Aggregation Pheromone of *Tribolium freemani* HINTON (Coleoptera: Tenebrionidae)

I. Identification of the Aggregation Pheromone¹

Takahisa SUZUKI, Hiroshi NAKAKITA² and Yasumasa KUWAHARA

Institute of Applied Biochemistry, University of Tsukuba, Sakura, Niihari, Ibaraki 305, Japan

²*Stored-Product Laboratory, National Food Research Institute, Yatabe, Tsukuba, Ibaraki 305, Japan*

(Received January 23, 1987)

The presence of the aggregation pheromone of *Tribolium freemani* HINTON was confirmed. The pentane eluate of Porapak Q column from male beetles attracted both sexes. In the cross-attraction tests among *T. castaneum*, *T. confusum* and *T. freemani*, *T. castaneum* was attracted to *T. freemani* volatile, whereas *T. freemani* did not respond to *T. castaneum* pheromone. *T. freemani* was also attracted to *T. confusum* pheromone. However, the aggregation pheromone of *T. freemani* was identified as 4,8-dimethyldecanal on the basis of GLC and MS. This compound was a common aggregation pheromone among these three species, and was termed "Tribolure." The activity of "Tribolure" to *T. freemani* seemed to be largely dependent upon the composition ratios of the stereoisomers of the compound as well as other *Tribolium*.

INTRODUCTION

In a previous paper (SUZUKI and MORI, 1983), SUZUKI concluded that the aggregation pheromone of *T. castaneum* and *T. confusum* is (4*R*, 8*R*)-(—)-4,8-dimethyldecanal itself, on the basis of the bioassay result using the four stereoisomers of 4,8-dimethyldecanal (I) prepared by MORI et al. (1983). LEVINSON (LEVINSON and MORI, 1983) also obtained a similar result using the same samples prepared by MORI et al. (1983). Since then, however, SUZUKI et al. (1984) found that a mixture of (4*R*, 8*R*)-I and (4*R*, 8*S*)-I in a ratio of 8:2 was ten times more active than (4*R*, 8*R*)-I alone. This fact strongly suggests that the aggregation pheromone of *T. castaneum* may be a mixture of the stereoisomers of I.

Recently, NAKAKITA et al. (1981) rediscovered *T. freemani* HINTON (HINTON, 1948), another *Tribolium* species, in Japan in a shipment of corn imported from Brazil, after an interval of one century. *T. freemani* is also a potential stored-product pest, and is more closely related to *T. castaneum* than to *T. confusum*, because F₁ hybrids (sterile) are produced between *T. freemani* and *T. castaneum*, but not between *T. freemani* and *T. confusum* (NAKAKITA et al., 1981).

In the present work, we investigated the aggregation pheromone of this new species in the laboratory, and report the structure determination and the bioassay result of interspecific attraction among three species using their pheromones.

¹ This work was supported by funds of the Special Project on Instinct of the University of Tsukuba.

MATERIALS AND METHODS

Insects. Insects used were *T. freemani*, *T. castaneum* and *T. confusum*, and they were reared on wheat-feed (<20 mesh) containing 5% weight of dried brewer's yeast. *T. castaneum* and *T. confusum* were reared at 25°C, whereas *T. freemani* was reared at 30°C. This larvae of *T. freemani* were reared at a low density, 2 larvae per 1 g of feed in a 5-ml open vial, to prevent the crowding effect which causes inhibition of pupation (NAKAKITA, 1982). Pupae were sexed by observation of the genital lobes and reared in separate containers with medium until adult stage.

Chemicals. Racemic-I used in this study was synthesized by SUZUKI (1981). Optically pure (4*R*, 8*R*)-I was provided by Dr. K. MORI (MORI et al., 1985).

Bioassay procedure. The bioassay procedure was almost the same as described in a previous paper (SUZUKI and SUGAWARA, 1979), but is described here briefly. The method was two choice tests using plastic petri dishes (9 cm i.d.). Two paper disks, one of which was treated with the test material and the other a blank material or hexane only (control), were placed in diametrically opposite positions about 2.5 cm apart in the dish. Thirty beetles were released in the dish, and the number of beetles on each disk was recorded after ten minutes. Each test was repeated six times with new beetles. Attractiveness (%) was calculated from an equation $100 (S-C)/N$, where *S* and *C* are an average number of beetles on the treated and the control disks, respectively. *N* is the number of beetles used per test (=30).

Cross-attraction tests among three species, *T. freemani*, *T. castaneum* and *T. confusum*, were made by the same method as described above, although the latter two species were not sexed. The Porapak eluates of two species except *T. freemani* were obtained as by SUZUKI and SUGAWARA (1979).

Collection and purification of *T. freemani* pheromone. Insect volatiles were trapped by a method similar to that described previously (SUZUKI and SUGAWARA, 1979). The collection apparatus was composed of three 500-ml Erlenmeyer flasks and Porapak Q columns (4 mm×20 cm). Each flask containing (i) 570 virgin males plus 5 g wheat-feed, (ii) 650 virgin females plus 5 g feed, or (iii) 5 g feed only as a blank, was connected with a Porapak Q column. The apparatus was placed in a room at 25°C. Aeration was continued for 12 days by gentle suctioning with an aspirator. During this period, wheat-feed was renewed every 4 days. After 12 days, each Porapak column was eluted (back-washed) with 20 ml of pentane by suctioning with an aspirator. The male or female eluate was assayed against the blank eluate (control) at 10 adult equivalents (AE) per disk.

To obtain a larger amount of the pheromone, collection was further scaled up. A 500-ml Erlenmeyer flask containing 1850 virgin males (4- to 20- days-old) and 10 g wheat-feed was connected to a Porapak column, and aerated for 20 days as described above. During this period, wheat-feed was renewed every 4 days, and the column was eluted with 20 ml of pentane every 10 days. The combined eluates were concentrated to 0.5 ml by a rotary evaporator. The concentrate was then submitted to column chromatography using a 1.2×9 cm column of 20 g Wako-gel C-200. The column was developed successively with hexane, 3% ether-hexane, 5% ether-hexane and ether (40 ml each). Each fraction was bioassayed against control (hexane only) at 10–20 male equivalents (ME) per disk. The active fraction (3% ether-hexane) was concentrated to 0.1 ml, which was subjected to GLC analyses and mass spectrometry.

Gas liquid chromatography (GLC) and mass spectrometry (MS). GLC analyses were carried out on a Shimadzu GC-6A gas chromatograph equipped with a FID using a glass column (3 mm×2 m) packed with 5% SE-30 or 5% PEG-20M on Chromosorb WAW (both 80–100 mesh), a Hitachi 263-30 gas chromatograph with a fused silica CP-Sil 19 CB capillary column (0.22 mm×26 m, Chrompack), or a Yanaco G-180 gas chromatograph with a fused silica FFAP capillary column (0.22 mm×26 m).

The mass spectra were measured with a Hitachi double-focusing mass spectrometer model M-80 B coupled with a gas chromatograph model 367-0200. The operating conditions were as follows: column, OV-1 (packed); column temp., 120°C; He flow rate, 30 ml/min; ionization voltage, 70 eV; interface temp., 250°C; inj. temp., 200°C.

RESULTS

Presence of the male-secreted aggregation pheromone

The responses of male or female beetles of *T. freemani* to the Porapak eluates from both sexes against the blank eluate are given in Table 1, which clearly reveals the presence of male-secreted aggregation pheromone attracting both sexes. The pheromone appeared to be more attractive to females than males. Therefore, females were used for the assay thereafter.

Interspecific attraction among the three species

The result of the cross-attraction tests among the three species is shown in Table 2. *T. castaneum* was attracted to *T. freemani* pheromone, whereas *T. freemani* did not respond to *T. castaneum* pheromone. *T. freemani*, however, was attracted to *T. confusum* pheromone.

Identification of the pheromone

The gas chromatogram of the male volatile of *T. freemani* is shown in Fig. 1, and was identical with that of *T. castaneum*. The peak No. 4 ($t_R=10.22$ min) was present only in the male volatile, and was the aggregation pheromone, 4,8-dimethyldecanal. The chromatogram also showed the presence of the following components of the defense substances: 2-methylbenzoquinone (peak No. 1), 2-ethylbenzoquinone (2), 1-tetradecene (3), 1,6-pentadecadiene (5), 1-pentadecene (6), 1-hexadecene (7), 1,8-heptadecadiene (8) and 1-heptadecene (9), respectively (SUZUKI, unpublished). These eight

Table 1. Response of *T. freemani* to Porapak eluates from males or females

Porapak eluate from	Sex tested ^a	Dose ^b	Response ^c		Attractiveness (%) ^d
			Beetles + food (S)	Food (C)	
Male + food	Male	10	15.8±3.2	5.7±2.6	33.7*
Male + food	Female	10	20.0±1.8	5.2±2.1	49.3*
Female + food	Male	10	10.7±3.4	9.0±3.0	5.7 NS
Female + food	Female	10	8.0±4.8	7.8±2.8	0.7 NS

^a Thirty virgin beetles (4- to 10-days-old) were used in each test.

^b Adult equivalent (AE)/disk.

^c Average no. of beetles gathering on each disk (6 replicates, 10 min exposure; $\bar{x} \pm SE$).

^d Value of 100 (S-C)/30.

* Significant at 1% level; NS: not significant.

Table 2. Interspecific attraction among females of *T. freemani*, *T. castaneum* and *T. confusum*

Porapak eluate from	Assay insect	Dose ^a	Response		Attractiveness (%)
			Beetles+food (S)	Food (C)	
<i>T. freemani</i>	<i>T. castaneum</i>	10	17.8±3.4	5.7±2.1	40.3*
	<i>T. confusum</i>	10	9.8±4.4	10.0±5.9	-0.7 NS
<i>T. castaneum</i>	<i>T. freemani</i>	10	15.2±6.8	8.0±1.9	12.0 NS
	<i>T. castaneum</i>	10	20.7±2.7	4.7±2.0	53.0*
<i>T. confusum</i>	<i>T. freemani</i>	20	15.0±1.4	3.2±1.5	39.3*
	<i>T. castaneum</i>	20	18.8±1.5	7.0±2.1	39.3*

^a Male equivalent (ME)/disk. Fig. 1. Gas chromatogram of male volatile of *T. freemani*. Condition: column, CP-Sil 19 CB capillary column; column temp., 90–140°C, 4°C/min. Numbers refer to the text.

components were the same as those found in the other two species (SUZUKI et al., 1975). Other unidentified peaks in Fig. 1 were present in the blank eluate.

The retention times (t_R) of the pheromones of *T. freemani* and *T. castaneum* measured under different conditions are given in Table 3. These t_R values of the pheromone of *T. freemani* were in agreement with those of *T. castaneum* and 4,8-dimethyldecanal.

The mass spectrum of the pheromone obtained from the active fraction eluted with 3% ether-hexane is shown with that of racemic-I in Fig. 2. The MS spectrum of *T. freemani* pheromone was identical with that of I.

Response of *T. freemani* females to (4R, 8R)-I

(4R, 8R)-I was active to *T. freemani* females at more amounts than 25 ng/disk, whereas the racemic-I was inactive even at 100 ng/disk (Table 4).

Table 3. Retention times (t_R) of the natural and synthetic pheromones

Pheromone source	GLC condition				
	1	2	3	4	5
<i>T. freemani</i>	5.00 min	4.12	18.93	10.22	14.53
<i>T. castaneum</i>	5.04	4.12	18.95	10.25	14.50
Racemic-I	5.00	4.12	18.92	10.32	14.60
(4 <i>R</i> , 8 <i>R</i>)-I	4.95	4.10	18.91	10.22	14.52

Condition 1: 5% SE-30, 3 mm×2 m (packed), 140°C.

Condition 2: 5% PEG-20M, 3 mm×2 m (packed), 140°C.

Condition 3: fused silica CP-Sil 19 CB capillary column, 0.22 mm×26 m, 100°C.

Condition 4: CP-Sil 19 CB capillary column, 90–140°C, 4°C/min.

Condition 5: fused silica FFAP capillary column, 0.22 mm×26 m, 100°C.

Fig. 2. Mass spectra of *T. freemani* pheromone and 4,8-dimethyldecanal.

Table 4. Response of *T. freemani* females to (4*R*, 8*R*)-I and racemic-I

Sample	Dose (ng/disk)	Response		Attractiveness (%)
		Sample (<i>S</i>)	Control (<i>C</i>)	
(4 <i>R</i> , 8 <i>R</i>)-I	1.0	6.4±2.4	5.0±2.1	5.0 NS
	10	10.2±2.1	6.2±2.4	13.3 NS
	25	15.5±3.9	8.2±3.6	24.3*
	100	18.5±1.4	4.7±1.5	46.0*
Racemic-I	100	8.5±2.4	4.8±2.4	12.0 NS

DISCUSSION

The male-secreted aggregation pheromone of *T. freemani* was identical with those of *T. castaneum* and *T. confusum*, which was identified as 4,8-dimethyldecanal on the basis of GLC and MS, although its absolute configuration remained to be determined. Consequently, this is a common aggregation pheromone among these three species and we termed this compound "Tribolure." Tribolure has two asymmetric carbon atoms, and four stereoisomers could be present.

Interspecific attraction among these three species gave a very interesting result which is illustrated in Fig. 3. *T. castaneum* and *T. confusum* were cross-attractive to each other as reported previously (SUZUKI and SUGAWARA, 1979). It is noticeable that *T. freemani*, a more closely related species to *T. castaneum* than *T. confusum*, did not respond to *T. castaneum*, but was attracted to the *T. confusum* pheromone. FAUSTINI et al. (1982) reported that *T. brevicornis* was highly attractive to *T. castaneum* and *T. confusum*, but the latter two species did not respond to *T. brevicornis*. HINTON (1948) subdivided *Tribolium* into five species-groups: *brevicornis*, *confusum*, *alcine*, *castaneum* and *myrmecophilum*. *T. freemani* belongs to the *castaneum* group. *T. brevicornis* is regarded as the most primitive.

The cross-activity of each pheromone of the three species used in the present study seemed to be largely dependent upon the composition ratios of the stereoisomers of 4,8-dimethyldecanal. There are several supporting evidences: (1) no other peak except 4,8-dimethyldecanal was found as a pheromone constituent in the male volatiles of these three species; (2) in silicic acid column chromatography, only the 3% ether-

Fig. 3. Correlation of cross-attraction among the three species.

hexane fraction was biologically active; (3) as reported previously (SUZUKI et al., 1984), the activity of the mixture of (4*R*, 8*R*)-I and (4*R*, 8*S*)-I in a ratio of 8:2 was ten times more active than (4*R*, 8*R*)-I alone to *T. castaneum*; (4) the reaction product of 4,8-dimethyldecanal (racemate) with (*R*)-(+)- α -methoxy- α -tri-fluoromethylphenylacetic acid chloride (MTPA) or (–)-menthyl chloroformate, optical purity analysis reagent, was not separable in GLC analyses using fused silica capillary columns (SUZUKI, unpublished); (5) *T. freemani* was significantly attracted to (4*R*, 8*R*)-I at amounts greater than 25 ng/disk (Table 4) and (6) (4*R*, 8*R*)-I showed activity equal to the natural pheromone of *T. castaneum* when used alone (SUZUKI and MORI, 1983).

By these evidences the aggregation pheromone of the three species used in this study was concluded to be a mixture of (4*R*, 8*R*)-I (main active component) and more than one other stereoisomer. A similar example was found in the sawflies (MATSUMURA et al., 1979; KIKUKAWA et al., 1982; OLAIFA et al., 1984). In this case, their sex pheromones, 2,3-dimethylpentadecanoates (II), were composed of (2*S*, 3*S*, 7*S*)-II and its diastereomers (2*S*, 3*R*, 7*R*)- or (2*S*, 3*R*, 7*S*)-II, in combination with their acetate or propionate.

The bioassay using four optically pure stereoisomers of 4,8-dimethyldecanal is now in progress.

REFERENCES

- FAUSTINI, D. L., J. R. ROWE and W. E. BURKHOLDER (1982) A male-produced aggregation pheromone in *Tribolium brevicornis* (LECONTE) (Coleoptera: Tenebrionidae) and interspecific responses of several *Tribolium* species. *J. Stored Prod. Res.* **18**: 153–158.
- HINTON (1948) A synopsis of the genus *Tribolium* MACLEAY, with some remarks on the evolution of its species-groups (Coleoptera, Tenebrionidae). *Bull. Entomol. Res.* **39**: 13–55.
- KIKUKAWA, T., F. MATSUMURA, M. KRAEMER, H. C. COPPEL and A. TAI (1982) Field attractiveness of chirally defined synthetic attractants to males of *Diprion similis* and *Gilpinia frutetorum*. *J. Chem. Ecol.* **8**: 301–314.
- LEVINSON, H. Z. and K. MORI (1983) Chirality determines pheromone activity for flour beetles. *Naturwissenschaften* **70**: 190–192.
- MATSUMURA, F., A. TAI, H. C. COPPEL and M. IMAIDA (1979) Chiral specificity of the sex pheromone of the red-headed pine sawfly, *Neodiprion lecontei*. *J. Chem. Ecol.* **5**: 237–249.
- MORI, K., M. KATO and S. KUWAHARA (1985) New synthesis of (4*R*, 8*R*)-4,8-dimethyldecanal, the aggregation pheromone of *Tribolium castaneum*, and its (4*R*, 8*S*)-isomer. *Liebigs Ann. Chem.* **1985**: 861–865.
- MORI, K., S. KUWAHARA and H. UEDA (1983) Synthesis of all the four possible stereoisomers of 4,8-dimethyldecanal, the aggregation pheromone of the flour beetles. *Tetrahedron* **39**: 2439–2444.
- NAKAKITA, H. (1982) Effect of larval density on pupation of *Tribolium freemani* HINTON (Coleoptera: Tenebrionidae). *Appl. Ent. Zool.* **17**: 269–276.
- NAKAKITA, H., O. IMURA and R. G. WINKS (1981) Hybridization between *Tribolium freemani* HINTON and *Tribolium castaneum* (HERBST), and some preliminary studies on the biology of *Tribolium freemani* (Coleoptera: Tenebrionidae). *Appl. Ent. Zool.* **16**: 209–215.
- OLAIFA, J. I., T. KIKUKAWA, F. MATSUMURA and H. C. COPPEL (1984) Response of male jack pine sawfly, *Neodiprion pratti banksianae* (Hymenoptera: Diprionidae), to mixtures of optical isomers of the sex pheromone, 3,7-dimethylpentadecan-2-ol. *Environ. Entomol.* **13**: 1274–1277.
- SUZUKI, T. (1981) A facile synthesis of 4,8-dimethyldecanal, aggregation pheromone of flour beetles, and its analogues. *Agric. Biol. Chem.* **45**: 2641–2643.
- SUZUKI, T. and K. MORI (1983) (4*R*, 8*R*)-(–)-4,8-Dimethyldecanal: The natural aggregation pheromone of the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae). *Appl. Ent. Zool.* **18**:

134–136.

- SUZUKI, T. and R. SUGAWARA (1979) Isolation of an aggregation pheromone from the flour beetles, *Tribolium castaneum* and *T. confusum* (Coleoptera: Tenebrionidae). *Appl. Ent. Zool.* **14**: 228–230.
- SUZUKI, T., J. KOZAKI, R. SUGAWARA and K. MORI (1984) Biological activities of the analogs of the aggregation pheromone of *Tribolium castaneum* (Coleoptera: Tenebrionidae). *Appl. Ent. Zool.* **19**: 15–20.
- SUZUKI, T., T. SUZUKI, V. M. HUYNH and T. MUTO (1975) Hydrocarbon repellents isolated from *Tribolium castaneum* and *T. confusum* (Coleoptera: Tenebrionidae). *Agric. Biol. Chem.* **39**: 2207–2211.