J Chem Ecol (2009) 35:1202–1211
DOI 10.1007/s10886-009-9693-6

Monoalkenes as Contact Sex Pheromone Components of the Woodwasp *Sirex noctilio*

Katalin Böröczky · Damon J. Crook · Tappey H. Jones · Joshua C. Kenny · Kelley E. Zylstra · Victor C. Mastro · James H. Tumlinson

Received: 14 April 2009 / Revised: 14 August 2009 / Accepted: 15 September 2009 / Published online: 3 October 2009
© Springer Science + Business Media, LLC 2009

Abstract A pheromone on the cuticle of females of the woodwasp *Sirex noctilio,* a recently introduced pest of pines in North America, induces conspecific males to attempt copulation. Dead females washed with hexane did not elicit copulation attempts from males, whereas reapplication of a female hexane body wash onto the cuticle of dead females elicited copulation attempts by 65% of males tested. Analysis of the hexane extract revealed saturated and unsaturated hydrocarbons as major components of the female cuticle. Behavior-guided fractionation of the female body wash led to the identification of three components, (*Z*)-7-heptacosene, (*Z*)-7-nonacosene, and (*Z*)-9-nonacosene, of the sex pheromone of *S. noctilio* that elicited copulatory responses from males.

K. Böröczky · J. H. Tumlinson
Center for Chemical Ecology, Department of Entomology,
The Pennsylvania State University,
University Park, PA 16802, USA

D. J. Crook · V. C. Mastro
USDA, APHIS, PPQ, CPHST, Otis Laboratory,
Bldg. 1398, W. Truck Rd.,
Buzzards Bay, MA 02542, USA

T. H. Jones · J. C. Kenny
Virginia Military Institute Chemistry Department,
Lexington, VA 24450, USA

K. E. Zylstra
USDA, APHIS, PPQ, CPHST,
374 Northern Lights Drive,
North Syracuse, NY 13212, USA

Present Address:
K. Böröczky (✉)
Department of Entomology, North Carolina State University,
Box 7613, Raleigh, NC 27695, USA
e-mail: katalin.by@gmail.com

Keywords *Sirex noctilio* · Mating behavior · Cuticular lipids · Contact sex pheromone components · Hymenoptera · Siricidae

Introduction

The woodwasp *Sirex noctilio*, Fabricius (Hymenoptera: Siricidae) is native to southern Europe, North Africa, and parts of Asia. It was first detected as a pest at the beginning of the last century in New Zealand and has since spread to Australia, South America, South Africa, and North America (Hoebeke et al. 2005; Hurley et al. 2007). Four major host species in Canada and the Northeastern United States are Jack pine (*Pinus banksiana*), Scots pine (*P. sylvestris*), red pine (*P. resinosa*), and white pine (*P. strobes*). Female wasps attack weakened, stressed trees and deposit up to 400 eggs in the outer sapwood of the host tree (Madden 1981). The trees eventually die due to the disruption of their vascular system caused by the mucus and fungus injected by the female woodwasp when probing or ovipositing (Coutts and Dolezal 1969; Fong and Crowden 1973). Adults typically emerge the following year, but the larvae can spend 2 or 3 years in the tree making galleries as they feed (Taylor 1981).

Observing the mating behavior of *S. noctilio* in the wild is especially challenging since mating occurs in the upper canopy. Males emerge first, rest, and then move up to the canopy. Although they perform periodic short flights, they typically stay close to the site of emergence. Females emerge a few days later, after which they disperse (Morgan and Stewart 1966). The sex ratio of emerging wasps largely favors males, sometimes being as high as about ten to one (Morgan 1968). Morgan and Stewart (1966) reported that when a female lands on a tree, she starts ovipositing while

walking up the tree bole. They suggested that the female initiates courtship by walking close to a male on the upper branches of the canopy. The male follows the female, tapping her body with the forelegs and the antennae. This description by Morgan and Stewart (1966) strongly suggested the presence of a contact signal on the female cuticle.

In a preliminary study in the laboratory, *Sirex noctilio* males, when presented with freeze-killed females, responded with abdominal bending but did not do so after the cuticular coating of the dead females was removed by dipping the female in hexane (D.J.C., unpublished data). These preliminary results indicated the relevance of female cuticular compounds in the mating behavior. This is further supported by the work of Crook et al. (2008), who reported a high abundance of contact chemoreceptors on the antennae of *S. noctilio*.

Cuticular lipids are key compounds involved in the recognition of mates, caste, and kin, in a number of insect species (Howard 1993; Wyatt 2003). Hydrocarbons of the epicuticle, in particular, have been described as recognition cues, kairomones, chemical mimicry agents, and sex pheromone components throughout the insect taxa (Blomquist et al. 1993; Howard and Blomquist 2005). While semiochemicals of hymenopterans in the sub order Apocrita frequently are reported, we know much less about the chemical ecology of insects in the other sub order, Symphyta, in which the family Siricidae belongs (Ayasse et al. 2001; Keeling et al. 2004). The primary focus of this study was to analyze the cuticular lipid coating of male and female *S. noctilio*, and to identify the contact pheromone that induces males to attempt copulation with females.

Methods and Materials

Insects Adults were reared out of Scots pine logs harvested near Syracuse, NY, USA. The first batch of infested trees were taken down in December 2007 and cut into approximately 60 cm-long logs. The logs were kept in barrels with a mesh lid at 25°C at the USDA PPQ rearing facility in Syracuse. Emergence of males started at the end of February 2008, followed thereafter by the emergence of females in March. A second batch of logs was taken into the rearing facility in May 2008. Emergence started in July and lasted until the beginning of August. Barrels were checked twice a week, and emerging insects were identified and separated by sex. Males and females were kept individually in screw cap glass jars, with a piece of paper towel inside, until used.

Solvent Dipping For the analysis of cuticular lipids, insects were freeze killed at age 1–6 days. On the day of analysis, bodies were thawed for 30 min at room temperature. Each wasp was placed individually into a 10 ml screw cap vial and extracted × 3, for 2 min, with 3 ml of hexane (Burdick & Jackson, 95%, Morristown, NJ, USA). The extracts were combined in a separate 10-ml screw cap vial. Solutions were held at 4°C until subsequent fractionation or analysis. For analysis, the body wash was evaporated under a gentle stream of nitrogen. The residue was redissolved in 300 µl of a 50 ng/µl solution of 16-methyl hexatriacontane in hexane.

For the bioassay experiments, 1 to 6-d-old females were freeze killed the night before the experiment. The bodies were thawed for 30 min at room temperature. Extraction was performed as described above. The extracts were combined in a 10-ml screw cap vial, and the body wash was evaporated under a gentle stream of nitrogen. Samples were redissolved with the appropriate amount of hexane to give body wash solutions of 1/3, 1, or 3 female equivalents (FE) per 100 µl. Washed females were dried in a fume hood for 30 min before application of any treatment.

Analysis of the Cuticular Washes Amounts of compounds were quantified with an Agilent 6890 gas chromatograph-flame ionization detector (GC-FID) system equipped with an Equity-5 column (30 m×0.2 mm×0.2 µm; Supelco, Bellefonte, PA, USA). Quantification of compounds was based on their peak area values obtained from the data acquisition and analysis software (Chemstation D.01.00, Agilent). The output values were corrected with the relative response factors to calculate percentage composition and amounts of the identified compounds (Böröczky et al. 2008). For identification, selected samples were injected into an Agilent 6890N GC, equipped with an identical Equity-5 column, coupled with a 5973N Mass Selective Detector (MSD) in EI mode (+70 eV). For all the analyses, the oven temperature program was initially 50°C (held for 1 min) then programmed to 210°C at 20°C min^{-1}, then to 320°C at 3°C min^{-1} (and held for 25 min). The injector temperature was 280°C, in both systems, and the FID (in the GC) and transfer line (in the GC-MS) temperatures were 300°C. Samples were injected splitless (purge set at 0.75 min), and the carrier gas was helium at an average linear flow velocity of 25 cm s^{-1}.

Identification of the compounds was based primarily on their EI mass spectra (NIST05, Masslib) and their Kovats indices on the Equity-5 column (Van Den Dool and Kratz 1963; Kovats 1965). Position of the methyl branching of mono-, and dimethylalkanes was determined by using characteristic even- and odd-mass fragments of their respective mass spectra (Nelson, 2001) as well as by calculated retention indices (Carlson et al., 1998). Position of double bonds was determined by examining the mass spectral fragmentation of the dimethyl disulfide (DMDS)

adducts of the alkenes (Francis and Veland 1981; Vincenti et al. 1987). Identification of wax esters is tentative based on their molecular weight and the weight of the $[RCO\text{-}1]^+$ and $RCOOH_2^+$ fragments from their mass spectra. High resolution MS (HR-MS) analysis of the natural and synthetic compounds was performed at the Proteomics and Mass Spectrometry Core Facility at The Huck Institutes of the Life Sciences of The Pennsylvania State University.

Contact Behavioral Assay Behavioral experiments were performed between March 20 and April 18, and between July 16 and August 1, 2008. Live males used in the assays were 1-6-d-old. Treatments were prepared fresh each morning. Dead females (unwashed and hexane washed) were mounted, at the thorax, on a piece of wire. Treatment solutions were applied to previously washed dead females with a 10 µl syringe, thus ensuring that the solution covered the bodies evenly. Treated females were placed in a fume hood for 10 min to allow evaporation of the solvent. In the bioassay, the responses of males were tested by presenting a female 1–2 cm away from the males. The typical sequence of responses to an active treatment was, walking up to the treated dead female, antennation of the abdomen and/or grabbing the dead female with the forelegs, mounting, and either abdominal bending or a copulation attempt. Each observation lasted 20–25 sec or was terminated earlier if the male attempted copulation or flew away. We counted copulation attempts as being a positive response. Each morning a new group of males was tested with an unwashed dead female (UDF), and only the positively responding males were used on that day in the bioassay (6–12 males per day). Each set of treatments was replicated 17–24 times over 3–4 d. The order in which the treatments were presented varied each day. A treatment was presented to all the males before switching to the next treatment, with males allowed to rest half an hour between treatments. Each set of treatments included a negative and a positive control. The negative control (C) was prepared by evaporating 9 ml hexane and redissolving the residue in 100 µl of hexane. For the positive control, an unwashed dead female (UDF) or a female with reapplied whole body wash (WB) was used. The sets of treatments tested were the following: a) Whole body wash reapplied at three concentrations: 1/3, 1, and 3FE (WB1/3, WB, and WB3, respectively); and the controls C and UDF. b) Fractions of the female body wash at 1FE: alkanes (A), monoalkenes (MA), dialkenes (DA), and more polar lipids (P) (see below); and the controls C and WB. c) Synthesized components of MA at 1FE: (*Z*)-7-heptacosene (Z7C27), (*Z*)-9-heptacosene (Z9C27), (*Z*)-7-nonacosene (Z7C29), and (*Z*)-9-nonacosene (Z9C29) individually (see below), a mix of all four (Mix4), MA; and the controls C and UDF. d) Binary combinations of the three most active components at 1FE: Z7C27+Z7C29, Z7C27+Z9C29, and Z7C29+Z9C29, a mix of the three components (Mix3), Mix4, MA; and the controls C and UDF.

For each treatment within a set, the significance of difference from the positive control was determined by using the 2-tail values of the Fisher's exact test.

Fractionation of the Female Body Wash Body washes of ten females were combined and evaporated to dryness, which resulted in approximately 4 mg solid material. This was redissolved in 50 µl of hexane and loaded onto a small flash chromatography column, consisting of a Pasteur pipette, with a ball of glass wool as a plug and approximately 300 mg of silica gel (Sigma-Aldrich, 230–400 mesh, 60 Å). Compounds were eluted with hexane in two 1-ml fractions, followed by three 1-ml fractions of diethyl-ether. The hexane fractions were combined to provide a hydrocarbon mix, while the combined diethyl-ether fractions gave a mixture of more polar lipids. The latter was evaporated to dryness and redissolved in 1 ml of hexane resulting in a 1FE/100 µl solution (P).

The hydrocarbon fraction was further separated on 300 mg of silver nitrate-treated silica (Li et al. 1995). The fraction, in hexane, was loaded onto the column, and the alkanes were eluted with hexane (two 1-ml fractions), the monoalkenes with dichloromethane (B&J, High Purity Solvent; five 1-ml fractions), and the dialkenes with acetonitrile (EMD, Omnisolv, High Purity Solvent; two 1-ml fractions). Fractions containing compounds of the same class were combined and evaporated to dryness. Residues were redissolved in 1 ml of hexane, resulting in the following 1FE /100 µl solutions: alkanes (A), monoalkenes (MA), and dialkenes (DA).

Syntheses Z9C27 was prepared as the main geometric isomer from the Wittig reaction of nonyltriphenyl phosphonium bromide and octadecanal. A significant amount of the (*E*)-isomer was formed by this route and required additional chromatography for purification. Partial hydrogenation of the appropriate alkynes was used to provide alkenes Z7C27, Z7C29, and Z9C29. The corresponding alkanes were produced in 3–5% as a result of over-reduction, but they were easily removed by flash chromatography. The alkynes were conveniently prepared by condensing the appropriate alkyl iodides with either 1-decynyl lithium or 1-octynyl lithium in the presence of 1, 3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone (DMPU) (Bengtsson and Liljefors 1988). The identity of the compounds was confirmed by their mass spectra and the exact mass of the molecular ion determined by HR-MS. Melting points are uncorrected.

Preparation of 7-Heptacosyne A solution containing 0.33 g (2 mmol) of distilled 1-octyne in 10 ml of tetrahydrofuran

(THF) was cooled to −70°C and treated with 2 ml of 1.6 M *n*-BuLi. After 45 min, a solution containing 0.39 g (1 mmol) of 1-iodononadecane (Marukawa et al., 2001) in 3 ml of DMPU was added dropwise, and the mixture was allowed to warm to room temperature overnight. The mixture was treated with 5 ml of saturated NH_4Cl and taken up in 70 ml of petroleum ether. The organic layer was washed × 3 with 10 ml of water, and dried over anhydrous $MgSO_4$. After filtration, the solvent was removed in vacuum, and the residue was purified by flash chromatography (hexane/silica gel) to provide 0.19 g of 7-heptacosyne as a waxy solid: mp 35–37°C, EI-MS *m/z* 376 [M^+](2), 292(1), 291(2), 278(2), 277(1), 250(1), 222(1), 208(1), 207(2), 194(1), 180(2), 179(2), 166(10), 165(10), 152(5), 151(7), 138(9), 137(11), 124(15), 123(24), 111(15), 110(30), 109(58), 97(37), 96(82), 95(88), 83(48), 82(83), 81(100), 80(12), 79(16), 71(14), 69(40), 68(30), 67(73), 57(41), 55(56), 54(32), 43(57), 41(39). HR-MS *m/z* 376.4080; calculated for $C_{27}H_{52}$, 376.4069.

Preparation of 7-Nonacosyne Coupling 1-octyne and 1-iodoheneicosane (Mori and Wu, 1992), as described for 7-heptacosyne, provided 0.18 g of 7-nonacosyne as a waxy solid: mp 41–43°C, EI-MS *m/z* 404 [M^+](2), 320(1), 319 (2), 306(2), 305(1), 207(1), 180(3), 179(2), 166(11), 165 (11), 152(6), 151(7), 138(11), 137(12), 124(17), 123(25), 111(18), 110(33), 109(60), 97(41), 96(90), 95(89), 85(10), 83(51), 82(87), 81(100), 80(12), 79(15), 71(18), 69(44), 68(32), 67(74), 57(45), 55(56), 54(30), 43(59), 41(36). HR-MS *m/z* 404.4369; calculated for $C_{29}H_{56}$, 404.4382.

Preparation of 9-Nonacosyne Coupling 1-decyne and 1-iodononadecane (Marukawa et al., 2001), as described for 7-heptacosyne, provided 0.3 g of 9-nonacosyne as a waxy solid: mp 35–37°C, EI-MS *m/z* 404 [M^+](2), 347(1), 278 (2), 277(1), 264(1), 222(1), 208(2), 207(2), 195(1), 194(5), 193(5), 152(5), 151(6), 138(14), 137(22), 124(23), 123(26), 111(16), 110(20), 109(38), 97(40), 96(74), 95(72), 83(49), 82(93), 81(100), 80(12), 79(15), 71(15), 69(38), 68(25), 67(64), 57(46), 56(10), 55(58), 54(26), 43(52), 41(33). HR-MS *m/z* 404.4382; calculated for $C_{29}H_{56}$, 404.4382.

Preparation of Z7C27 A solution containing 100 mg of 7-heptacosyne, 2 drops of quinoline, and 75 mg of Lindlar catalyst (Aldrich Chemical Co.), in 25 ml of ethanol and 2 ml of ethyl acetate, was cooled to 10°C and hydrogenated at atmospheric pressure. The reaction products were followed by GC-MS analysis; this indicated the absence of starting alkyne after 3 h. The mixture was filtered through celite, and after the solvent was removed *in vacuo*, flash chromatography (hexane/silica gel) provided 85 mg of Z7C27, EI-MS *m/z* 378 [M^+](5), 237(1), 223(1), 222(1), 210(1), 209(2), 208(1), 196(1), 195(2), 194(1), 182(2), 181(3), 180(2), 167(6), 153(9), 139(15), 125(30), 112(12), 111(54), 110(10), 99(11), 98(17), 97(91), 96(22), 85(37), 84(27), 83(88), 82(29), 81(15), 71(50), 70(35), 69(68), 68(13), 67(17), 57(100), 56(39), 55(81), 54(14), 43(68), 41(39). HR-MS *m/z* 378.4232; calculated for $C_{27}H_{54}$, 378.4226.

Preparation of Z7C29 Hydrogenation of 7-nonacosyne, as described above, provided Z7C29, EI-MS *m/z* 406 [M^+](4), 251(1), 250(1), 237(1), 236(1), 224(1), 223(2), 222(1), 210(1), 209(2), 208(1), 196(1), 195(3), 194(1), 183(1), 182(2), 181(4), 180(2), 167(6), 153(11), 139(18), 125(34), 112(12), 111(58), 110(10), 99(13), 98(18), 97(96), 96(25), 95(10), 85(39), 84(26), 83(90), 82(30), 81(16), 71(54), 70(35), 69(69), 68(15), 67(20), 57(100), 56(34), 55(71), 54(13), 43(74), 41(37). HR-MS *m/z* 406.4522, calculated for $C_{29}H_{58}$, 406.4539.

Preparation of Z9C29 Hydrogenation of 9-nonacosyne, as described above, provided Z9C29, EI-MS *m/z* 406 [M^+](4), 251(1), 250(1), 237(1), 236(1), 224(1), 223(2), 222(1), 210(1), 209(2), 208(1), 196(1), 195(3), 194(1), 183(1), 182(2), 181(4), 180(2), 167(6), 153(11), 139(18), 125(34), 112(12), 111(58), 110(10), 99(13), 98(18), 97(96), 96(25), 95(10), 85(39), 84(26), 83(90), 82(30), 81(16), 71(54), 70(35), 69(69), 68(15), 67(20), 57(100), 56(34), 55(71), 54(13), 43(74), 41(37). HR-MS *m/z* 406.4544; calculated for $C_{29}H_{58}$, 406.4539.

After purification, compounds Z7C27, Z7C29, and Z9C29 were at least 99% pure (*Z*)-isomers based on the peak area values by GC.

Preparation of Z9C27 A suspension of 0.86 g (2 mmol) of nonyltriphenylphosphonium bromide in 10 ml THF was cooled to −10°C under argon and treated with 1.5 ml of 1.6 M butyl lithium in hexanes to provide an orange mixture. After 30 min, a solution containing 0.54 g of octadecanal in 5 ml THF was added dropwise, and the mixture was stirred overnight and allowed to warm to room temperature. The mixture was partitioned between diethyl ether and aqueous NH_4Cl. The ether layer was dried over anhydrous $MgSO_4$, filtered, and the solvent removed *in vacuo*. The residue was taken up in petroleum ether and filtered through celite, and the fitrate concentrated *in vacuo*. Flash chromatography (hexane/silica gel) provided 200 mg of a 5:1 mixture of (*Z*)- and (*E*)-isomers; EI-MS *m/z* 378 [M^+](2), 237(1), 210(1), 209(2), 208(1), 196(1), 195(2), 194(1), 182(2), 181(3), 180(2), 167(6), 153(9), 139(15), 125(30), 112(12), 111(54), 110(10), 99(11), 98(17), 97(91), 96(22), 85(37), 84(27), 83(88), 82(29), 81(15), 71(50), 70(35), 69(68), 68(13), 67(17), 57(100), 56(39), 55(81), 54(14), 43(68), 41(39). HR-MS *m/z* 378.4232, calculated for $C_{27}H_{54}$, 378.4226. A small portion (4 mg) of the 5:1 (*Z*)/(*E*)-mix was purified further on a silver nitrate treated silica

column (400 mg) by sequential elution with hexane, 5% dichloromethane in hexane, 10% dichloromethane in hexane, and 50% dichloromethane in hexane, all in 1-ml fractions. Fractions 2–6, in 10% dichloromethane in hexane, and fractions 1–3, in 50% dichloromethane in hexane, were combined, which resulted in 3 mg of Z9C27, 98% pure (*Z*)-isomer based on the peak area values by GC.

Results

Cuticular Lipids of S. noctilio The hexane-extractable cuticular lipids of *S. noctilio* consisted predominantly of alkanes (C21-C33), mono- and dimethyl alkanes (C23-C37), mono- and dialkenes (C23-C31), and wax esters (C36-C44). (Fig. 1, Table 1). The hydrocarbon components of the cuticle were sexually dimorphic. While *n*-alkenes were one of the major groups of the hexane body wash of females (29% in females, 4% in males), methyl-branched alkanes were more abundant in the male body wash (28% in males, 2% in females). Additonally, a few lower molecular-weight (C23-C25) monoalkenes were present on the male cuticle. Wax esters and *n*-alkanes were abundant in both sexes (37% and 31% in males, and 38% and 31% in females, respectively).

Activity-Guided Bioassay UDF and WB positive controls, respectively, elicited copulation attempts in 65% and 60% of males (not significantly different, Fisher's exact test). However, WB controls at one-third the amount (1/3WB) elicited a significantly weaker response from males than when the same treatment was tested at 1 FE (Fig. 2a). Negative C controls did not elicit copulation attempts in any of the males tested across all experiments (Fig. 2), thus demonstrating that hexane washing removed the cuticular signals.

The A fraction did not elicit a positive response in any of the males (N=17) tested, whereas a weak response was observed to the DA and more polar P fractions. The response to the MA fraction was not different statistically

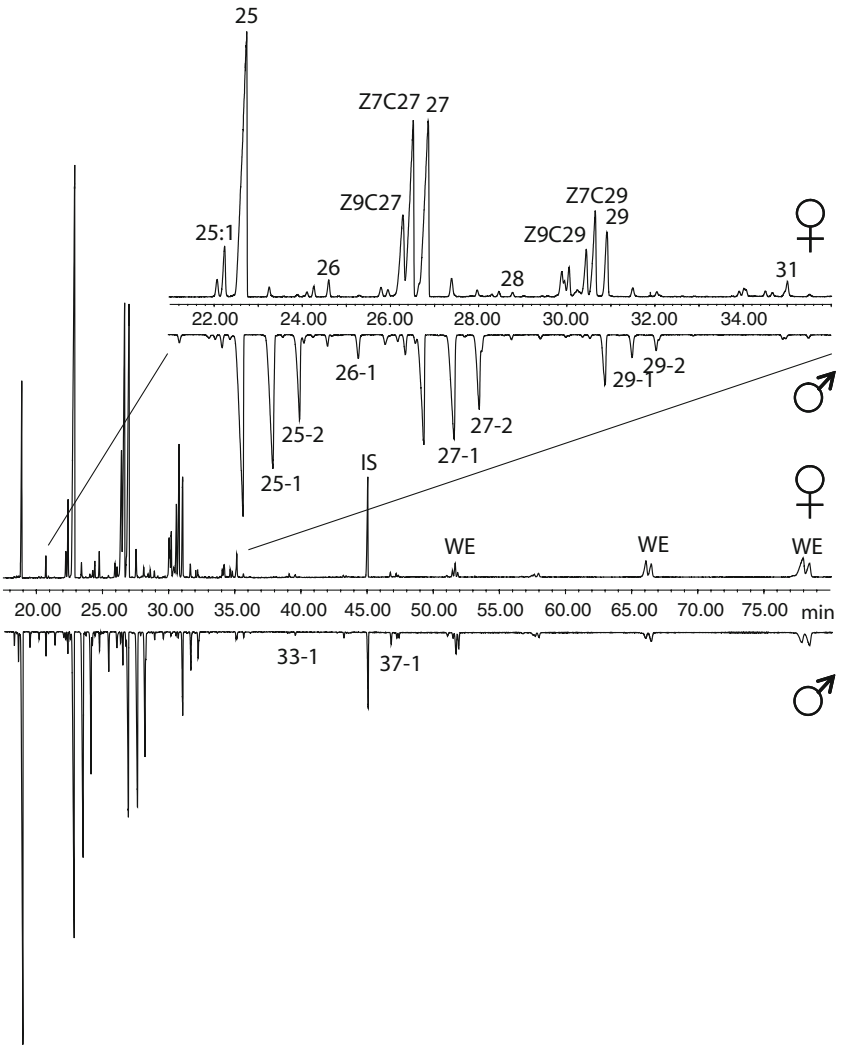

Fig. 1 Representative gas chromatograms of the whole body wash of female (*top*) and male (*bottom*) *Sirex noctilio*. Each sample is a hexane extract of one individual with 50 ng/μl 16-methyl hexatriacontane as internal standard. Coding is the same as in Table 1, for example, pentacosane (25), pentacosene (25:1), (*Z*)-9-nonacosene (Z9C29), methyl heptacosane (27-1), dimethyl pentacosane (25-2), and wax ester (WE), etc.

Table 1 Cuticular lipids of the male and female *S. noctilio*

Code	Compound[a,b]	RI[c]	Amount (μg/wasp)[d,e]			
			Males		Females	
			Mean	S.E.M.	Mean	S.E.M.
21	Heneicosane	2100	0.24	0.07	0.13	0.03
22	Docosane	2200	0.19	0.05	0.12	0.03
23:1	?-Tricosene	2279	0.12	0.09	tr	
U	Unknown	2284	0.54	0.27	0.18	0.06
23	Tricosane	2300	21.68	5.76	11.98	2.47
23-1	11-/9-Methyl tricosane	2332	0.43	0.14	tr	
	7-Methyl tricosane	2336	tr		nd	
23-2	9,13-Dimethyl tricosane	2366	tr		nd	
	7,11-Dimethyl tricosane	2370	0.15	0.06	tr	
24	Tetracosane	2400	0.72	0.16	0.76	0.09
24-1	12-/11-/10-/9-/8-Methyl tetracosane	2433	0.41	0.14	nd	
24-2	8,12-Dimethyl tetracosane	2466	0.23	0.08	nd	
25:1	9-Pentacosene	2473	0.59	0.18	1.07	0.25
	7-Pentacosene	2481	2.10	1.20	3.48	0.82
25	Pentacosane	2500	20.96	4.67	33.43	7.71
25-1	13-/11-/9-Methyl pentacosane	2538	13.86	3.99	0.49	0.12
U	Unknown	2549	0.12	0.04	nd	
25-2	9,13-Dimethyl pentacosane	2568	4.63	2.17	0.10	0.04
	7,13-Dimethyl pentacosane	2573	0.35	0.18	nd	0.00
26:1	9-Hexacosene	2576	nd		0.33	0.08
	7-Hexacosene	2583	nd		0.69	0.17
25-2	5,15-/5,17-Dimethyl pentacosane?	2583	0.22	0.06	nd	
26	Hexacosane	2600	0.75	0.17	1.07	0.18
26-1	13-/12-/11-/10-/9-/8-Methyl hexacosane	2633	1.65	0.49	tr	
27:2	8,18-/?-Heptacosadiene	2657	nd		0.72	0.16
26-2	10,14-/11,15-Dimethyl hexacosane	2662	0.62	0.30	nd	
27:2	7,19-Heptacosadiene	2664	nd		0.56	0.11
27:1	*(Z)-9-Heptacosene, Z9C27*	*2676*	*1.19*	*0.45*	*11.71*	*2.91*
	(Z)-7-Heptacosene, Z7C27	*2684*	*3.51*	*1.79*	*27.54*	*6.67*
27	Heptacosane	2700	13.03	2.91	23.94	5.96
27-1	13-/11-/9-Methyl heptacosane	2737	15.16	3.36	1.20	0.26
U	Unknown	2749	0.14	0.06	nd	
27-2	11,15-/9,13-Dimethyl heptacosane	2764	6.30	2.61	0.47	0.08
28:1	9-Octacosene	2777	nd		0.23	0.05
	7-Octacosene	2786	nd		0.40	0.09
28	Octacosane	2800	0.37	0.09	0.48	0.10
28-1	14-/13-/12-/11-Methyl octacosane	2831	0.36	0.09	nd	
29:2	9,19-Nonacosadiene	2854	nd		3.66	0.77
	7,17-Nonacosadiene	2859	nd		1.58	0.31
28-2	14,18-/11,15-Dimethyl octacosane?	2859	0.11	0.02	nd	
29:2	7,19-Nonacosadiene	2862	nd		4.03	0.85
	?-Nonacosadiene, 14-/13-/12-/11-/10-Nonacosene	2871	nd		1.86	0.40
29:1	*(Z)-9-Nonacosene, Z9C29*	*2878*	*0.40*	*0.13*	*5.52*	*1.10*
	(Z)-7-Nonacosene, Z7C29	*2886*	*1.07*	*0.70*	*8.44*	*1.82*
29	Nonacosane	2900	5.75	1.76	9.42	3.05

Table 1 (continued)

Code	Compound[a,b]	RI[c]	Amount (μg/wasp)[d,e]			
			Males		Females	
			Mean	S.E.M.	Mean	S.E.M.
29-1	15-/13-/11-Methyl nonacosane	2932	2.43	0.43	0.77	0.15
29-2	13,17-Dimethyl nonacosane	2958	1.42	0.55	0.57	0.11
30	Triacontane	3000	0.12	0.02	tr	
31:2	9,19-Untriacontadiene	3050	nd		0.82	0.11
	7,17-Untriacontadiene	3056	nd		1.03	0.09
	7,19-Untriacontadiene	3059	nd		0.91	0.20
	?-Untriacontadiene	3063	nd		0.17	0.04
	?-Untriacontadiene	3069	nd		0.15	0.04
31:1	?-Untriacontene	3072	nd		0.12	0.04
	9-Untriacontene	3081	nd		0.45	0.09
	7-Untriacontene	3088	nd		0.31	0.10
31	Untriacontane	3100	0.63	0.11	0.65	0.28
31-1	15-/13-Methyl untricontane, (Cholesterol)	3130	0.56	0.10	0.34	0.06
33	Tritriacontane	3300	0.14	0.01	0.14	0.03
33-1	13-Methyl tritriacontane, (Stigmasterol)	3333	0.36	0.05	0.28	0.07
35-1	17-/15-/13-Methyl pentatriacontane	3531	0.71	0.12	0.27	0.02
37-1	17-/15-/13-Methyl heptatriacontane	3715	1.93	0.27	0.84	0.08
WE	C17:1CO-OC18:0	3737	1.49	0.08	0.98	0.09
	C17:1CO-OC18:0	3745	0.98	0.24	0.52	0.05
	C17:1CO-OC20:0	3937	1.74	0.33	2.14	0.16
	C17:1CO-OC20:0	3947	8.44	2.17	7.62	1.34
	C17:1CO-OC20:0	3955	4.51	1.72	2.54	0.53
	C17:1CO-OC22:0	4136	2.87	0.89	3.06	1.15
	C17:1CO-OC22:0	4144	4.00	1.65	2.44	0.48
	C17:1CO-OC24:0	4348	12.15	4.23	13.85	2.40
	C17:1CO-OC24:0	4358	13.05	4.72	11.61	2.02
	C17:1CO-OC26:0	>4400	17.78	6.46	34.35	5.73
	C17:1CO-OC26:0	>4400	14.88	6.01	18.62	3.57

[a] Compounds in italics were synthesized and tested in a bioassay.

[b] Partial identification of wax esters (WE) is based on their MS spectra in EI mode.

[c] Kovats indices on the Equity-5 column (for references see Methods).

[d] All averages are means of four.

[e] 'nd': not detected, 'tr': <0.10 μg/wasp.

from the response to WB (Fig. 2b), hence, we investigated that fraction further. The major components of MA were identified as pentacosenes, heptacosenes, and nonacosenes based on their retention indices and mass spectra. The position of the double bond of the most abundant compounds was shown to be 7 and 9, based on the mass spectra of the DMDS adducts. Additional isomers in lower amounts were nonacosenes with the double bond being more toward the middle of the chain (Table 1). The four most abundant components of MA, 7- and 9-heptacosene, and 7- and 9-nonacosene, were synthesized, and the geometries of the double bonds in the natural compounds were determined by comparison of the retention times on the Equity-5 column. For all four monoalkenes, the natural compounds were the (*Z*)-isomers. Purification of the crude synthetic products by flash chromatography on silica and silver nitrate-treated silica led to 98–99% pure (*Z*)-isomers, which were tested in our bioassay at 1FE (for amounts see Table 1). One of the compounds, Z9C27, did not elicit significant responses from males (N=24), while the other

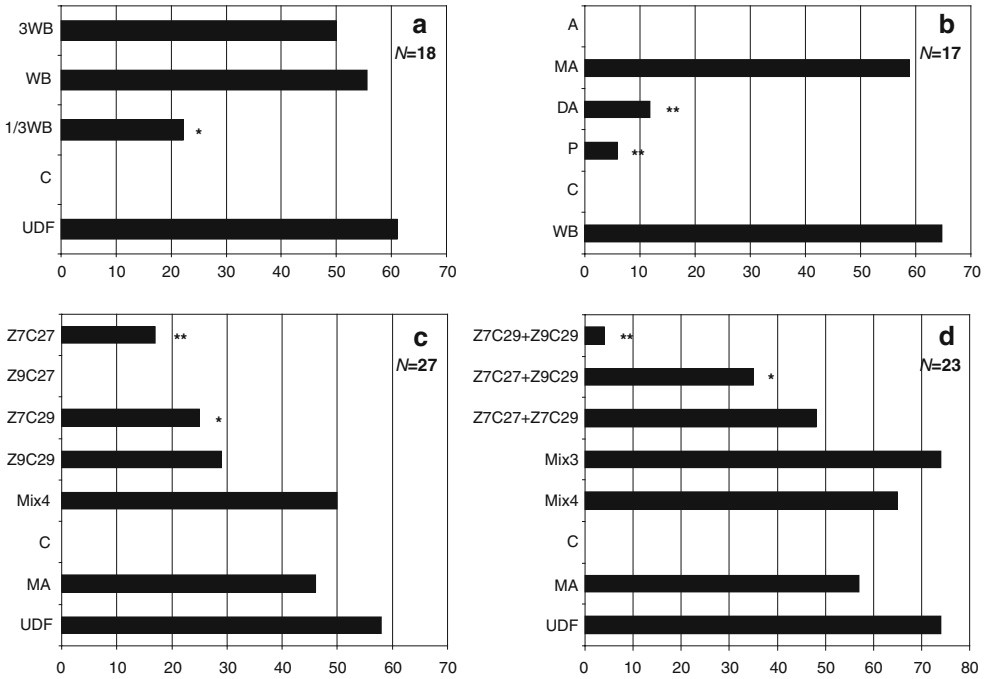

Fig. 2 Percentages of males that attempted copulation with dead females having various treatments applied in four sets of treatments. (**a**) Three concentrations of the female whole body wash: 3 female equivalents (FE) (3WB), one FE (WB), and one third FE (1/3WB). (**b**) Fractions of the body wash at one FE: alkane fraction (A), monoalkene fraction (MA), dialkene fraction (DA), and the fraction of more polar cuticular lipids (P). (**c**) Four synthetic components of MA: (*Z*)-7-heptacosene (Z7C27), (*Z*)-9-heptacosene (Z9C27), (*Z*)-7-nonacosene (Z7C29), and (*Z*)-9-nonacosene (Z9C29). (**d**) Binary combinations of the active synthesized monoalkenes. Positive control was either an unwashed dead female (UDF; **a**, **c**, and **d**) or whole body wash reapplied (WB; **b**). Negative control was hexane reapplied (C). Within each set the statistical significance of the difference of a treatment from the positive control was calculated using Fisher's exact test; *: $P<0.05$, **; $P<0.01$

three did (Fig. 2c). The mixture of the three active components at 1 FE (Mix3), and also the binary mixture of Z7C27 and Z7C29, elicited activity from males not different statistically from that elicited by UDF. The results of testing binary combinations of the three active monoalkenes indicated that Z7C27 was an essential component of the blend (Fig. 2d).

Discussion

We identified three major components, Z7C27, Z7C29, and Z9C29, of the cuticular lipids of female *S. noctilio* that elicited copulation attempts in conspecific males. The responses of males to a binary mixture of synthetic Z7C27 and Z7C29 or to a mixture of these three compounds were not different statistically from the responses to unwashed dead females, indicating that these compounds accounted for most, if not all, the cuticular activity. When Z7C27 was not present in the blend, the responses of males dropped significantly (Fig. 2d, $P<0.01$). Further work on these compounds should test more rigorously for possible additive and/or synergistic effects in mixtures of these compounds.

Cuticular monoalkenes are well-known to act as contact sex pheromone components and sex recognition compounds in some cerambicid beetle (Ginzel et al. 2003, 2006) and *Drosophila* species (Howard 1993; Howard and Blomquist 2005). These compounds are odd chain-length alkenes, ranging in chain length from 25 to 33 carbons, with (*Z*) geometry across the double bond. Among hymenopterans, various parasitic wasps are reported to use a mix of cuticular alkenes and, more often, alkadienes as volatile sex pheromones (Keeling et al. 2004). In the suborder Symphyta, the family Diprionidae (conifer sawflies) is the most extensively studied. Many of the species use female-produced volatile sex pheromones that are methyl-branched secondary alcohols and esters thereof (Keeling et al. 2004). However, no reports are available on the role of cuticular compounds in mating behavior. To our knowledge this is the first description of cuticular hydrocarbons that serve as contact sex pheromone components in this ancient hymenopteran suborder.

In our bioassays, 65% of males attempted copulation with UDF controls, although all males tested had responded positively during the selection process earlier in the day. That we did not observe 100% positive responses to our positive control treatment is likely due to some males being

affected by frequent handling. Also, defrosted females sometimes had their wings in a twisted position, which restricted the movements of the male; males can copulate with a live female only after pressing down the wings of the female tight to her body (unpublished observation). Moreover, we only counted attempted copulation, the clearest behavioral response, as a positive response for our observations, and we did not include other positive responses, such as agitated wing-flapping and abdominal bending. Other variation observed in responses of males to UDF across treatment sets is likely due to differences in age (1-6-d-old males were used), changes in barometric pressure, relative humidity, temperature, and light conditions.

Our laboratory bioassay tested only contact cuticular chemicals for activity in mating. It seems likely that other factors (olfactory, visual, tactile, and auditory) may provide additional cues to males during mate location and selection. To date no evidence has been found for the existence of a long-range sex pheromone component in the Siricidae. Some hymenopterans are hill-topping insects and use rendezvous sites to find their mate (Alcock and Dodson 2008). Similar behavior has not been observed with *S. noctilio*. Although females may fly into male swarms, there are no behavioral data published that investigate the cause of such behavior; it is difficult to determine in a laboratory bioassay what type of cue (olfactory, visual, and/or auditory) may be used in such behavior. After emergence, both sexes are positively phototactic (Morgan and Stewart 1966), and it is likely that both sexes are attracted by host volatiles to the same area since their antennae are responsive to volatile terpenes produced by pines (Simpson 1976). Indeed, females can be caught using girdled pine trees as lures; however, males are not found in the same traps that are usually placed way under the canopy. Thus, it remains to be investigated how the two sexes locate each other.

Acknowledgements This research was supported by USDA APHIS PPQ. We thank Robert D. Minard at the Proteomics and Mass Spectrometry Core Facility at The Huck Institutes of the Life Sciences of The Pennsylvania State University very kindly for providing the high resolution mass spectrometry data.

References

ALCOCK, J. and DODSON, G. 2008. The diverse mating systems of hilltopping insects. *Am. Entomol.* 54:80–87.

AYASSE, M., PAXTON, R. J., and TENGÖ, J. 2001. Mating behavior and chemical communication in the order of Hymenoptera. *Annu. Rev. Entomol.* 46:31–78.

BENGTSSON, M. and LILJEFORS, T. 1988. DMPU: An alternative to HMPT in moth sex pheromone synthesis. *Synthesis* 1988:250–252.

BLOMQUIST, G. J., TILLMAN-WALL, J. A., GUO, L., QUILICI, D. R., GU, P., and SCHAL, C 1993. Hydrocarbon and hydrocarbon derived sex pheromones in insects: biochemistry and endocrine regulation, pp. 317–351, in D. W. Stanley-Samuelson, D. R. Nelson (eds.). Insect Lipids: Chemistry, Biochemistry and Biology. University of Nebraska Press, Lincoln, Nebraska.

BÖRÖCZKY, K., PARK, K. -Ch., MINARD, R. D., JONES, T. H., BAKER, Th. C., and TUMLINSON, J. H. 2008. Differences in the cuticular lipid composition of the antennae of *Helicoverpa zea*, *Heliothis virescens*, and *Manduca sexta*. *J. Ins. Physiol.* 54: 1385–1391.

CARLSON, D. A., BERNIER, U. R., and SUTTON, B. D. 1998. Elution patterns from capillary GC for methyl-branched alkanes. *J. Chem. Ecol.* 24:1845–1865.

COUTTS, M. P. and DOLEZAL, J. E. 1969. Emplacement of fungal spores by the woodwasp, *Sirex noctilio*, during oviposition. *Forest Sci.* 15:412–416.

CROOK, D. J., KERR, L. M., and MASTRO, V. C. 2008. Sensilla on the antennal flagellum of *Sirex noctilio* (Hymenoptera: Siricidae). *Ann. Entomol. Soc. Am.* 101:1094–1102.

FONG, L. K. and CROWDEN, R. K. 1973. Physiological effects of mucus from the wood wasp, *Sirex noctilio* F., on the foliage of *Pinus radiata* D. Don. *Australian J. Biol. Sci.* 26:365–378.

FRANCIS, G. W. and VELAND, K. 1981. Alkylthiolation for the determination of double-bond positions in linear alkanes. *J. Chrom.* 219:379–384.

GINZEL, M. D., MILLAR, J. G., and HANKS, L. M. 2003. (*Z*)-9-Pentacosene — contact sex pheromone of the locust borer, *Megacyllene robiniae*. *Chemoecology* 13:135–141.

GINZEL, M. D., MOREIRA, J. A., RAY, A. M., MILLAR, J. G., and HANKS, L. M. 2006. (*Z*)-9-Nonacosene—major component of the contact sex pheromone of the beetle *Megacyllene caryae*. *J. Chem. Ecol.* 32:435–451.

HOEBEKE, E. R., HAUGEN, D. A., and HAACK, R. A. 2005. *Sirex noctilio*: Discovery of a Palearctic siricid woodwasp in New York. *Newsletter of the Michigan Entomol. Soc.* 50:24–25.

HOWARD, R. W. 1993. Cuticular hydrocarbons and chemical communication, pp. 179–226, in D. W. Stanley-Samuelson, D. R. Nelson (eds.). Insect Lipids: Chemistry, Biochemistry and Biology. University of Nebraska Press, Lincoln, Nebraska.

HOWARD, R. W. and BLOMQUIST, G. J. 2005. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annu. Rev. Entomol.* 50:371–93.

HURLEY, B. P, SLIPPERS, B., and WINGFIELD, M. J. 2007. A comparison of control results for the alien invasive woodwasp Sirex noctilio, in the southern hemisphere. *Agric. Forest Entomol.* 9: 159–171.

KEELING, Ch. I., PLETTNER, E., and SLESSOR, K. N. 2004. Hymenopteran semiochemicals. *Top. Curr. Chem.* 239:133–177.

KOVATS, E. sz. 1965. Gas chromatographic characterization of organic substances in the retention index system, pp. 229–247, in J. C. Giddings, R. A. Keller (eds.). Advances in Chromatography, M. Dekker Inc, New York.

LI, T. -S., LI, J. -T., and LI, H. -Z. 1995. Modified and convenient preparation of silica impregnated with silver nitrate and its application to the separation of steroids and triterpenes. *J. Chrom. A* 715:372–375.

MADDEN, J. L. 1981. Egg and larval development in the woodwasp, Sirex noctilio F. *Aust. J. Zool.* 29: 493–506.

MARUKAWA, K., TAKIKAWA, H., and MORI, K. 2001. Synthesis of the enantiomers of some methyl-branched cuticular hydrocarbons of the ant *Diacamma* sp. *Biosci. Biotechnol. Biochem.* 65:305–314.

MORGAN, F. D. 1968. Bionomics of Siricidae. *Annu. Rev. Entomol.* 13:239–256.

MORGAN, F. D. and STEWART, N. C. 1966. The biology and behaviour of the woodwasp *Sirex noctilio* F. in New Zealand. *Trans. roy. Soc. N. Z., Zool.* 7:195–204.

MORI, K. and WU, J. 1992. Synthesis of the four possible stereoisomers of 13,17-dimethylnonatriacontane, a kairomone for the wasp *Trichogramma nubilale*. *Liebigs Ann. Chem.* 1992:83–85.

NELSON, D. R. 2001. Discovery of novel trimethylalkanes in the internal hydrocarbons of developing pupae of *Heliothis virescens* and *Helicoverpa zea*. *Comp. Biochem. Physiol. Part B* 128:647–659.

SIMPSON, R. F. 1976. Bioassay of pine oil components as attractants for *Sirex noctilio* (Hymenoptera: Siricidae) using electroantennogram techniques. *Ent. Exp. Appl*. 19:11–18.

TAYLOR, K. L. 1981. The sirex woodwasp: Ecology and control of an introduced forest insect, pp. 231–248, in R. L. Kitching and R. E. Jones (eds.). The Ecology of Pests — Some Australian Case Histories. CSIRO, Melbourne, Australia.

VAN DEN DOOL, H. and KRATZ, P. D. 1963. A generalization of the retention index system including linear temperature programmed gas—liquid partition chromatography. *J. Chrom.* 11:463–471.

VINCENTI, M., GUGLIELMETTI, G., CASSANI, G., and TONINI, C. 1987. Determination of double bond position in diunsaturated compounds by mass spectrometry of dimethyl disulfide derivatives, *Anal. Chem.* 59:694–699.

WYATT, T. D., 2003. Pheromones and Animal Behavior: Communication by Smell and Taste, pp. 70–73, pp. 102–109. Cambridge University Press, Cambridge, UK.