A new component of attractive aggregation pheromone in the bean bug, *Riptortus clavatus* (Thunberg) (Heteroptera: Alydidae)

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(Received 9 May 2006; Accepted 8 August 2006)

Abstract
The attractive aggregation pheromone from males of the bean bug, *Riptortus clavatus*, has been identified as a blend of \((E)-2\)-hexenyl \((E)-2\)-hexenoate, \((E)-2\)-hexenyl \((Z)-3\)-hexenoate and tetradecyl isobutyrate \(3\ mix\)). When intact male bodies were extracted with hexane, octadecyl isobutyrate was detected in addition to the known 3 mix. Octadecyl isobutyrate was also detected in airborne volatiles using the glass-beaker collection method. In field experiments, the attractiveness of tetradecyl isobutyrate or 3 mix to *R. clavatus* was increased by the addition of octadecyl isobutyrate. These results suggest that octadecyl isobutyrate is one of the components of the attractive aggregation pheromone of *R. clavatus*, and may act as a synergistic composition in this bug.

Key words: *Riptortus clavatus*; octadecyl isobutyrate; tetradecyl isobutyrate; attractive aggregation pheromone

INTRODUCTION

The bean bug, *Riptortus clavatus* (Thunberg) (Heteroptera: Alydidae), is an important soybean pest in Japan (Tabaru and Nagai, 1981; Ikeda and Fukazawa, 1983; Setokuchi et al., 1986). Adult males attract both conspecific males and females in field experiments (Numata et al., 1990). A three-component-mixture (3 mix) of \((E)-2\)-hexenyl \((E)-2\)-hexenoate, \((E)-2\)-hexenyl \((Z)-3\)-hexenoate and tetradecyl isobutyrate \(3\ mix\)) has been identified as the attractive aggregation pheromone of male *R. clavatus* (Leal et al., 1995). It attracts not only adults of both sexes but also nymphs. Successive experiments revealed that male and female *R. clavatus* were captured in traps baited with 14:iBu alone as well as with the 3 mix (Mizutani et al., 1997). The synergistic effect of E2-6:E2Hz and E2-6:Z3Hz was confirmed in Japan (Endo et al., 2005) and Korea (Huh et al., 2005) when these compounds were mixed with 14:iBu.

This paper reports a new component from male *R. clavatus* that enhances attraction to the traps baited with 14:iBu or the 3 mix.

MATERIALS AND METHODS

Insects. *R. clavatus* adults were collected from soybean fields in Tsukuba and maintained on soybean seeds, red clover (*Trifolium pretense*) seeds, and water at 25°C with a 14L : 10D photoperiod in the laboratory. The emerged adults were segregated by sex, and males were separately transferred to other cages and used within 10–14 d old for whole-body extraction and collection of airborne volatiles as described below.

Preparation of whole-body extracts. Intact male individuals were separately dipped into 2 ml of hexane for 3 h at room temperature. The hexane so-
olution of n-hexadecane (2 μg) was added as an internal standard to each sample. The extract was decanted from the male body into a glass vial. The residual body was rinsed with 1 ml of hexane, and the rinse was added to the extract. The extracts were stored under −20°C until gas chromatography-mass spectrometry (GC-MS) analyses. The extracts of hexane solution were concentrated to ca. 100-μl volumes just before GC-MS analyses. Four males were extracted in this experiment.

Collection of airborne volatiles with glass beakers. A male was put into a stainless-mesh cage (6.0 cm outside diameter × 6.0 cm height) with a dried soybean and a piece of cotton containing water. The cage was fixed with stainless steel wire (0.55 mm outside diameter) inside a 300-ml glass beaker (7.8 cm inside diameter × 10.5 cm height), and covered with another 300-ml glass beaker (Fig. 1). The male was held here for 18 h between 17 : 00 and 11 : 00 the following day with 16L : 8D (light from 04 : 00) under artificial lighting (ca. 2,000 lx) at ca. 25°C in the laboratory. The hexane solution of n-hexadecane (10 ng) was added as an internal standard to the beakers. The inside of the beaker was rinsed with ca. 1 ml of hexane twice. The rinses were combined and stored under 20°C. The extracts of hexane solution were concentrated to ca. 10-μl volumes just before GC-MS analyses. The males used in these collections were extracted as described above in the preparation of whole-body extracts. Six males were used in this experiment.

GC-MS analyses. GC-MS analyses were performed on an Agilent 6890N GC with an HP-INNOWax column (30 m length × 0.25 mm inside diameter × 0.25 μm film thickness) by on-column injection combined with an Agilent 5973 Network Mass Selective Detector. Injection was made directly onto the capillary column through the cool-column injector and the injector temperature was programmed at oven temperature plus 3°C. Helium was used as the carrier gas. The initial GC oven temperature was 50°C (1 min hold), increased to 180°C at 10°C min⁻¹, increased again to 240°C at 20°C min⁻¹, and then held for 5 min.

Chemicals. E2-6:E2Hx, E2-6:Z3Hx and 14:iBu were obtained from the Fuji Flavor Co., Ltd., and were 91.7, 88.6 and 99.7% pure, respectively. Octadecyl isobutyrate (18:iBu) was obtained from 1-octadecanol reacted with isobutyric anhydride and pyridine. 18:iBu was more than 99% pure in GC-MS analyses.

Synthetic chemicals for the field experiments were impregnated into gray septa made of halobutyl isoprene blend elastomer (8 mm outside diameter, West Pharmaceutical Services Singapore Pte Ltd., Singapore) by applying hexane solution into the depression. Each septum was placed in a draft chamber for ca. 12 h at room temperature to allow the solvents to evaporate. For the negative control, a septum containing hexane was used.

Field experiment 1. Field experiments to check the additional effect of 18:iBu on the attractiveness of the synthetic 14:iBu or the 3 mix were conducted in November 2004. The field experiments were conducted in 6 different fields in Kagoshima and Okinawa Prefectures. A grassy field surrounded by a coniferous grove in Kawanabe-cho in Kagoshima Prefecture was ca. 2,000 m² (Kawanabe). Two different fields were located at the Kagoshima Agricultural Experiment Station in Kagoshima-city in Kagoshima Prefecture (the Kagoshima Agricultural Experiment Station was reorganized into the Kagoshima Prefectural Institute for Agricultural Development, and moved to Minamisatsuma-city in 2006). A roadside with coniferous trees was ca. 200 m length along a paddy field after harvest (Kagoshima 1), and a potato and strawberry field was ca. 4,400 m² (Kagoshima 2). Three fields were located at the Okinawa Prefectural Agricultural Experiment Station in Naha-city in Okinawa Prefecture (the Okinawa Prefectural Agricultural Experiment Station was reorganized into the Okinawa Prefectural Agricultural Research Center, and moved to Itoman-city in 2006). Three grassy roadsides with garden trees were ca. 200 m length (Naha 1, Naha 2 and Naha 3).
Double-sided sticky plates (18 cm×25.5 cm, Fieldcatch, Fuji Flavor Co., Ltd.) were set at a height of ca. 1 m from the ground and each septum impregnated with test chemicals was fixed at the center position on one side of the sticky plate. Three replicates of each chemical (18 traps) were set in each field at about 10-m intervals from 10 to 13 and from 13 to 22 November in Kawanabe and Kagoshima 1 and Kagoshima 2, and from 8 to 11 and from 19 to 26 November in Naha 1, Naha 2 and Naha 3. The trap locations were swapped on 12 and 15 November for Kawanabe and Kagoshima 1 and Kagoshima 2, and 10 and 22 November for Naha 1, Naha 2 and Naha 3. The numbers of *R. clavatus* caught in a trap were pooled for each treatment.

**Field experiment 2.** Additional field experiments to clarify the additional effect of 18:iBu to 14:iBu on the attractiveness were conducted from 27 August to 2 September, 2005 in a ca. 50,000 m² grassy field at the National Agricultural Research Center for Kyushu Okinawa Region in Koshi, Kumamoto Prefecture, Japan. Sticky plates were set in the same way as in field experiment 1. Three replicates of each chemical (12 traps) were set in fields at about 15 m intervals. The numbers of *R. clavatus* caught in a trap (x) were transformed to log(x+0.5) for two-way-ANOVA.

**Field experiment 3.** Additional field experiments to clarify the additional effect of 18:iBu to the 3 mix on attractiveness were conducted from 20 to 24 July 2005 in a ca. 50,000 m² grassy field of the National Agricultural Research Center for Kyushu Okinawa Region in Koshi, Kumamoto Prefecture, Japan. Sticky plates were set as in field experiment 1. Three replicates of each chemical (12 traps) were set in fields at about 15 m intervals. The numbers of *R. clavatus* caught in a trap (x) were transformed to log(x+0.5) for two-way-ANOVA.

**RESULTS**

**Analysis of components in extracts**

Hexane extracts of four individual *R. clavatus* males were separately analyzed with GC-MS. Six dominant peaks and one peak (t<sub>R</sub>=10.49 min) for n-hexadecane as an internal standard were observed in each extract (Fig. 2). Five peaks were identified as (E)-2-hexenal (t<sub>R</sub>=5.65 min), (E)-2-hexenyl hexanoate (t<sub>R</sub>=11.36 min), E2-6:Z3Hx (t<sub>R</sub>=12.14 min), E2-6:E2Hx (t<sub>R</sub>=12.93 min) and 14:iBu (t<sub>R</sub>=16.01 min). These five compounds have been previously identified in male *R. clavatus* (Leal and Kadosawa, 1992; Leal et al., 1995). One remaining peak (t<sub>R</sub>=18.54 min) was estimated to be an isomer of an 18-carbon saturated alcohol ester of a 4-carbon carboxylic acid, which showed the following characteristic fragment ions: m/z 89 (base peak) and 340 (C<sub>22</sub>H<sub>44</sub>O<sub>2</sub>, M+, 2.7%) (Fig. 3). When authentic octadecyl n-butyrate or octadecyl isobutyrate (18:iBu) were analyzed with the same methods, the 18:iBu showed identical retention times, whereas octadecyl n-butyrate showed longer retention times; therefore, the peak (t<sub>R</sub>=18.54 min) was identified as 18:iBu. The contents of (E)-2-hexenal, (E)-2-hexenyl hexanoate, E2-6:Z3Hx, E2-6:E2Hx, 14:iBu and 18:iBu in the extracts of four individual males are shown in Fig. 4.

To examine whether 18:iBu was released by *R. clavatus*, airborne volatiles from six individual males collected in glass beakers were analyzed with GC-MS. The two compounds, 14:iBu (t<sub>R</sub>=16.01 min) and 18:iBu (t<sub>R</sub>=18.54 min), were observed in each of the extracts of both upper and lower beakers (Fig. 5). 14:iBu were collected at a mean of 66.5 (±27.2 SE) and 50.5 (±20.4 SE) ng/18 h in upper and lower beakers, respectively (n=6). 18:iBu were collected at a mean of 15.5 (±6.3 SE) and 45.3 (±7.8 SE) ng/18 h in upper and lower beakers, respectively (n=6).
Field experiment 1

Field experiments to check the additional effect of 18:iBu on the attractiveness of synthetic 14:iBu or the 3 mix were conducted. Traps baited with 3 mix + 18:iBu captured more adult R. clavatus than traps baited with 3 mix, and traps baited with 14:iBu + 18:iBu captured more adult R. clavatus than traps baited with 14:iBu in Kawanabe and Naha 1 in field experiment 1 (Fig. 6), although the increasing effect of 18:iBu was not confirmed. To confirm the increasing effect of 18:iBu, additional sample field experiments were conducted in one test field.

Field experiments 2 and 3

Traps baited with 14:iBu + 18:iBu captured significantly more R. clavatus than traps baited with 14:iBu, 18:iBu and the control (Fig. 7A). The effect of 14:iBu on the number of captured R. clavatus was significant ($p<0.01$), while the effect of 18:iBu was not ($p>0.05$). The interaction between 14:iBu and 18:iBu was significant ($p<0.01$).

Traps baited with 3 mix + 18:iBu captured more R. clavatus than traps baited with 3 mix, 18:iBu and the control (Fig. 7B). The effects of 3 mix and 18:iBu on the number of captured R. clavatus were significant ($p<0.01$ for 3 mix; $p<0.05$ for 18:iBu). The interaction between 3 mix and 18:iBu was not significant ($p>0.5$).
**DISCUSSION**

A 5:1:1 blend of E2-6:E2Hx, E2-6:Z3Hx and 14:iBu has been identified as the attractive aggregation pheromone of male *R. clavatus* (Leal et al., 1995). In previous reports (Mizutani et al., 1997; Endo et al., 2005; Huh et al., 2005), 14:iBu was identified as an essential component, and E2-6:E2Hx and E2-6:Z3Hx as synergistic components of the *R. clavatus* attractive aggregation pheromone. In the present report, octadecyl isobutyrate (18:iBu) was released from male *R. clavatus* (Figs. 2, 3, 4 and 5) and the attractiveness of 14:iBu or the 3 mix was increased by the addition of 18:iBu (Figs. 6 and 7). These results suggested that 18:iBu may act as a synergist when mixed with 14:iBu or the 3 mix.

In the previous study (Mizutani et al., 1997), 50 mg of 14:iBu was attractive to *R. clavatus* as well as the 3 mix. In the present study, however, 10 mg of 14:iBu was not attractive (Fig. 7A), in which the trap catch was almost the same level as that of the control. This means that the dose of 10 mg of 14:iBu alone was too low to show attractiveness.

When blends of 18:iBu and/or 3 mix were tested, 18:iBu alone was significantly attractive (*p*<0.05) (Fig. 7B), although another experiment showed that the same dose of 18:iBu alone was not attractive (Fig. 7A). Further experiments will be necessary to reveal the attractiveness of 18:iBu alone.

In the present study (Fig. 4), the contents and/or ratio of components in the extracts showed great diversity between individual males. In particular, Nos. 1 and 4 males had no E2-6:Z3Hx; the reason for this is not clear.

In other species of the Alydidae family, attractive pheromones were identified in two species. Males of the rice bug, *Leptocorisa chinensis* (Dallas) were attracted to a 5:1 mixture of (E)-2-octenyl acetate and 1-octanol, which was detected in extracts of both conspecific males and females (Leal...
Males of the broad-headed bug, *Alydus eurinus* (Say), were attracted to blends of 2-methylbutyl butyrate and \((E)\)-2-methyl-2-butenyl butyrate, which were detected in extracts of conspecific females (Aldrich et al., 2000). No common compounds were found between the three species in Alydidae.

Some studies revealed that insect-related volatiles including pheromones are adsorbed to various substrates, including glass (Colwell et al., 1978; Baker et al., 1980, 1981; Pope et al., 1984; Yasui et al., 2003), filter paper (Phillips et al., 1996) and leaves (Noldus et al., 1991; Karg et al., 1994). 14:iBu and 18:iBu were detected in hexane solutions of rinses on the inner surface of glass beakers into which male *R. clavatus* were placed (Fig. 5). As insects in this experiment could not touch the beakers (Fig. 1) and these two components were detected in both beakers, it appears that these components were volatile emissions not adhered by direct touch with bugs and/or as liquid excretion. However, components such as E2-6:Z3Hx and E2-6:E2Hx could not be absorbed onto the glass surface (Fig. 5), although these components were detected in the solvent extracts of bodies of the same individuals (data not shown). Further experiments will be necessary to reveal whether these components are released from male bugs, and attraction components should be examined in detail.

The glass-beaker method is a simple and useful collection system for volatiles from individual insects. Whole body extraction is the usual sampling method, but the individuals extracted must be dead. In many semiochemical studies, absorbents made from a porous polymer such as Porapak Q and Tenax TA were used for volatile collection from living individuals. These absorbents have many advantages such as high absorptivity even with chemicals with high volatility; however, it is necessary to clean the absorbents by time-consuming processing, and it is difficult to deal with many samples. The emission rates of volatiles such as 14:iBu and 18:iBu could be measured from living individuals using the simple glass beaker method. With this method, the relation between the amount of pheromones and the attractiveness of individual insects could be investigated in detail.

**ACKNOWLEDGEMENTS**

We thank the staff of the Kagoshima Prefectural Institute for Agricultural Development and the Okinawa Prefectural Agricultural Research Center for their kind help with the experiments. We also thank Sadao Wakamura, Toshiharu Akino, Hiroe Yasui and Midori Fukaya of the National Institute of Agrobiological Sciences and Takashi Wada of National Agricultural Research Center for Kyushu Okinawa Region for their helpful discussions. Improvement of the manuscript by Serge Glushkoff is also appreciated.

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Aggregation Pheromone in *Riptortus clavatus*

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