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DETERMINATION OF CHIRALITY IN 5-HYDROXY-4-METHYL-3-HEPTANONE, THE AGGREGATION PHEROMONE OF *Sitophilus oryzae* (L.) AND *S. zeamais* Motschulsky[1]

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Abstract—The chirality of the pheromone of the rice weevil, *Sitophilus oryzae* (L.), and the maize weevil, *S. zeamais* (Motschulsky), 5-hydroxy-4-methyl-3-heptanone, was determined using an acetyl lactate derivatization procedure. Maize weevils were shown to produce >98% 4*S*,5*R*. Determination was more difficult with rice weevils due to a smaller quantity of insect extract, but they were shown to produce at least 92% 4*S*,5*R*.

The attractancy of the four synthetic stereoisomers of 5-hydroxy-4-methyl-3-heptanone was tested using rice and maize weevils. As expected, both species were most strongly attracted to the 4*S*,5*R* enantiomer. Maize weevils also showed low but significant responses ($P < 0.05$) to both 4*R*,5*R* and 4*S*,5*S*. Rice weevils showed a highly significant ($P < 0.01$) response to 4*R*,5*S*, although it was only about one third the response to 4*S*,5*R*. Thus, (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone is clearly the major component of the pheromone of both *S. zeamais* and *S. oryzae*.

[1] This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or a recommendation for its use by USDA.

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Key Words—Chirality, enantiomers, 5-hyroxy-4-methyl-3-heptanone, stereoisomer, *Sitophilus oryzae*, *Sitophilus zeamais*, rice weevil, maize weevil, aggregation pheromone, Coleoptera, Curculionidae.

INTRODUCTION

Sitophilus spp. weevils cause severe damage to cereal grains throughout the world, through direct feeding on grain kernels. The male-produced aggregation pheromone of the rice weevil, *Sitophilus oryzae* (L.) and the maize weevil, *S. zeamais* Motschulsky, has been identified as (*R**,*S**)-5-hydroxy-4-methyl-3-heptanone,[2] of unknown enantiomeric composition (Schmuff et al., 1984; Phillips et al., 1985). Some response to the *R**,*R** diastereomer by both species was observed in the laboratory, but this compound was present at less than 0.5% in natural pheromone samples.

5-Hydroxy-4-methyl-3-heptanone possesses two asymmetric centers and thus can exist as four different stereoisomers (Figure 1). The asymmetric center at position four is adjacent to the ketone and as such is susceptible to racemization by either acid or especially base. The hydroxyl group beta to the ketone makes the molecule even more labile in that retro aldol cleavage or facile dehydration can occur with complete loss of stereochemistry. At the outset of this work we were unsure as to whether these properties would prevent the determination of the chirality through racemization or elimination of the required acetyl lactate diastereomeric ester. The objective of this work was to determine the natural enantiomeric ratios in maize and rice weevils, and to examine the ability of both species to distinguish between the four synthetic stereoisomers.

METHODS AND MATERIALS

Insects. Rice and maize weevils were obtained from laboratory stock cultures and were reared according to procedures previously described (Phillips and Burkholder, 1981; Walgenbach et al., 1983).

Weevils for experimental use were collected on day of emergence by sieving and were separated by sex according to dimorphic rostral characteristics (Halstead, 1963). Weevils were held on wheat at constant densities on a 16:8 light–dark regime and 27° ± 1°C and 65 ± 5% relative humidity.

Extracts. Collections of pheromone from individual virgin male maize and rice weevils were made as previously described (Phillips and Burkholder, 1981). Parallel collections from cracked wheat controls were made at the same time.

[2] *R**,*S** represents an unknown mixture of the 4*R*,5*S* and 4*S*,5*R* enantiomers. Similarly, *R**,*R** represents an unknown mixture of 4*R*,5*R* and 4*S*,5*S*.

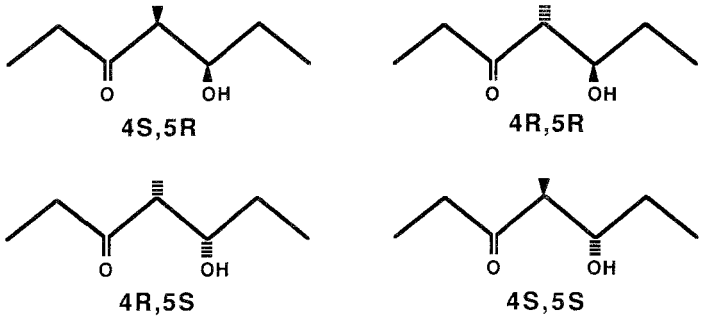

FIG. 1. Stereoisomers of 5-hydroxy-4-methyl-3-heptanone.

Glass wool-filtered hexane extracts containing 1152 insect-day equivalents (IDE) (two samples) for maize weevils, 1464 and 1500 IDE for rice weevils, and 1000 DE (two samples) for the cracked wheat control were each divided into two portions. Half the material was purified on Florisil by elution with hexane–diethyl ether (1 : 1) as previously described (Phillips et al., 1985). The samples were concentrated under N_2 to approximately 200 μl and placed in silanized screw-top vials fitted with a Teflon liner. The four samples for each species and cracked wheat control, plus the chiral standards, were shipped via air express to Vancouver, in a container maintained at $-78.5°C$ by dry ice. On arrival, samples were transferred to a low-temperature freezer for continued maintenance at $-78.5°C$.

Synthetic Standards. A racemic sample of 5-hydroxy-4-methyl-3-heptanone containing approximately equal amounts of *R*,S** and *R*,R** diastereomers (Phillips et al., 1985) was used to establish reaction conditions, capillary gas chromatograph column selection, and the operating conditions. The four optically pure stereoisomers of 5-hydroxy-4-methyl-3-heptanone used as standards in this study were synthesized by Mori and Ebata (1986). Chemical purity for all four samples was >98%.

Analyses. Splitless capillary gas chromatography was carried out on a Hewlett Packard HP 5890 using a 30-m × 0.25-mm ID fused silica column with injector and detector temperatures of 250°C. The column, SP-2340 (Supelco Inc., Bellefonte, Pennsylvania), was temperature programmed as follows: 100°C for 2 min, 20°C/min to 150°C, and isothermal at 150°C. Flame ionization detection was employed with a helium carrier and makeup gas. All samples were chromatographed to ascertain concentrations and ensure that no underlying impurity existed at the retention time of the acetyl lactate derivatives. Splitless gas chromatography–mass spectroscopy (GC-MS) was carried out on a Hewlett Packard HP 5985B in which the same SP-2340 column had been

installed. The temperature program was identical, except the initial temperature of 100°C was held for only 1 min.

Derivatization of Samples. To the sample contained in 10 μl of hexane, a benzene solution (5 μl) containing dimethylaminopyridine (250 μg) was added followed immediately by the acetyl lactate reagent [8 μl of methylene dichloride solution containing 200 μg of the acetyl lactyl chloride prepared as previously described (Slessor et al., 1985)]. When larger or smaller aliquots were used, the reagent amounts were scaled proportionately. The reactions were sealed in micro vials and kept at room temperature for 24–48 hr. Work-up involved the addition of 10 μl hexane followed by washes with 1% aqueous HCl (1× 50 μl), 5% aqueous NaOH (4× 50 μl), 5% aqueous $NaHCO_3$ (1× 50 μl), and finally water (1× 50 μl).

Bioassay. The dual-choice pitfall bioassay developed by Phillips and Burkholder (1981) was used to test the attractancy of synthetic stereoisomers. Bioassays were conducted as previously described (Walgenbach et al., 1983) except that they ran for 30 min.

For the first experiment, (4*R*,5*S*), (4*S*,5*R*), and *RS*, a racemic mixture of the two enantiomers, were tested. In the second experiment, (4*R*,5*R*), (4*S*,5*S*), and *RR* (racemic mixture) were tested, with a 4*S*,5*R* series included as a positive control.

Test samples were diluted in UV-grade hexane such that 2 μl of solvent was applied. The same amount of solvent was used in the control vessel of each pitfall chamber. For rice weevils, 38 ng of each stereoisomer or mixture were tested. Maize weevils were tested using 100 ng.

Statistical Analysis. Response (treatment − control) was calculated and paired *t*-tests were used to determine the attractancy of each isomer. Isomers were compared using an analysis of variance (ANOVA). Significance was accepted at $P < 0.05$ for both tests. When the *F* test proved significant, means were compared using a Duncan's (1955) multiple-range test, also at $P < 0.05$. If male and female responses were statistically equivalent, results were pooled.

The sum of treatment and control was also calculated and was used as a measure of insect activity. Insect activities generated by the isomers were compared using ANOVA at $P < 0.05$.

RESULTS

Determination of Chirality. Concentrations of the pheromone samples ranged from 20 to 24 ng/μl for the maize weevil and 7 to 32 ng/μl for the rice weevil. All insect extracts showed the later eluting *R**,*S** diastereomer to be predominant, indicating that little, if any, epimerization had occurred on preparation and transfer of the samples.

Acetyl lactate formation and work-up of the dilute synthetic pheromone produced a sample which exhibited four baseline separated peaks when gas chromatographed on a 30-m SP-2340 capillary column. Other columns used gave varying degrees of separation, none of which were as good as the SP-2340. GC-MS showed virtually identical fragmentation patterns for all of the four diastereomers, with intense ions at *m/z* 115(71); 97(45); 87(75); 70(40); 57(100); 43(35).

Derivatization of the maize weevil pheromone in both purified and crude preparations showed 90–99% of the 4*S*,5*R* stereoisomer (Table 1), with a small amount of material running marginally faster than the 4*R*,5*R*. Examination of the GC-MS of maize weevil sample 2, Florisil acetyl lactate derivative (96%), revealed the latter peak to be primarily an impurity [*m/z* 133(100); 57(43)] and comprised of at most 50% 4*R*,5*R* isomer. The maize weevil pheromone is accordingly 98% or higher (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone.

The lower pheromone amounts produced by the rice weevil yielded samples with larger impurity concentrations for a given amount of pheromone. This led to much more varied (55–97%) and less accurate determinations, but all showed high 4*S*,5*R* chirality. Again, the marginally faster peak was present in the region of the 4*R*,5*R* isomer and in about the same absolute amount found in maize weevil samples. GC-MS of rice weevil sample 4, crude acetyl lactate derivative, indicated the same impurity containing no more than 50% 4*R*,5*R* isomer. These results indicate the chirality to be at least 92% 4*S*,5*R*, and rice weevil sample 2, Florisil acetyl lactate derivative, showed this value to be at least 97%. These results indicate that the rice weevil pheromone is greater than 92% (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone.

Weevil Response to Synthetic Stereoisomers. In the first experiment, maize weevils were most strongly attracted to 4*S*,5*R* with a mean response of 7.1 ($P < 0.001$; Figure 2a). The response to 4*R*,5*S* was not significant ($\bar{X} = 1.2$; $P > 0.05$). The response to the *RS* mixture fell between the responses to the two enantiomers and was highly significant ($\bar{X} = 4.3$; $P < 0.001$). ANOVA indicated that the responses to the *RS* mixture and 4*S*,5*R* were significantly higher than the response to 4*R*,5*S*. However, all activity values were high ($\bar{X}$ range 6.5–8.3) and were not significantly different from one another. The 4*R*,5*S* isomer is apparently close enough to the pheromone to get the insects moving, but not close enough to direct them, and they fall randomly into the treatment and control traps.

In experiment 2, 4*S*,5*R* again proved strongly attractive ($\bar{X} = 6.3$; $P < 0.001$) (Figure 2b). Low, but significant responses to 4*S*,5*S* ($\bar{X} = 1.7$; $P < 0.05$) and 4*R*,5*R* ($\bar{X} = 1.0$; $P < 0.05$) were observed. However, response to the *RR* mixture was not significant ($\bar{X} = 0.6$; $P > 0.05$). The activity values were correspondingly low. Responses to (4*R*,5*R*), (4*S*,5*S*), and *RR* were statistically equivalent, but 4*S*,5*R* was significantly more attractive than all others.

TABLE 1. RETENTION TIMES AND INTEGRATOR COUNTS FOR SITOPHINONE STEREOISOMERS AND THEIR *S*-(+)-ACETYL LACTATE DERIVATIVES[a]

	Underivatized		Acetyl lactate derivative	
Source and preparation	Time	Counts	Time	Counts
Synthetic 4*S*,5*R*	5.37		15.10	
4*R*,5*S*	5.37		15.39	
4*R*,5*R*	5.12		15.69	
4*S*,5*S*	5.13		16.05	
MW 1, Florisil	5.37	101	15.11	63
			15.59	<1
MW 2, Florisil	5.12	2	15.07	118
	5.37	119	15.59	5
MW 3, Crude	5.12	2	15.06	106
	5.37	107	15.57	5
MW 4, Crude	5.12	1	15.02	114
	5.36	114	15.52	8
RW 1, Florisil	5.37	35	15.13	160
			15.62	47
RW 2, Florisil	5.37	125	15.13	105
			15.64	3
RW 3, Crude	5.37	74	15.04	38
			15.55	10
RW 4, Crude	5.12	3	15.11	133
	5.37	162	15.61	14
CW 1, Florisil			15.59	4
CW 2, Florisil			15.61	4
CW 3, Crude	5.37	6	15.12	2
CW 4, Crude	5.36	4		

[a] MW = maize weevil; RW = rice weevil; CW = cracked wheat control. Integrator counts are proportional to peak area.

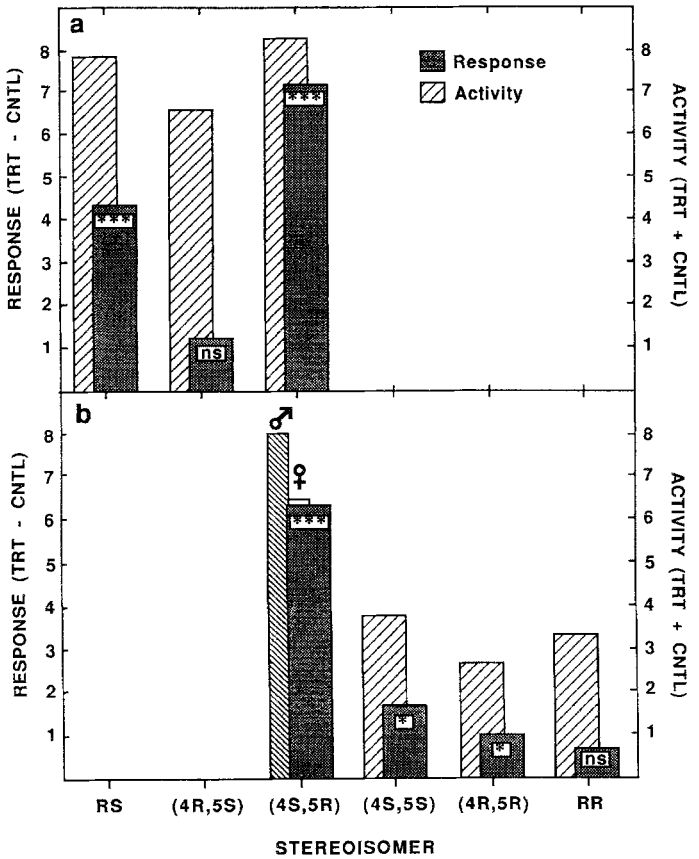

FIG. 2. Mean response (treatment − control) and activity (treatment + control) of maize weevils to stereoisomers of 5-hydroxy-4-methyl-3-heptanone. Male and female results combined for analysis, unless significantly different, as indicated. ***$P < 0.001$; *$P < 0.05$; ns, not significant, Student's t test for paired data. (a) experiment 1; (b) experiment 2.

The rice weevil profile for both experiments was similar to that of the maize weevil (Figure 3). In experiment 1, response was strongest to $4S,5R$ ($\bar{X} = 7.5$; $P < 0.001$), followed closely by the RS mixture ($\bar{X} = 5.8$; $P < 0.001$). The $4R,5S$ enantiomer proved least attractive, but in contrast to the maize weevil, the lower response still proved highly significant ($\bar{X} = 2.6$; $P < 0.01$). The response to $4R,5S$ was significantly lower than the response to either the RS mixture or $4S,5R$. This trend held true for activity as well. All activity values were high (>6.4), but activation by both RS and $4S,5R$ was significantly greater than for $4R,5S$.

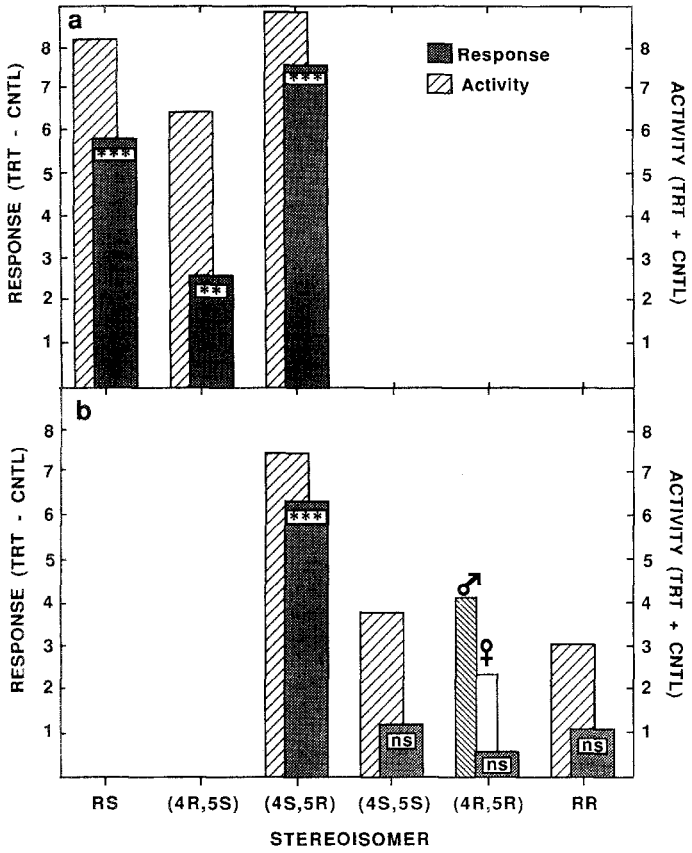

FIG. 3. Mean response (treatment − control) and activity (treatment + control) of rice weevils to stereoisomers of 5-hydroxy-4-methyl-3-heptanone. Male and female results combined for analysis, unless significantly different, as indicated. ***$P < 0.001$; **$P < 0.01$; ns, not significant, Student's t test for paired data. (a) experiment 1; (b) experiment 2.

In experiment 2, rice weevil results were again similar to those of the maize weevil (Figure 3b). The $4S,5R$ isomer generated a mean response of 6.3 ($P < 0.001$). However, rice weevils did not respond significantly to $4S,5S$ ($\bar{X} = 1.2$; $P > 0.05$) nor $4R,5R$ ($\bar{X} = 0.6$; $P > 0.05$), as had maize weevils. Neither was there a significant response to the RR mixture ($\bar{X} = 1.1$; $P > 0.05$). These results were paralleled by low activity values. As with maize weevils, the response to $4S,5R$ was significantly higher than the responses to all other isomers.

DISCUSSION

It is clear that the pheromone of the *S. zeamais* and *S. oryzae* is defined by its optical as well as chemical properties. This is well-documented in other beetle species as well as in other groups of insects (Silverstein, 1979; Mori, 1984). Both maize and rice weevils responded most strongly to the 4*S*,5*R* isomer of 5-hydroxy-4-methyl-3-heptanone, and it has been proven to be the major component of the aggregation pheromone of both species. In addition, some interesting differences were observed in the specificity of the response of the two species to the other three stereoisomers.

In both species, the *RS* enantiomeric mixture produced somewhat lower responses than 4*S*,5*R* alone. However, only half as much 4*S*,5*R* was tested in the *RS* mixture, and thus the effect could simply be due to the lower concentration of this enantiomer.

The low responses observed by both species to the *RR* enantiomeric mixture contradict previous work (Phillips et al., 1985), where both species showed comparable responses to (*R**,*S**), (*R**,*R**), and a racemic mixture of the diastereomers. Since the present study indicated that optically pure 4*S*,5*S* and 4*R*,5*R* generated responses much lower than to the 4*S*,5*R* stereoisomer or the *RS* mixture, contamination of the mixtures used in the 1985 study is implied. Indeed, examination of gas chromatograph traces of *R**,*R** used in the previous study suggest the presence of 2–4% *R**,*S**. This represents <1–8 ng of 4*S*,5*R* in the 1985 work, but maize weevils are known to respond to amounts this low (Walgenbach et al., 1983).

Walgenbach and Burkholder (1986) showed that weevil activity (treatment + control) was a valuable additional tool in interpreting pitfall bioassay results. In the present study, activity appears to be a gauge of the relative "closeness" of the stereoisomer to 4*S*,5*R*. For instance, for these species, both response and activity to 4*S*,5*R* were high, indicating that many weevils were trapped in the treatment receptacle and few in the control receptacle. Similarly, low responses and activities were observed for the 4*R*,5*R* and 4*S*,5*S* stereoisomers, indicating that few insects were trapped in either receptacle. However, activities to 4*R*,5*S* were high compared to relatively low responses. This indicates that many insects left the pitfall chamber and were trapped, but they fell more or less randomly into both receptacles. Using this activity information in conjunction with response data may thus be helpful in interpreting which characteristics of the molecule are important in receptor specificity. When response and activity are both high, it is an indication that the weevils are not only activated, but their response is directional, since few incorrect (i.e., control) choices are made. Almost all responses are to the treatment, so response (T − C) and activity (T + C) are very close. For compounds where the receptor fit is interpreted to be nearly correct, high activities, but low responses are observed. The compound

is able to "activate" the insects, but is not able to direct them to its source. Thus, for maize and rice weevils, the need for the chiral centers to have opposite configurations is nearly as important to receptor binding as the specific orientation at each center.

The major component of the pheromone of both *S. zeamais* and *S. oryzae* has herein been shown to be (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone. While the differential attractiveness of the other stereoisomers may simply reflect imperfect receptor specificity, it is possible that these responses reflect differences in the makeup of the maize and rice weevil pheromones, which could have important implications in reproductive isolation, as well as interspecific competition.

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