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EFFECT OF REARING CONDITIONS ON PRODUCTION OF STERNAL GLAND SECRETION, AND IDENTIFICATION OF MINOR COMPONENTS IN THE STERNAL GLAND SECRETION OF THE PREDATORY STINK BUG *Eocanthecona furcellata*

HSIAO-YUNG HO,* YUN-CHE HSU, YA-CHUN CHUANG, and YIEN-SHING CHOW

Institute of Zoology, Academia Sinica, Taipei
Taiwan 115, Republic of China

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Abstract—Production of the male specific compound, 6,10,13-trimethyltetradecyl isovalerate by the predatory stink bug *Eocanthecona furcellata* (Wolff) was dramatically affected by rearing conditions. Male bugs kept isolated after eclosion produced an average of 1,948 ng of 6,10,13-trimethyltetradecyl isovalerate per bug, whereas male bugs reared in groups of 5–8 bugs produced an average of only 4 ng of 6,10,13-trimethyltetradecyl isovalerate per bug. Same-sex or mixed-sex pairs of bugs produced less than 50 ng per bug. Male bugs kept isolated for 1 wk and then grouped for 1 wk produced 3 ng of 6,10,13-trimethyltetradecyl isovalerate per bug, whereas male bugs grouped first and then isolated produced 135 ng of 6,10,13-trimethyltetradecyl isovalerate. A total of 11 minor components in relative amounts of less than 1% of the major 6,10,13-trimethyltetradecyl isovalerate were found in the sternal gland secretion. These included 6,10,13-trimethyltetradecanol, acetate, propionate, and butyrate esters of 6,10,13-trimethyltetradecanol, and isovalerate or valerate esters of homologs of 6,10,13-trimethyltetradecanol.

Key Words—*Eocanthecona furcellata*, Pentatomidae, 6,10,13-trimethyltetradecyl isovalerate, 6,10,13-trimethyltetradecyl acetate, 6,10,13-trimethyltetradecyl propionate, 6,10,13-trimethyltetradecyl butyrate, sternal gland, rearing conditions.

* To whom correspondence should be addressed. E-mail: shine@gate.sinica.edu.tw

INTRODUCTION

In the study of semiochemicals from the predatory stink bug *Eocanthecona furcellata* (Wolff), 6,10,13-trimethyltetradecyl isovalerate extracted from sternal glands (SG) was found to be present in only 5% of mature virgin male bugs (Ho et al., 2003). In a related species, *Perillus bioculatus* (Fabricius), production of 6,10,13-trimethyltetradecyl isovalerate as an attractant pheromone was highest when males were starved first and then fed. It was proposed that stimulation of isovalerate production after feeding occurred in order to attract conspecifics to food sources (Aldrich et al., 1986). In another report (Aldrich and Lusby, 1986), a subset of male bugs of *Mineus strigipes* Herrich–Schaeffer and *Oplomus severus* Breddin with SG setae never produced a detectable amount of secretion, but starvation for 2–3 d after ecdysis followed by feeding was the most effective way of stimulating secretion.

In our initial attempts to obtain enough of the SG secretion from *E. furcellata* for bioassays, starving followed by feeding failed to stimulate the secretion of SG compounds from *E. furcellata*. Because 6,10,13-trimethyl-1-tetradecanol from the SG of *Stiretrus anchorago* (Fabricius) was found to be an aggregation pheromone (Kochansky et al., 1989), and SG of males of the male predatory stink bug *E. furcellata* produce compounds similar to that of *S. anchorago*, we hypothesized that the SG secretion of *E. furcellata* may be an aggregation pheromone as well. We further postulated that isolated bugs should produce more aggregation pheromone than bugs in groups because isolated bugs would use the chemical to attract conspecifics. The male-specific SG secretion may also have a role as a sex pheromone to attract female bugs. Thus, we hypothesized that male bugs grouped with females should produce minimal SG secretion to attract the opposite sex, whereas males grouped with males would still produce more SG secretion.

In this report, the amount of 6,10,13-trimethyltetradecyl isovalerate from SGs of virgin male bugs reared under different conditions, including being reared in isolation or in groups, and being reared in single or mixed sex pairs, was quantified and compared. Furthermore, because relatively large amounts of SG secretion could be collected from male bugs reared in isolation, other minor compounds in the SG secretion, mainly homologs of the major compound—6,10,13-trimethyltetradecyl isovalerate, were also identified.

METHODS AND MATERIALS

Insects. Bugs were reared on blowfly larvae, *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) in the laboratory under a 14:10 L:D regime at 23 $\pm$ 3°C and 70% relative humidity. The bugs were fed on alternate days *ad libitum*. Blowfly larvae were purchased from a fishing supply store. The colony was

maintained as described earlier in the literature (Chu and Chu, 1975a,b). Nymphs were kept in transparent plastic cups of 7 cm height $\times$13 cm diam. cup in groups of 10 bugs per cup. Adults were kept under different conditions as described later in this paper. The cups were covered with cheesecloth and a sugar–water impregnated cotton ball was placed at the top of the cup as the water source for the bugs.

Bugs Under Different Conditions. For the group-reared treatment, adults of the same sex were kept in groups of 5–8 bugs in 7 cm height $\times$13 cm diam. cup containers after eclosion. For the isolated rearing treatment, adults were kept individually in 250 ml transparent ice-cream cups after eclosion. For tests with pairs of bugs, two bugs, either both males of the same age or one male and one female of the same age, were kept in 250 ml ice-cream cups after eclosion. All these bugs were analyzed for the contents of the SG secretions 2 wk after eclosion.

To test the effects of grouping on previously isolated bugs, five bugs were kept individually for 1 wk and then brought together in a 7 cm height $\times$13 cm diam. cup for 1 wk before the extraction of SG compounds. For the converse test of grouped and then isolated adults, the bugs were kept in groups of five in the 7 cm height $\times$13 cm diam. cup for 1 wk, and then isolated individually in the 250 ml ice cream cups for 1 wk.

Extraction of Sternal Gland Contents of Adults. The bugs from various treatments were anesthetized with CO_2, and the ventral surface was washed with 40 μl of hexane. The extracts were analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). The amount of 6,10,13-trimethyltetradecyl isovalerate was quantified vs. benzophenone as an internal standard as described by Ho et al. (2003).

Chemical Analysis. The extracts were analyzed by splitless coupled GC–MS with a Thermo Quest Trace GC, interfaced with a Finnigan Trace MS (electron impact ionization, 70 eV). The GC was held at 60°C for 1 min, then programmed at 10°C/min to 300°C, with injector and transfer line temperatures of 200 and 250°C, respectively. A DB-1 column (30 m $\times$ 0.32 mm ID, J & W Scientific, Folsom, CA) was used in the GC–MS analyses with helium carrier gas.

Hydrolysis of 6,10,13-Trimethyltetradecyl Isovalerate. Ten micrograms of 6,10,13-trimethyltetradecyl isovalerate (authentic sample from Dr. J. R. Aldrich) were hydrolyzed in 200 μl of ethanol and 20 μl of 1N NaOH at 50°C for 8 hr. The hydrolyzed product, 6,10,13-trimethyltetradecanol, was extracted into hexane and analyzed by GC-MS, then used for esterification as described below.

Formation of Esters of 6,10,13-Trimethyltetradecanol. Acetyl chloride (50 μl) was added to a solution of 6,10,13-trimethyltetradecanol (1 μg) in hexane at 0°C. After 5 min, water was added to the reaction mixture, and the product was extracted with hexane. After filtration through anhydrous $MgSO_4$, the extract was concentrated for GC-MS analysis. Analogous procedures were used to esterify aliquots of the 6,10,13-trimethyltetradecanol solution with propionyl chloride, isobutyryl chloride, butyryl chloride, and valeryl chloride, respectively.

TABLE 1. AMOUNT OF 6,10,13-TRIMETHYLTETRADECYL ISOVALERATE FOUND IN THE SG OF VIRGIN MALE *E. furcellata* REARED UNDER DIFFERENT CONDITIONS. PAIRS OF TREATMENTS WERE COMPARED WITH *t*-TESTS

Rearing condition	Amount of 6,10,13-trimethyltetradecyl isovalerate (ng) (mean ± SE)	*P*
Grouped	4 ± 3 ($N = 20$)	$P < 0.001$
Isolated	1948 ± 525 ($N = 27$)	
Grouped first, then isolated	135 ± 109 ($N = 24$)	$P = 0.07$
Isolated first, then grouped	3 ± 3 ($N = 19$)	
Paired with another male	22 ± 13 ($N = 12$)	$P = 0.57$
Paired with another female	48 ± 43 ($N = 15$)	

RESULTS

Amounts of Isovalerate from Bugs Reared under Different Conditions. The amounts of 6,10,13-trimethyltetradecyl isovalerate produced by male bugs reared in isolation, in pairs, or in groups are shown in Table 1. Almost 500 times as much isovalerate was extracted from bugs reared in isolation as compared to bugs reared in groups (*t*-test, $P < 0.001$). Although the mean amount of isovalerate that was produced by bugs that were grouped first and then isolated was more than 40 times greater than the mean amount produced by bugs that were isolated first and then grouped, the two groups were not significantly different ($P = 0.07$), probably due to the extreme variability in the amounts produced per bug. There was also no difference in the mean amount of isovalerate recovered from males paired with other males or with females ($P = 0.57$).

Identification of Homologs of 6,10,13-Trimethyltetradecyl Isovalerate. Several compounds with mass spectra similar to that of 6,10,13-trimethyltetradecyl isovalerate were found in the SG extracts. Spectral data, possible structures, percentage relative to the most abundant compound, 6,10,13-trimethyltetradecyl isovalerate, and Kovats retention indices are listed in Table 2, and a chromatogram of the gland extract is shown in Figure 1. Detailed identification data are presented in subsequent paragraphs.

Peak 1: The compound gave a base peak of m/z 97, a molecular ion at m/z 256, and an M-18 peak (m/z 238), very similar to the mass spectrum of 6,10,13-trimethyltetradecanol in the SG secretion of *Oplomus dichrous* (Aldrich and Lusby, 1986). The mass spectrum and GC retention time matched those of an authentic standard generated by hydrolysis of 6,10,13-trimethyltetradecyl isovalerate, confirming the structure.

Peak 2: The mass spectrum of this compound was similar to that of 6,10,13-trimethyltetradecyl isovalerate, but its GC retention time was shorter. A prominent fragment at m/z 61 (McLafferty, 1980) suggested that it might be the acetate analog

TABLE 2. RELATIVE PERCENTAGES AND MASS SPECTRAL DATA OF COMPOUNDS IN THE SG SECRETION OF MALE *E. furcellata*. DIAGNOSTIC IONS ARE SHOWN IN BOLD

Peak No.[a]	Retention time (min)	Compound	Relative percentage[b]	Mass spectral data	Kovats index[c]
1	15.35	6,10,13-trimethyltetradecanol	0.12 ± 0.03	57 (96), 69 (62), 83 (33), **97 (100)**, 111 (19), 126 (14), 139 (1.6), 168 (1), 182 (3), 210 (0.1), **238 (1), 256 (0.01, M^+)**	1817
2	16.58	6,10,13-trimethyltetradecyl acetate	0.16 ± 0.08	43 (100), 57 (97), **61 (21)**, 69 (53), 83 (29), 97 (76), 111 (18), 126 (9), 182 (3), 213 (3), **256 (1), 298 (0.03, M^+)**	1940
3	17.33	IVb - 2 CH_2	0.04 ± 0.02	57 (100), 69 (44), 85 (68), **97 (88), 103 (78)**, 112 (13), 168 (2), 182 (2), **255 (0.3), 312 (0.3, M^+)**	2016
4	17.45	6,10,13-trimethyltetradecyl propionate	0.38 ± 0.09	57 (100), 69 (32), **75 (30), 97 (54)**, 111 (13), 126 (6), 154 (1.3), 182 (2.3), 210 (0.2), 238 (0.07), **283 (0.41), 312 (0.02, M^+)**	2028
5	18.17	IVa - CH_2	0.66 ± 0.07	57 (100), 69 (44), 85 (75), **97 (80), 103 (77)**, 111 (14), 126 (5), 168 (2), 182 (1), 196 (1), 224 (1), **269 (1), 326 (trace, M^+)**	2101
6	18.24	6,10,13-trimethyltetradecyl butyrate	0.06 ± 0.02	57 (100), 71 (69), **89 (60), 97 (93)**, 111 (18), 126 (8), 182 (3), 207 (0.29), **326 (0.02, M^+)**	2110
7	18.32	IVb - CH_2	0.18 ± 0.05	57 (100), 69 (42), 85 (52), **97 (75), 103 (78)**, 111 (11), 168 (2), 182 (0.13), 196 (0.75), 224 (0.6), **269 (0.7), 326 (0.03, M^+)**	2120
IV	18.72	6,10,13-trimethyltetradecyl isovalerate	100	57 (88), 69 (44), 85 (45), **97 (100), 103 (64)**, 111 (46), 126 (25), 182 (11), 210 (1), 238 (1), **283 (2), 325 (0.26), 340 (0.02, M^+)**	2163
8	19.01	IV isomer (IVa)	1.03 ± 0.07	57 (100), 69 (36), 85 (41), **97 (69), 103 (58)**, 111 (12), 126 (5), 182 (1.3), 210 (0.6), 238 (0.5), **283 (0.6), 340 (0.03, M^+)**	2205
9	19.13	IV isomer (IVb)	0.08 ± 0.02	57 (100), 69 (49), 85 (62), **97 (82), 103 (78)**, 111 (15), 126 (4), 182 (1), 210 (0.6), 238 (0.4), **283 (1.2), 340 (0.43, M^+)**	2217
10	19.51	IV + CH_2	0.02 ± 0.00	57(100), 71 (32), 85 (37), **97 (50), 103 (68)**, 111 (21), 126 (11), 182 (3), 196 (1), 252 (0.6), **297 (0.4), 354(trace, M^+)**	2263
11	19.82	IVa + CH_2	0.34 ± 0.05	57 (100), 71 (42), 85 (58), **97 (57), 103 (52)**, 111 (21), 126 (7), 182 (1), 196 (0.7), 224 (0.42), 252 (0.34), **297 (0.51), 354 (0.08, M^+)**	2299
12	20.37	IV + 2 CH_2	0.19 ± 0.02	45 (100), 57 (81), 69 (29), 85 (35), **97 (49), 103 (45),** 111 (13), 125 (9), 182 (0.77), 210 (0.5), 238 (0.47), 266 (0.23), **311 (0.05), 368 (0.01, M^+)**	2367

[a] Peak number corresponds to the peaks indicated in Figure 1.
[b] Relative percentage is to the major compound (6,10,13-trimethyltetradecyl isovalerate, abbreviated IV). $N = 3$, mean ± SE.
[c] Kovats indices calculated in relation to straight-chain alkanes.

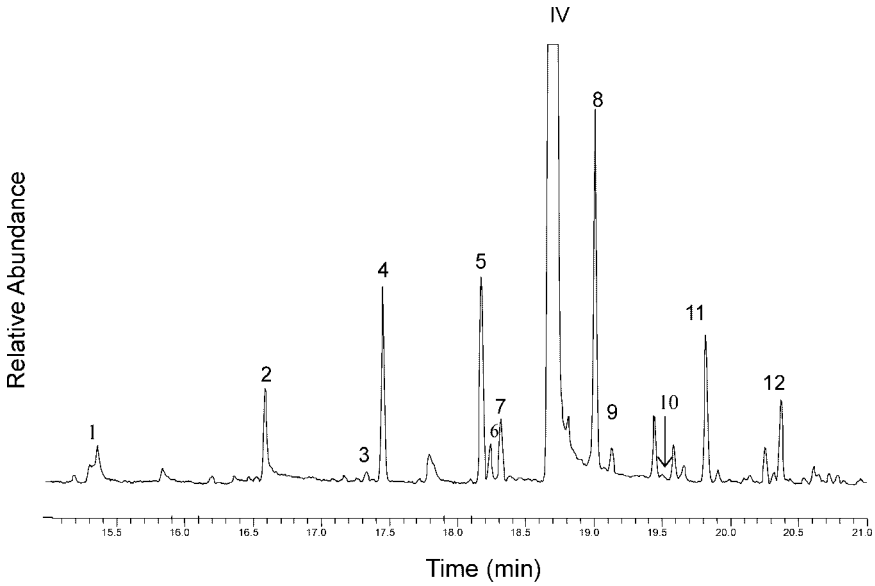

FIG. 1. Total ion chromatogram of the SG secretion of male *E. furcellata*. Compounds: **1**: 6,10,13-trimethyltetradecanol; **2**: 6,10,13-trimethyltetradecyl acetate; **3**: IVb - $2CH_2$; **4**: 6,10,13-trimethyltetradecyl propionate; **5**: IVa $-$ CH_2; **6**: 6,10,13-trimethyltetradecyl butyrate; **7:** IVb $-$ CH_2; **IV**: 6,10,13-trimethyltetradecyl isovalerate; **8**: IVa isomer; **9**: IVb isomer; **10**: IV $+$ CH_2; **11**: IVa $+$ CH_2; **12**: IV $+$ $2CH_2$. Column: DB-1. GC conditions are described in "Methods and Materials."

of 6,10,13-trimethyltetradecyl isovalerate, and the identification was confirmed with an authentic standard.

Peak 4: Because of the distinct ion at m/z 75, characteristic of fragmentation of a propionate ester (McLafferty, 1980), and a mass spectrum similar to that of 6,10,13-trimethyltetradecyl isovalerate, peak 4 was identified as 6,10,13-trimethyltetradecyl propionate, again confirmed with an authentic standard.

Peak 6: The fragment ion at m/z 89, characteristic of a butyrate or isobutyrate ester (McLafferty, 1980), and an otherwise similar mass spectrum to that of 6,10,13-trimethyltetradecyl isovalerate, suggested that this compound was 6,10, 13-trimethyltetradecyl butyrate or isobutyrate. The peak was confirmed as 6,10,13-trimethyltetradecyl butyrate with a standard.

Peaks 8 and 9: The mass spectra of peaks 8 and 9 were similar to that of 6,10,13-trimethyltetradecyl isovalerate, but these peaks had different retention times, suggesting that they might be isomers. The valerate ester of 6,10,13-trimethyltetradecanol was synthesized, but its retention time (19.08 min) was slightly different than that of peak 8 (19.01 min) and peak 9 (19.13 min). Thus, these

two compounds appear to be isomers of 6,10,13-trimethyltetradecyl isovalerate, but the exact structures are unknown.

Peaks 3, 5, 7, 10, 11, and 12: These six components had similar mass spectra in the lower mass ranges to that of 6,10,13-trimethyltetradecyl isovalerate, but different fragment ions in the higher mass ranges. All of these peaks had a significant *m/z* 103 fragment ion, typical of isovalerate or valerate esters. For peak 3, an ion at *m/z* 255 and a molecular ion of *m/z* 312, as compared with the analogous ion of *m/z* 283 and molecular ion of *m/z* 340 of the 6,10,13-trimethyltetradecyl isovalerate, suggested that peak 3 might be the isovalerate or valerate ester of an alcohol with similar structure but two methylene units fewer than 6,10,13-trimethyltetradecanol. Similarly, for peaks 5 and 7, with significant ions at *m/z* 269, and molecular ions of *m/z* 326, the compounds are proposed to be isovalerate or valerate esters of an alcohol with one methylene fewer than 6,10,13-trimethyltetradecanol. For peaks 10 and 11, which gave ions with *m/z* 297 and 354, the compounds are proposed to be the isovalerate or valerate ester of an alcohol with one methylene unit more than 6,10,13-trimethyltetradecanol. Similarly, for peak 12, with ions of *m/z* 311 and 368, the compound was suggested to be the isovalerate or valerate ester of an alcohol with two methylene units more than 6,10,13-trimethyltetradecanol.

These isomers with different chain lengths were further grouped into three series of homologs according to the Kovats retention indices. Peaks 3 and 7 were homologs of peak 9 (IVb) with one or two methylene units less than peak 9 (Kovats indices are 2016, 2120, and 2217, respectively). Peaks 5 and 11 were homologs of peak 8 (IVb) with one methylene unit less and one unit more than peak 8 (Kovats indices are 2101, 2299, and 2205, respectively). Peaks 10 and 12 are homologs of IV with one or two more methylene units than IV (Kovats indices are 2263, 2367, and 2163, respectively).

Thus, the evidence suggests that these six compounds are isovalerate or valerate esters of homologs of 6,10,13-trimethyltetradecanol, with extra or fewer methylene units in the carbon backbone, but their exact structures remain to be fully elucidated.

DISCUSSION

Amount of 6,10,13-Trimethyltetradecyl Isovalerate Produced by Bugs Reared under Different Conditions. Production of SG secretions of some asopine hemipterans was stimulated by first starving and then feeding adult bugs after eclosion (Aldrich and Lusby, 1986; Aldrich et al., 1986). In contrast, in our study, we found that rearing density was the key factor affecting the production of the SG secretion, with isolated bugs producing more than two orders of magnitude more of the isovalerate ester than bugs reared in groups. The dramatic decrease in production of the isovalerate ester in the SG secretion from grouped bugs in

comparison to isolated bugs may be triggered by a variety of cues, such as physical contact with other bugs, or visual, olfactory, or acoustic signals. This remains to be explored further experimentally.

A comparison of the amount of isovalerate from bugs grouped first and then isolated with that from bugs isolated first and then grouped suggested that a period of isolation stimulated the production of SG secretion, even in bugs that were initially held in a group before being isolated. However, comparison of the amount of isovalerate from male bugs paired with either a male or a female indicated that the sex of the companion did not affect the production of SG secretion. Thus, even though SG secretion is only produced by male bugs, and they are the only sex to possess the setiferous patches on the ventral abdominal surface for release of the pheromone, the pheromone may be an aggregation pheromone for attracting both sexes, rather than being for sexual attraction alone. Possible roles for the SG secretion currently are being tested in bioassays in our laboratory.

6,10,13-Trimethyltetradecanol and the corresponding isovalerate have been found from the SG of several asopine stink bugs (Aldrich and Lusby, 1986; Aldrich et al., 1986), and the alcohol alone was demonstrated to be an aggregation pheromone of *S. anchorago* (Kochansky et al., 1989). Our results showing that isolated bugs produce much more of the SG secretion than grouped bugs support the hypothesis of the SG secretion being used for aggregation purposes.

Homologs of 6,10,13-Trimethyltetradecyl Isovalerate. Of the minor compounds found in the SG secretion, only 6,10,13-trimethyltetradecanol has been reported before (Aldrich and Lusby, 1986; Kochansky et al., 1989). All the homologs of the isovalerate, including the acetate, propionate, butyrate, valerate, and isovalerate esters with different alcohol chain lengths, have never been reported from natural sources. Although the amounts of the homologs were small (less than 1% relative to the major isovalerate), it is possible that they have a biological function.

There were actually two series of homologs of the major isovalerate ester. In the first series, the acid portion of the esters were variable, and the structures in this series were confirmed as acetate, propionate, and butyrate esters of 6,10,13-trimethyltetradecanol (peaks 2, 4, and 6). In the second series, the acid portion was constant (isovaleric or possibly valeric acid), whereas the alcohol portion appeared to have a variable chain length, although the structures of these peaks (peaks 3, 5, 7, 10, 11, and 12) have not yet been confirmed. It remains unclear whether this plethora of minor homologs of the main component represents imprecision in the biosynthesis of the major compound, or whether these trace components of the SG secretion indeed have a biological function.

In conclusion, we demonstrated that rearing conditions can have a profound effect on the production of stink bug SG secretions. The generality of this observation is being studied for other bugs. In particular, if it is indeed a fairly general phenomenon that a simple change in the rearing conditions can dramatically

increase pheromone production for a variety of bug species, it will make it easier to collect sufficient quantities of secretions for identification and bioassays. As a case in point to be noted, the method of collecting relatively large amounts of the SG secretion and the identification of the homologs of isovalerate in the resulting SG secretion has accelerated studies on the function of the SG secretion that are currently in progress in our laboratory.

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