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SHORT COMMUNICATION

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Methyl 2-(methylthio)benzoate: the unique sulfur-containing sex pheromone of *Phyllophaga crinita*

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Abstract The female-produced sex pheromone of *Phyllophaga crinita* (Burmeister) (Coleoptera: Scarabaeidae: Melolonthinae; the adult has no common name) is identified as methyl 2-(methylthio)benzoate. This is the first identification of a sulfur-containing, long-distance, female-produced sex attractant from any insect taxa. The root-feeding larvae of this species are serious pests in many crops in Texas and Mexico. In field tests, many *P. crinita* males were captured in traps baited with the authentic compound. Interestingly, a heteroatom analog, methyl 2-methoxybenzoate, also captured *P. crinita* males, but only at a dose 10,000 times higher than the lowest tested dose of the authentic pheromone.

Introduction

The larvae of *Phyllophaga crinita* (Burmeister) (Coleoptera: Scarabaeidae: Melolonthinae) are root-feeding pests of some economic consequence. There is no common name for this species. In Texas, the larvae have been reported feeding in rangeland pasture, ornamental turf, sugarcane, grain sorghum, cotton, and wheat, as well as corn, parsley, and cabbage (Reinhard 1940; Teetes 1973, 1976; Huffman et al. 1976; Huffman and Harding 1980). *P. crinita* larvae are also serious pests in Mexico (Rodriguez-del-Bosque 1984, 1995). In the USA, *P. crinita* is reported from Texas, Louisiana, Mississippi, and Alabama (Luginbill and Painter 1953). Until recently, the most southerly known distribution of *P. crinita* was San Fernando, Tamaulipas, Mexico, 137 km south of the Mexico–USA border (Rodriguez-del-Bosque et al. 1995). However, Rodriguez-del-Bosque (2003) recently reported *P. crinita* larvae feeding on grain sorghum in Tampico, southern Tamaulipas, 275 km south of San Fernando, and indicates that the larvae of this insect have the potential to become a pest of some significance there.

P. crinita has long been regarded as a member of the genus Phyllophaga (sensu stricto), and as such, is one of about 200 species of *Phyllophaga* (sensu lato) found in North America (Woodruff and Beck 1989). However, a recent publication (Coca-Abia 2002) hypothesized the monophyly and subsequent re-establishment of the genus *Trichesthes* and removed *P. crinita* from the genus *Phyllophaga* to *Trichesthes*. We report here the identification of the unique sulfur-containing female sex pheromone of *P. crinita* as methyl 2-(methylthio)benzoate (CAS number 3704-28-7).

Materials and methods

Pheromone collections

Third instar larvae of *P. crinita* were collected by digging in Dallas, Texas, in April 2000 and were shipped to Geneva, New York, via overnight mail. Larvae were individually housed in ~30-ml plastic cups in a 3:1 mixture of greenhouse sand and screened peat moss raised to about 12% moisture. The cups were kept in a controlled environment room maintained at 25°C during the 16-h photophase and 20°C during the 8-h scotophase. After pupation and adult emergence, females were placed in observation cages. When the females were observed calling (abdominal pheromone gland everted) (Leal et al. 1993) during the scotophase, they were removed from the cages and their glands excised and soaked in 200 µl of dichloromethane. After 20 min, the glands were removed and the extract concentrated under a nitrogen stream to a volume of about 20 µl.

In memory of M.G. Villani, who died on 15 May 2001. His enthusiasm for soil-dwelling scarab beetles and contributions to their research are greatly missed.

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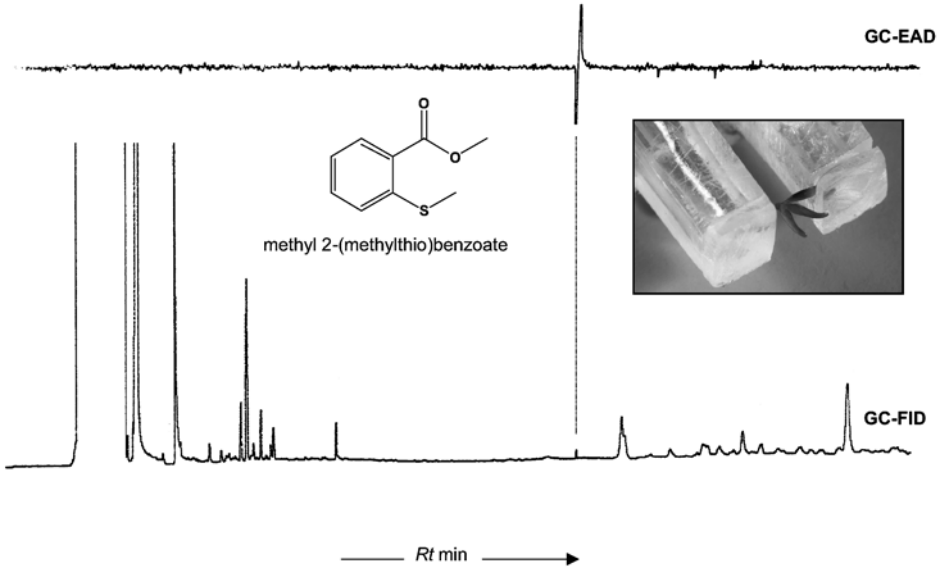

Fig. 1 Simultaneous responses of GC-FID and GC-EAD of the male antenna of *P. crinita* to the female pheromone gland extract on a nonpolar capillary column. Photograph of antenna in holder apparatus

Coupled gas chromatographic-electroantennographic detection (GC-EAD) analysis

The GC instrumentation used in this study was the same as that described by Nojima et al. (2003a). A Hewlett Packard 5890 series II gas chromatograph equipped with either a nonpolar SPB-1 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, Pa.) or a polar EC-WAX econo-cap capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Alltech, Deerfield, Ill.) was used for analysis in splitless mode. Nitrogen was used as the carrier gas at a head pressure of 138 Pa (flow rate, 2.0 ml/min). The oven temperature was programmed 40°C for 2 min, increased at 15°C/min to 250°C and held for 10 min. Injector and detector temperatures were set at 250 and 280°C, respectively.

The column effluent was combined with nitrogen make-up gas (30 ml/min) and then split 1:1 to the flame ionization detector (FID) and EAD. The EAD outlet was secured in a charcoal-filtered and humidified airstream, refrigerated by a modified condenser flushed with 0°C water, flowing at 500 ml/min over the antennal preparation.

EAD recordings

An antenna plucked from the head of a beetle was bridged between two slits in a custom-made acrylic holder in such a position that the lamellae were held open (Fig. 1). Each slit was filled with 0.9% NaCl saline, to which pure gold indifferent and recording electrodes were connected, respectively. The holder was placed inside of the cooling condenser and maintained at about 5°C. Good recordings could often be made for several hours on these antennal preparations.

The output signal from the antenna was amplified 100× by a customized single-step high-output impedance DC amplifier (Nojima et al. 2003a). The signal was filtered by a simple resistance/capacitor high-pass filter with a cutoff frequency of about 0.5 Hz and recorded on an HP 3390A integrator synchronized with the GC integrator. A total of seven antennae from different males (2–8 runs/antenna) were used for the GC-EAD analysis.

Chemical analysis

GC-MS analyses were performed on a Shimadzu GC-17A equipped with a nonpolar DB-1 ms capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, Calif.) or a polar EC-WAX econo-cap capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Alltech). The GC was coupled to a Shimadzu QP-5050A quadrupole mass spectrometer running in the EI (electron ionization at 70 eV) scan mode. Helium was used as the carrier gas at an initial head pressure of 53.8 Pa at constant flow rate (1.0 ml/min). The time for splitless injection was 1 min. Oven temperature was programmed 40°C for 2 min, increased at 15°C/min to 250°C and held for 10 min. Injector and interface temperatures were set at 280 and 250°C, respectively. The EAD active component was tentatively identified by matches to library spectra followed by comparisons of retention time and mass spectra with that of an authentic sample. The antennal activity of the synthetic compound was confirmed by GC-EAD.

Chemicals

Methyl 2-(methylthio)benzoate and methyl 4-(methylthio)benzoate were purchased from Lancaster Synthesis (Pelham, N.H.) and Maybride Chemical Company. (UK), respectively. Methyl 3-(methylthio)benzoate was prepared from 3-mercaptobenzoic acid (Toronto Research Chemical, Canada) by methylation with trimethylsilyl diazomethane (Aldrich, Milwaukee, Wis.). Methyl 2-methoxybenzoate was also obtained from Aldrich.

Field evaluation of compounds

A total of six treatments were field-tested in 2001. These treatments included methyl 2-(methylthio)benzoate at 100, 300, and 1,000 μg, and methyl 2-methoxybenzoate at 1,000 and 1,000,000 μg, as well as a solvent-only treatment. The 100, 300, and 1,000 μg treatments were made by dissolving each chemical in hexane (10 μg/μl) and applying the appropriate amount into 5 mm rubber stopper septa (Thomas Scientific, Swedesboro, N.J.) and allowing the hexane to evaporate in a fume hood. The 1,000,000 μg lure was made by applying 1.0 g of neat methyl 2-methoxybenzoate into an evaporative dispenser (GenPore, Reading, Pa.).

Four sets of the six treatments of cross-vane traps and lures were deployed at four locations in and around Dallas, Texas, in May 2001. Traps were placed about 20 m apart in a line. Traps were hung on stakes such that the trap bottom was about 60 cm from the ground. Treatment positions were randomized at deployment and once each week thereafter. Traps were checked and beetles counted a total of 24 times between 31 May and 19 August. Lures were replaced every 4 weeks.

Statistics

The total numbers of beetles caught in each trap at each location over the 24 count dates were combined. Data were log transformed (x+1) before analysis to establish homogeneity of variance. Treatments were compared with the Student-Newman-Keuls multiple range test (at $P<0.05$).

Results

Identification of the sex pheromone component

GC-EAD analyses of female *P. crinita* gland extracts on nonpolar and polar columns consistently revealed a single EAD-active component (Fig. 1). Mass spectral matches to library spectra tentatively identified the compound as methyl 2-(methylthio)benzoate. The identity was confirmed by comparison of mass spectra, GC retention times and EAD activity on both polar and nonpolar columns with the authentic standard. The retention times and MS fragment patterns of the other isomers, methyl 3-(methylthio)benzoate or 4-(methylthio)benzoate, were different from the natural compound.

Field tests

Results from the field test can be seen in Fig. 2. A total of 8,664 male beetles were captured. No females were captured. The 1,000 μg dose of the synthetic pheromone (methylthio) captured 337 times more beetles than the oxygen analog (methoxy) at the same dose and was significantly different from it ($F_{5,15}=88.04$; $P>0.0001$). The oxygen analog, when presented to the beetles at 1,000,000 μg (1.0 g), caught only slightly more than half the beetles than the pheromone did at 100 μg, a rate 10,000 times smaller. No significant differences were found between those two treatments.

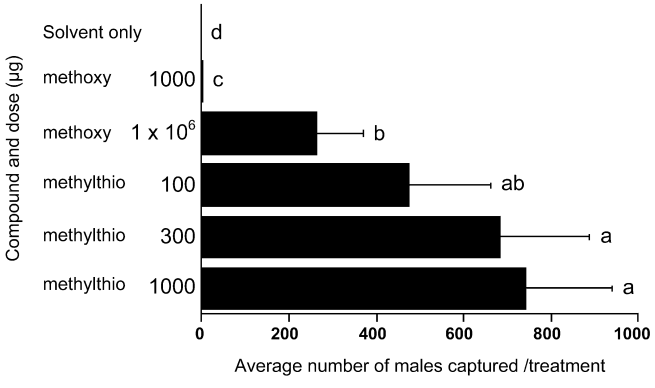

Fig. 2 Catch (Mean ± SE) of male beetles in traps baited with various rates of methyl 2-methoxybenzoate and methyl 2-(methylthio)benzoate. Treatments flanked by the same *letter* are not significantly different at $P<0.05$; Student-Neuman-Keuls multiple range test

Discussion

This is the only sulfurous, long-distance, female-produced sex attractant that has been identified from any insect taxa. Field data indicate that the presence of the sulfur atom is important for pheromonal activity. Interestingly, this compound is crystalline at room temperature. A short-range, sulfur-containing, male-produced sex attractant has been described from the cockroach, *Nauphoeta cinerea* (Sreng 1990; Sirugue et al. 1992). Leal recently summarized the current knowledge regarding scarab beetle sex pheromones (Leal 1998, 1999). The ruteline beetles generally employ sex pheromones that are fatty acid derivatives reminiscent of moth sex pheromone biosynthetic pathways. Melolonthine beetles, however, have been found to utilize a more diverse suite of compounds than the rutelines. Although melolonthine pheromones do include fatty acid derivatives such as (*R,Z*)-7,15-hexadecadien-4-olide (Leal et al. 1996a) and 2-tetradecanone (Zhang et al. 2003) as sex attractants, they also make use of compounds such as methyl esters of L-valine and L-isoleucine (Leal et al. 1992, 2003; Zhang et al. 1997), L-leucine methyl ester (Nojima et al. 2003b), phenol (Henzell and Lowe 1970), anisole (Leal et al. 1996b; Ward et al. 2002), linalool (Leal et al. 1993), and the alkaloid 1,3-dimethyl-2,4-(1H,3H)-quinazolinedione (Leal et al. 1997). To this group of compounds is now added methyl 2-(methylthio)benzoate. There is a paucity of information on the biosynthetic pathways by which most melolonthine sex pheromone compounds are produced. The sex pheromone of the grass grub, *Costelytra zealandica*, identified as phenol (Henzell and Lowe 1970), was ultimately found to be produced by endosymbiotic bacteria (Hoyt and Osborne 1971). Whether other melolonthines such as *P. crinita* also employ symbiotic bacteria to produce semiochemicals will be the subject of our future research. Methyl 2-(methylthio)benzoate is inexpensive and commercially available off the shelf. It is hoped that the identification of this compound as the sex attractant of *P. crinita* will find some use in detection and/or management systems for this pest.

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References

Coca-Abia MM (2002) Reestablishment of the genus *Trichesthes* Erichson, 1847 (Coleoptera: Scarabaeidae, Melolonthinae) based on phylogeny. J N Y Entomol Soc 110(1):95–114

Henzell RF, Lowe MD (1970) Sex attractant of the grass grub beetle. Science 168:1005–1006

Hoyt CP, Osborne GO (1971) Production of an insect sex attractant by symbiotic bacteria. Nature 230:472–473

Huffman FR, Harding JA (1980) Biology of *Phyllophaga crinita* in Lower Rio Grande Valley sugarcane. Southwest Entomol 5(1):59–64

Huffman FR, Fuchs TW, Harding JA (1976) Insecticidal control of the white grub, *Phyllophaga crinita*, on sugarcane. Southwest Entomol 1(3):122–124

Leal WS (1998) Chemical ecology of phytophagous scarab beetles. Annu Rev Entomol 43:39–61

Leal WS (1999) Scarab beetles. In: Hardie J, Minks AK (eds) Pheromones of non-lepidopteran insects. New York, CABI, pp 51–68

Leal WS, Matsuyama S, Kuwahara Y, Wakamura S, Hasegawa M (1992) An amino acid derivative as the sex pheromone of a scarab beetle. Naturwissenschaften 79(4):184–185

Leal WS, Sawada M, Matsuyama S, Kuwahara Y, Hasegawa M (1993) Unusual periodicity of sex pheromone production in the large black chafer *Holotrichia parallela*. J Chem Ecol 19(7):1381–1391

Leal WS, Kuwahara S, Ono M, Kubota S (1996a) (*R,Z*)-7,15-hexadecadien-4-olide, sex pheromone of the yellowish elongate chafer, *Heptophylla picea*. Bioorg Med Chem 4:314–321

Leal WS, Yadava CPS, Vijayvergia JN (1996b) Aggregation of the scarab beetle *Holotrichia consanguinea* in response to female-released pheromone suggests a secondary function hypothesis for semiochemical. J Chem Ecol 22:1557–1566

Leal WS, Zarbin PHG, Wojtasek H, Kuwahara S, Hasegawa M, Ueda Y (1997) Medicinal alkaloid as a sex pheromone. Nature 385:213

Leal WS, Oehlschlager AC, Zarbin PHG, Hidalgo E, Shannon PJ, Murata Y, Gonzalez L, Andrade R, Ono M (2003) Sex pheromone of the scarab beetle *Phyllophaga elenans* and some intriguing minor components. J Chem Ecol 29(1):15–25

Luginbill P, Painter HR (1953) May beetles of the United States and Canada. USDA Technical Bulletin 1060

Nojima S, Linn CE Jr, Morris BD, Zhang A, Roelofs WL (2003a) Identification of host fruit volatiles from hawthorn (*Crataegus* spp.) attractive to hawthorn-origin *Rhagoletis pomonella* flies. J Chem Ecol 29:319–334

Nojima S, Robbins PS, Salsbury GA, Morris BD, Roelofs WL, Villani MG (2003b) L-leucine methyl ester: the female-produced sex pheromone of the scarab beetle *Phyllophaga lanceolata*. J Chem Ecol 29

Reinhard HJ (1940) The life history of *Phyllophaga lanceolata* (Say) and *Phyllophaga crinita* (Burmeister). J Econ Entomol 33(3):572–578

Rodriguez-del-Bosque LA (1984) Oviposition of *Phyllophaga crinita* (Burmeister) in various crops in Northern Tamaulipas Mexico. Southwest Entomol 9(2):184–186

Rodriguez-del-Bosque LA, Crocker RL, Riley EJ (1995) Diversity and abundance of *Phyllophaga* and *Anomala* species in agroecosystems of northern Tamaulipas, Mexico. Southwest Entomol 20(1):55–59

Rodriguez-Del-Bosque LA, Avila-Valdez J, Garza-Urbina E (2003) *Phyllophaga crinita* (Coleoptera: Scarabaeidae): a new pest in the Huastecas area of Mexico. Southwest Entomol 28(2):155–156

Sirugue D, Bonnard O, Le Quere J-L, Farine J-P, Brossut R (1992) 2-methylthiazolidine and 4-ethylguaiacol, male sex pheromone components of the cockroach, *Nauphoeta cinerea* (Dictyoptera, Blaberidae): a reinvestigation. J Chem Ecol 18(12):2261–2276

Sreng L (1990) Seducin, male sex pheromone of the cockroach, *Nauphoeta cinerea*: isolation, identification, and bioassay. J Chem Ecol 16(10):2899–2912

Teetes GL (1973) *Phyllophaga crinita*: Damage assessment and control in grain sorghum and wheat. J Econ Entomol 66(3):773–776

Teetes GL, Wade LJ, McIntyre RC, Schaefer CA (1976) Distribution and seasonal biology of *Phyllophaga crinita* in the Texas High Plains. J Econ Entomol 69(1):59–63

Ward A, Moore C, Anitha V, Wrightman J, Rogers DJ (2002) Identification of the sex pheromone of *Holotrichia reynaudi*. J Chem Ecol 28(3):515–522

Woodruff RE, Beck BE (1989) The scarab beetles of Florida (Coleoptera: Scarabaeidae). II. The May or June beetles (genus *Phyllophaga*). (Arthropods of Florida and neighboring land areas 13) Florida Department of Agriculture, Gainesville, Fla.

Zhang A, Robbins PS, Leal WS, Linn CE Jr, Villani MG, Roelofs WL (1997) Essential amino acid methyl esters: major sex pheromone components of the cranberry white grub, *Phyllophaga anxia* (Coleoptera: Scarabaeidae). J Chem Ecol 23:231–245

Zhang A, Robbins PS, Averill AL, Weber DC, Linn CE, Roelofs WL, Villani MG (2003) Identification of the female-produced sex pheromone of the scarab beetle, *Hoplia equina*. J Chem Ecol 29(7):1635–1642