling *A. sociella* males, (*Z*)-nona-2,6-dien-4-olide (**3**, absolute configuration undetermined) and (*R*)-3,4-dihydro-8-hydroxy-3-methylisocoumarin [(*R*)-(−)-mellein] (**5**) were identified.[4] It was also noted that (*R*)-(−)-mellein (5 μg) tested on 2-day-old females evoked the same searching behavior as crude extract; however, no data were provided.

In search of an efficient monitoring and/or control method for *A. sociella*, it is essential to unravel unambiguously what sensory modality governs the premating behavior, necessitating a thorough examination of both chemical and acoustic signals. Here we report a detailed analysis of male wing gland secretion and male emanations using comprehensive GCxGC-TOF-MS, GC-FTIR, GC-EAD, EAG, enantioselective gas chromatography, and NMR spectroscopy.

Results and Discussion

The electroantennographic comparison of sensitivity of male and female antennae to male wing gland secretion showed that both sexes do perceive the pheromone. The responses of female and male antennae to air, *n*-hexane, the male wing gland extract, and standards indicated that female antennae were more sensitive to all stimuli than the male ones (Figure 1). Two gland equivalents and enantiomerically pure standards of lactone **3** elicited significantly higher EAG responses above controls (air and *n*-hexane) in both males and females. The antennal sensitivity of both sexes to male pheromone is in agreement with observed premating behavior in *A. sociella*, with females being attracted to procreate,[14,15] while males accumulate in groups or compete with one another.[9]

GC-EAD analyses (female antennae) of *A. sociella* male wing gland secretion showed seven EAD-active chromatographic peaks (Figure 2) corresponding to 1-hexanol (**1**), 2-phenylethanol (**2**), [(*R*),(*Z*)]-nona-2,6-dien-4-olide (**3**), [(*S*),(*Z*)]-nona-6-en-4-olide (**4**), mellein (**5**), phytone (**6**), and a mixture of octadecatrienoic,

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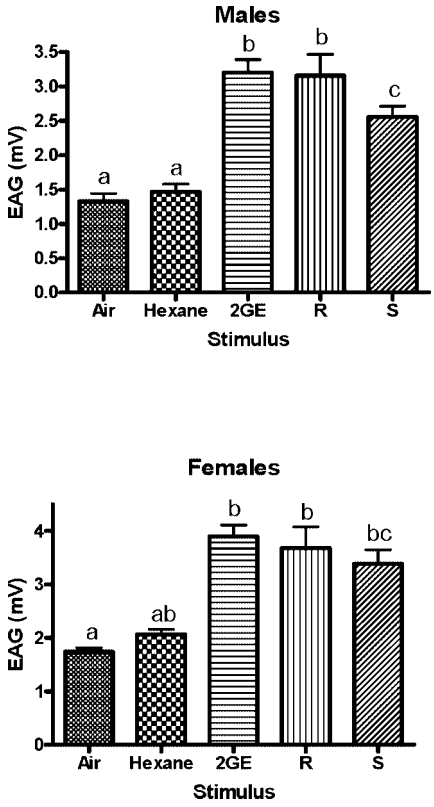


Figure 1. EAG testing of the male wing gland secretion (equivalent of 2 glands, 2GE), (*Z*)-nona-2,6-dien-4-olide (**3**, *R* and *S* enantiomers), with air and *n*-hexane as controls. Different letters above bars represent significant differences. Responses of male antennae (a) to all stimuli were significantly different ($P = 0.05$) from those of female antennae (b).

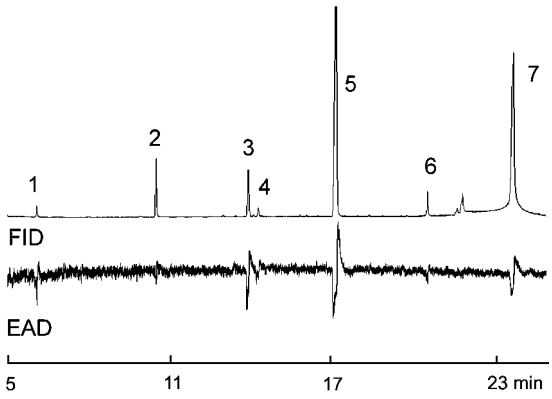


Figure 2. GC-EAD analysis of the male wing gland extract: the upper chromatogram represents FID response; the lower trace shows antennal response of an *A. sociella* female. The active peaks correspond to compounds **1**−**7** listed in the Results and Discussion.

octadecadienoic, and octadecenoic acids (**7**), respectively (Table 1). The identification was based on GCxGC-TOF-MS and GC-FTIR spectra, comparison of Kováts indices,[16,17] literature spectroscopic data, and synthetic standards. In addition, NMR spectra of the main component, mellein (**5**), were measured. Other compounds were not available in sufficient amounts for purification or NMR measurements.

Compounds **1** and **2** were identified as 1-hexanol (mass spectrum: *m*/*z* 84, M − H_2O) and 2-phenylethanol (mass spectrum: *m*/*z* 122, M^+; 91, $C_7H_7^+$), respectively, by comparison with authentic samples. Both compounds were present in the gland extract, but

Table 1. Comparison of Wing Gland Secretion and SPME of Volatiles Emanating from Calling Males

compound	Kováts index	relative abundance in extract, median (standard deviation) [%, $N = 3$]	relative abundance in SPME, median (standard deviation) [%, $N = 3$]
1-hexanol (**1**)	853	5.1 (1.6)	not present
2-phenylethanol (**2**)	1124	8.0 (1.6)	20.6 (1.4)
(*Z*)-nona-2,6-dien-4-olide (**3**) [90% ee for (*R*)]	1343	20.7 (1.1)	46.3 (8.5)
(*Z*)-nona-6-en-4-olide (**4**) [>99% ee for (*S*)]	1366	2.3 (0.3)	0.9 (0.7)
mellein (**5**)	1583	58.4 (0.5)	31.5 (9.0)
phytone (**6**)	1853	3.1 (0.7)	0.5 (0.2)
octadecadienoic acid[a]	2135	0.4 (0.2)	not present
octadecatrienoic acid[a]	2140	1.8 (1.4)	not present
octadecenoic acid[a]	2140	0.8 (0.7)	not present
octadecanoic acid[a]	2140	0.3 (0.2)	not present

[a] The acid ratios were verified after diazomethane methylation of the samples.

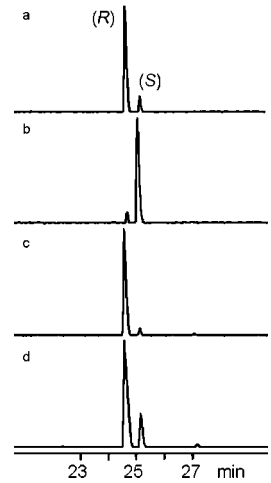


Figure 3. Enantioselective separation of (*Z*)-nona-2,6-dien-4-olide (**3**): (a) synthetic (*R*)-**3**; (b) synthetic (*S*)-**3**; (c) male wing gland extract, (d) co-injection of the male wing gland extract with synthetic (*S*)-**3**.

1-hexanol (**1**) was absent in the SPME sample. Compounds **1** and **2** are ubiquitous plant volatiles of various taxa and genera[16] semiochemicals in many insect species.[16] Particularly interesting within the context of our work is the occurrence of 2-phenylethanol (**2**) in male scents of several Lepidopteran species.[18,19] Within the subfamily Galleriinae, 2-phenylethanol (**2**) was reported as a component of the male wing gland pheromone in *A. gularis*.[12]

Compound **3** was identical with synthetic (*Z*)-nona-2,6-dien-4-olide[13] based on mass spectra (M^+ 152), infrared spectra (3018 cm^{-1}, =C−H; 1806 cm^{-1}, C=O in five-membered unsaturated lactone ring), and chromatographic properties. Enantioselective chromatography revealed an enantiomeric excess of 90% for the *R*-enantiomer in the gland secretion (Figure 3).

Compounds **3** and **4** displayed similar mass spectra to that of **4**, indicating saturation of one of the double bonds (M^+ 154). The carbonyl band in the IR spectrum of **4** (1813 cm^{-1}) suggested a saturated lactone, which was confirmed through comparison with commercial nonan-4-olide (ν_{CO} 1812 cm^{-1}). Absence of a band at 970 cm^{-1} pointed to *Z*-configuration of the double bond in the aliphatic chain. The structure was confirmed by reduction of **3** using $NaBH_4$ in the presence of $CuCl_2$, yielding **4** (Figure 4). The absolute configuration of **4** was determined as *S* (ee >99%), based on analogy with commercially available nonan-4-olide[20] and comparison of the reduction product of **3** on a chiral column.

The γ-lactones identified in the male wing gland secretion are relatively rare floral compounds.[21] In insects, (*Z*)-nona-2,6-dien-

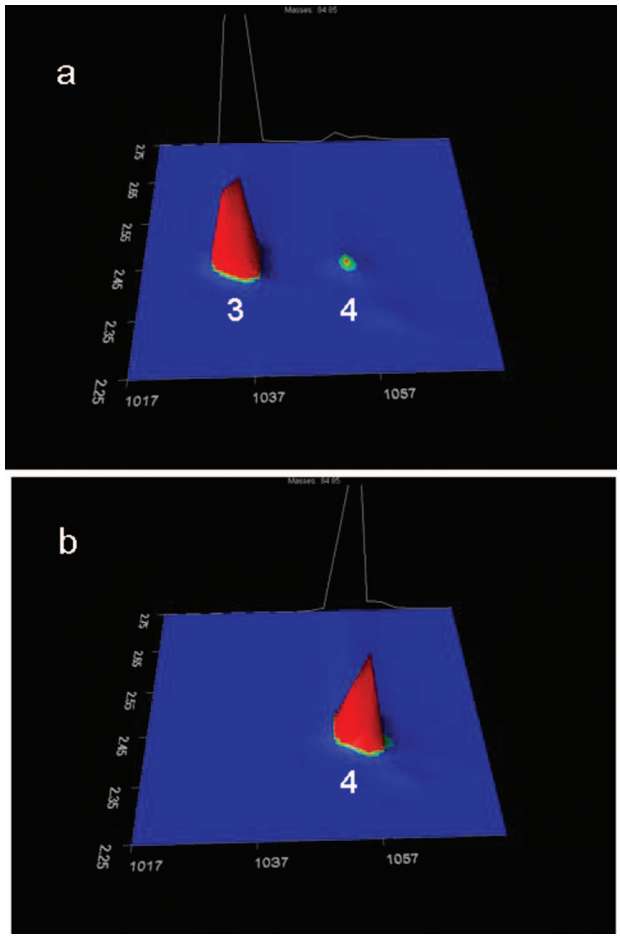
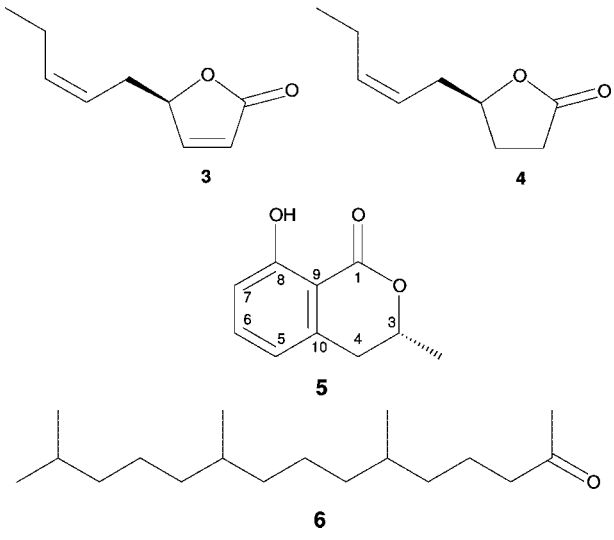


Figure 4. Reduction of (*Z*)-nona-2,6-dien-4-olide (**3**) and formation of (*Z*)-nona-6-en-4-olide (**4**). Two-dimensional gas chromatogram (GCxGC-MS) shows the area of lactones **3** and **4**: (a) male wing gland extract; (b) product of the reduction. *x*-Axis shows the elution time in the first dimension; *y*-axis represents elution in the second dimension.



4-olide (**3**) was reported as a major constituent of the male wing gland pheromone in a closely related species, *Aphomia gularis*,[12] and as a minor constituent in the analysis of the *A. sociella* pheromone.[4] In addition, lactone **3** was found in male scents of African milkweed butterflies (Danainae).[22] Here we report for the first time the absolute configuration of **3** and **4** identified in insects.

Compound **5** (main component of both the wing extract and SPME) was identified as mellein based on its spectroscopic data.[23] Its mass spectrum displayed a molecular ion at *m*/*z* 178. The IR spectrum showed characteristic absorption bands for a hydrogen-bonded hydroxy group (3146−3407) and carbonyl (1707), aromatic C=C (1589 and 1620), deformation CH_2 (1466) and CH_3 (1388), C_{arom}−O (1220), and lactone C−O−C (1100 cm^{-1}) functional groups. The ^1H NMR spectrum showed the presence of a $-CH_2-CH(CH_3)-O-$ fragment (δ 2.93 dt, 2H; δ 4.74 m, 1H; δ 1.53 d, 3H), three vicinal aromatic protons (δ 6.69, 6.89, and 7.41), and a downfield hydroxy proton resonance (its significantly low-field position δ 11.03 indicates a hydrogen-bonded phenolic hydroxy group). The ^{13}C NMR spectrum confirmed the presence of a $-CH_2-CH(CH_3)-O-$ fragment (δ 34.59, 76.08, and 20.76), six aromatic carbons of a trisubstituted aromatic ring (three methine carbons at δ 116.23, 117.87, and 136.13 and three quarternary carbons at δ 108.26, 139.36, and 162.16), and a lactone carbonyl carbon (δ 169.95). Assignment of all proton and carbon signals was achieved through 2D NMR analysis (Figure 5) and is reported here for the first time.[23]

Mellein (**5**) was originally identified as a phytotoxic compound produced by deuteromycetous fungal species *Aspergillus ochraceus* and *A. melleus*.[24] Subsequently, mellein was isolated from various insect species, where it serves functions such as sex pheromone or a defense substance in termites,[25] trail or alarm pheromones in ants,[26] or an alarm signal of Thysanoptera.[27] In the giant danaine butterfly, *Idea leuconoe*,[28] and *Grapholita molesta*,[29] mellein is released by courting males and serves as an aphrodisiac. It has been hypothesized that in *A. sociella* mellein is produced by fungi in the moth's gut and is subsequently sequestered by the gland to become a pheromonal component.[4]

Compound **6**, present in SPME and extract, was found to be phytone by comparison with a commercially available standard [M^+ 268, band of carbonyl group (1733 cm^{-1}) in the IR spectrum]. Phytone is an abundant floral compound in Orchidaceae, Asteraceae, and Hypericaceae (for a complete list see ref 16). Within the subfamily Galleriinae, phytone was found in the male wing gland pheromone of *Galleria mellonella*[30] and *Tirathaba mundella*.[31]

The GC peak at 23.46 min (**7**) was present only in the gland extract. It was identified as an inseparable mixture of C_{18} fatty acids. Esterification with diazomethane permitted the determination of the acid ratios, but the double-bond positions in the unsaturated acids were not established. GC-EAD analysis of commercial linolenic and oleic acid indicated overlapping retention time with EAD active area (**7**). EAG tests performed with acid standards revealed that 10 μg doses of linolenic and oleic acid elicited weak responses in *A. sociella* antennae (data not shown). Fatty acids were not detected in SPME sampling, suggesting they may not be part of pheromonal communication but rather extracted from membrane or body lipids.

SPME experiments provided evidence that most compounds extracted from the glandular secretions are released in detectable quantities by calling male moths. When the results from extraction and SPME were compared, qualitative and quantitative differences were observed (Table 1). The main component in both gland extract and SPME was mellein (**5**) (58% and 40% in extracts and SPME, respectively) followed by lactone **3** (21% and 58%) and 2-phenylethanol (2) (8% and 28%). The results reflect differences in composition of gland content versus emanations and differences in volatilities as well as in extraction selectivity of *n*-hexane versus the polydimethylsiloxane fiber. In SPME, the quantity and quality can also be affected by the sampling time.

The EAG responses to enantiomers of lactone **3** disclosed that both male and female antennae were more sensitive to the *R*- than to the *S*-enantiomer. This result is in agreement with the enantiomeric composition of lactone **3** in the gland extract (Figure 1).

Advances in insect semiochemical research, such as GC-EAD, has allowed for more detailed analyses of pheromones and glandular

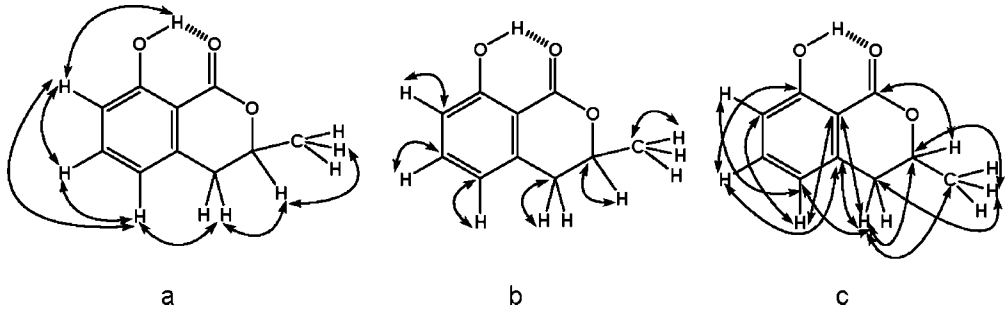


Figure 5. Structure of mellein. The arrows indicate (a) J(H,H) couplings observed in the 2D-H,H-COSY spectrum; (b) ^1J(C,H) couplings detected in the 2D-H,C-HMQC spectrum; (c) ^2J(C,H) and ^3J(C,H) couplings detected in the 2D-H,C-HMBC spectrum.

secretions.[4] Our data show that the composition of *A. sociella* male wing gland secretion and emanation is more complex than previously reported.[4] However, not all compounds present in the male wing gland extract or gland effluvia are necessarily part of the male pheromone signal.[32] Only behavioral experiments can determine which of the identified compounds constitute the sex pheromone in *A. sociella*.[32] Prior to the development of such behavioral tests, however, female dependence on male sound production has to be clarified. The recorded sound, in conjunction with the identified semiochemicals, should be used in bioassays to determine the true composition of the male *A. sociella* sex pheromone.

Experimental Section

Insects. Laboratory-started nests of buff-tailed bumblebee (*Bombus terrestris*)[33] were exposed to colonization by *A. sociella* in the field.[4] Larvae were allowed to develop and pupate in bumblebee hives. The newly emerged adults were collected and sexed. The sexes were kept separately in the dark under ambient laboratory temperature (20−23 °C). Adults used in the electrophysiological experiments were moved to a refrigerator with the temperature set to 5 °C one day postemergence and kept there in the high-humidity environment (90%) until used.

Chemicals. Commercially available standards were purchased from the following sources: hexan-1-ol, 2-phenylethanol, phytone (perhydrofarnesylacetone), nonan-4-olide, and $NaBH_4$ from Aldrich; linoleic acid from Fluka; stearic acid, linolenic acid, and oleic acid from Reachim; a series of *n*-alkanes to determine Kováts indices from Sigma. Standards and *n*-alkanes were dissolved in redistilled *n*-hexane (Merck) and co-injected with authentic samples. *R*- and *S*-Enantiomers of nona-2,6-dien-4-olide were kindly provided by Prof. K. Mori (University of Tokyo, Japan).

Pheromonal Gland Extraction. Wing glands of calling males were clipped out, soaked in redistilled *n*-hexane (Merck, 10 μL per gland), sonicated for 15 min, and extracted for 24 h. The extracts were filtered through purified glass wool, concentrated to double in initial concentration under argon flow, and stored at −20 °C.

Solid-Phase Microextraction (SPME). SPME using PDMS (polydimethylsiloxane, 100 μm) fiber (Supelco) was performed to determine which compounds of the male wing gland secretion are present in headspace volatiles. SPME fiber was exposed to a calling male located in a glass chamber for 20 min and analyzed using GCxGC-TOF-MS.

Electroantennographic Recordings (EAG). EAG was performed on isolated antennae. Glass Ag/AgCl microelectrodes filled with Ringer solution were used. Cut antennal tips were connected to a high-impedance amplifier (1014 ohms Syntech), and the signal was fed into a PC and analyzed using Syntech EAG software. A reference electrode was slipped over the antennal base and grounded. The antennal preparation was placed into a stream of clean air into which odor stimuli were injected from odor cartridges (1 mL of respective volatile within 1 s). The odor cartridges consisted of Pasteur pipettes containing filter paper discs (1 cm diameter), to which volatile solutions were applied. After solvent evaporation, the pipettes were sealed with Parafilm and stored in the freezer until use. Five cartridges were prepared for each stimulus type. Prior to each experiment, the Pasteur pipettes were removed from the freezer and allowed to equilibrate to ambient temperature for an hour. At least 10 antennae were used for each treatment. Stimuli were delivered at 1 min intervals, and the maximum of the EAG amplitude was evaluated. Results were subjected to statistical analysis using Student's t test. Using EAG we compared the antennal sensitivity of both sexes to the male wing gland extract (20 μL, 2 gland equivalents), the *R*- and *S*-enantiomers of nona-2,6-dien-4-olide (0.4 μg), and fatty acids (namely, stearic acid, linoleic acid, linolenic acid, and oleic acid; 10 μg). Significance of differences in responses was statistically evaluated by one-way ANOVA ($P = 0.05$).

Gas Chromatography with Electroantennographic Detection (GC-EAD). In GC-EAD experiments, isolated antennae of 2−7-day-old males and females were used as biological detectors in parallel to FID. Analyzed samples were injected splitless into a 5890A Hewlet-Packard gas chromatograph equipped with an Rxi-5Sil MS (Restek; 30 m × 0.25 μm i.d. × 0.25 μm film) column. The column was split at the end by a Graphpack 3D/2 four-arm splitter to divide the eluate to FID and EAD detectors. The GC was operated at an initial temperature of 50 °C for 2 min, then ramped up at a rate of 10 °C/min to 270 °C (with a 10 min hold). The temperature of the GC inlet and detector was set to 200 and 260 °C, respectively. A series of saturated C_8−C_{22} *n*-alkanes was co-injected with analyzed samples to allow for the calculation of Kováts indices (KI) of EAD active peaks and to check the correspondence of retention behavior and antennal activity of authentic compounds with synthetic standards.

Gas Chromatography−Mass Spectrometry (GCxGC-TOF-MS). Analyses were performed using a LECO Pegasus 4D instrument (LECO Corp., St. Joseph, MI) equipped with a nonmoving quad-jet cryomodulator. A slightly polar Rxi-5 Sil MS column (Restek, 30 m × 250 μm i.d. × 0.25 μm film) was used for GC in the first dimension. The second dimension analysis was performed on a polar BPX-50 column (SGE Inc., Austin, TX; 2 m × 100 μm i.d. × 0.1 μm film). Helium was used as a carrier gas at a constant flow of 1 mL/min. The temperature program for the primary GC oven was as follows: 40 °C for 2 min, then 40 to 320 °C at 10 °C/min, and finally 5 min hold at 320 °C. The program in the secondary oven was 5 °C higher than in the primary one and was operated in an iso-ramping mode. The modulation period, the hot-pulse duration, and the cool time between stages were set at 3.0, 0.6, and 0.9 s, respectively. The transfer line to the ion source was kept at 250 °C. The source temperature was 220 °C with a filament bias voltage of 70 eV. The data acquisition rate was 100 Hz (scans/s) for the mass range of 29−400 amu. The detector voltage was 1450 V. Samples (1 μL) were manually injected in splitless mode. The inlet temperature was 250 °C. The purge time was 30 s at a helium flow of 80 mL/min. Data were processed and consecutively visualized on the 2D and 3D chromatograms using the advanced LECO ChromaTOFTM software. As in GC-EAD experiments, saturated C_8−C_{22} hydrocarbon standards were analyzed under the same parameters to allow the comparison of Kováts indices (KI) with GC-EAD experiments.

Gas Chromatography−Infrared Spectroscopy (GC-FTIR). Infrared spectra were recorded in gas phase using an Agilent 6850 gas chromatograph connected to an Equinox 55 FT-IR spectrometer (Bruker Optics Inc., Ettlingen, Germany). A J&W Scientific column (30 m × 0.32 mm, film thickness 0.25 μm) was used for the separation. Injector temperature was 220 °C (splitless mode). Carrier gas was helium at a flow rate of 1.5 mL/min. The temperature program was set as follows: 40 °C for 2 min, then 5 °C/min to 240 °C (5 min). A liquid-nitrogen-cooled photoconductive mercury−cadmium−telluride (MCT) detector was used with FT-IR resolution of 8 cm^{-1}; light pipe temperature was 200 °C.

NMR spectra were recorded on a Bruker 600 AVANCE II spectrometer (^{1}H at 600.13 MHz and ^{13}C at 150.92 MHz) at 27 °C in $CDCl_3$ and referenced to the signal of solvent (7.26 ppm ^{1}H and 77.0 ppm ^{13}C). The homo- and heteronuclear 2D NMR spectra (2D-H,H-COSY, 2D-H,C-HSQC, and 2D-H,C-HMBC) were used for the structural assignment of proton and carbon signals.

Enantioselective Analysis. The absolute configuration of **3** and **4** was determined using a Thermo FOCUS GC connected to a Fisons MD-800 quadrupole mass spectrometer controlled with Xcalibur software. The analysis was performed on a heptakis(2,3-di-*O*-acetyl-6-*O*-TBDMS)-β-cyclodextrin column (60% in polysiloxane PS-268, 30 m × 250 μm i.d., a gift from the late Prof. W. König). Helium was used as a carrier gas at a constant flow rate of 1.5 mL/min. The temperature program was 70 °C for 2 min, then 2 °C/min to 150 °C and 10 min hold. The transfer line to the MS was heated to 150 °C. The source temperature was 150 °C with a filament bias voltage of 70 eV. The data acquisition rate was 1 scan/s for the mass range of 30−500 amu. Samples (1 μL) were manually injected in splitless mode. The inlet temperature was 150 °C. The purge time was 60 s at a helium flow of 20 mL/min. The elution order of the respective enantiomers was ascertained using synthetic *S*- and *R*-enantiomers of **3**. The elution order of enantiomers of **4** was determined according to the literature[20] and from the reduction product of **3**.

Esterification. Direct esterification of fatty acids from the male gland extract was performed to enable the fatty acid partial identification and quantification. Diazomethane in ether solution was added to the sample, and the products were subjected to GC-MS analysis.

1-Hexanol (1). Mass spectrum, *m/z*: 84 (M − H_2O) 56 (b.p.), 31 (CH_3O^+); KI 853.

2-Phenylethanol (2). Mass spectrum, *m/z*: 122 (M^+), 104 (M − H_2O), 91 (b.p., $C_7H_7^+$), 77, 65, 51. IR spectrum, ν_{max}: 3667 (OH), 3074, 3037 (aromatic C−H), 1047 (C−O), 742, 698 (aromatic C−H, deformation) cm^{-1}. KI 1124.

[(*R*),(*Z*)]-Nona-2,6-dien-4-olide (3). Mass spectrum, *m/z*: 152 (M^+), 107, 95, 84, 69, 55, 41 (b.p.). IR spectrum, ν_{max}: 3018 (=C−H), 1806 (C=O), 1147 (C−O) cm^{-1}. KI 1343. Elution order on chiral column: t_R 24.5 min *R*-enantiomer, t_R 25.2 min *S*-enantiomer. Enantiomeric excess: 90% (*R*).

[(*S*),(*Z*)]-Non-6-en-4-olide (4). Mass spectrum, *m/z*: 154 (M^+), 136, 94, 85, and 57. IR spectrum, ν_{max}: 3015 (=C−H), 1813 (C=O), 1161 (C−O) cm^{-1}. KI 1366. Elution order on chiral column: t_R 22.9 *S*-enantiomer, t_R 23.5 *R*-enantiomer. The *S*-enantiomer dominated; only a trace of *R*-enantiomer was detected. The accurate enantiomeric ratio in the sample could not be determined due to a low concentration.

Since a standard of this compound was not available, we prepared lactone **4** by the partial reduction of lactone **3** present in the gland secretion. The reduction was performed using a modified procedure of Dankwardt and co-workers.[34] The wing gland extract was dissolved in MeOH (0.7 mL), and a solution of $CuCl_2 \cdot 2H_2O$ (0.19 mg, 2 equiv) in MeOH (100 μL) and excess $NaBH_4$ (20 equiv) in MeOH (100 μL) were added. The reaction mixture was stirred at room temperature overnight. The resulting product (without purification, 1 μL) was analyzed by GCxGC-TOF-MS.

Mellein (5) was purified from the gland extract using preparative TLC. The crude extract was separated on precleaned glass plates (36 × 76 mm) coated with 0.2 mm of Adsorbosil-Plus (Applied Science Laboratories; with 12% gypsum) using an *n*-hexane/Et_2O/HCO_2H (80:20:1) mobile phase. TLC zones were made visible under UV light (254 nm). The zone of mellein (R_f = 0.3) was scraped off the TLC plate and eluted with diethyl ether. Mass spectrum, *m/z*: 178 (M^+), 160 (M − H_2O), 134 (b.p.), 106, 78, 51. IR spectrum, ν_{max}: 3100 broad weak (OH, intramolecular bridge), 1707 (C=O), 1589, 1620 (aromatic C=C), 1466 (CH_2 deformation), 1388 (CH_3 deformation), 1220 (C_{arom}−O), 1100 (C−O−C lactone) cm^{-1}. ^{1}H NMR (600.13 MHz; in $CDCl_3$): 1.53 (3H, d, J = 6.3 Hz, CH_3), 2.93 (2H, dm, J = 6.9, 1.0, 0.7 Hz, H-4); 4.74 (1H, tq, J = 6.9, 6.3 Hz, H-3); 6.69 (1H, dq, J = 7.4, 1.0 Hz, H-5); 6.89 (1H, m, J = 8.4, 1.0, 0.7, 0.5 Hz, H-7); 7.41 (1H, dd, J = 8.4, 7.4 Hz, H-6); 11.03 (1H, d, J = 0.5 Hz, OH). ^{13}C NMR (150.92 MHz; in $CDCl_3$): 20.76 (CH_3), 34.59 (C-4), 76.08 (C-3), 108.26 (C-9), 116.23 (C-7), 117.87 (C-5), 136.13 (C-6), 139.36 (C-10), 162.16 (C-8), 169.95 (C-1). KI 1585.

Phytone (6). Mass spectrum, *m/z*: 268 (M^+), 250 (M − H_2O), 58, 43 (b.p.). IR spectrum, ν_{max}: 1733 (C=O) cm^{-1}. KI 1853.

C18 Acids (7). 7 was an inseparable mixture of octadecatrienoic (M^+ 278), octadecadienoic (M^+ 280), octadecenoic (M^+ 282), and octadecanoic (M^+ 284) acids. After esterification, the proportion of methyl esters was determined as 11:1.1:4.4:1. The IR spectrum confirmed *Z*-configurations of double bonds: ν_{max} 3577 (OH), 3019 (=C−H), 1778 (C=O), 1126 (C−O), 715 (H−C=C−H, *Z*-configuration) cm^{-1}.

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