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UNUSUAL POLYMETHYL ALKENES IN TSETSE FLIES ACTING AS ABSTINON IN *Glossina morsitans*

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Abstract—The major alkene of the male tsetse fly, *Glossina morsitans morsitans*, was isolated for characterization by thin-layer and gas chromatography (GC). The mass spectra of the alkene and the alkene DMDS derivative indicated one isomer, 19,23-dimethyltritriacont-1-ene. The material is present at 1–2 μg/male fly and is partially transferred to the female preparatory to or during mating. A dose-dependent antiaphrodisiac effect was seen with exposed male flies using the isolated natural product, with 2 and 4 μg causing 80% loss of copulatory attempts, and 10 μg extinguishing the attempts. This effect was increased by addition of male-produced alkane. This compound and a 31-carbon homolog also appear in *G. m. submorsitans*. Similar quantities of alkenes that are species-specific appear in all tsetse males. Structures of male-produced trimethylalkenes that appear in two other species, *G. palpalis palpalis* and *G. fuscipes fuscipes*, were investigated.

Key Words—Mating inhibitor, abstinon, stimulatory, cuticle waves, diptera, extract, hydrocarbon, dimethylalkene, trimethylalkene, aphrodisiac, Diptera, Muscidae, *Glossina morsitans morsitans*, tsetse fly, 19,23-dimethyltritriacont-1-ene.

INTRODUCTION

Glossina morsitans morsitans (Westwood) males were reported to produce a chemical deterrent to mating (termed "abstinon"), that was hexane soluble and could be transferred to otherwise stimulatory decoys. Application of as little as

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0.5 and 0.25 male equivalent of crude extract caused 100% and 96% abolishment, respectively, of male *G. m. morsitans* response to freshly killed females. Application of up to 0.4 ml of solvent had no effect on the response of males. No chemical isolation accompanied this work, and no candidate material was identified (Schlein et al., 1981a).

Reports of the existence of antiaphrodisiac pheromones (abstinons) in tsetse flies were challenged by other workers who described solvent effects that moved cuticular hydrocarbon components about the cuticle of otherwise stimulatory female test flies (Coates and Langley, 1982). Recently, the physical masking of active sex pheromone by inactive compounds was described as more likely than the decline or loss of activity due to the addition of antiaphrodisiacs (Langley et al., 1987).

Sex-stimulant hydrocarbons are produced by female tsetse flies that stimulate the male to attempt copulation. Pheromones have been isolated, identified, and synthesized for *Glossina morsitans morsitans* (Westwood) including 15,19,23-trimethylheptatriacontane (morsilure, Carlson et al., 1978), and 13,23-dimethylpentatriacontane in *G. pallidipes* (Austen) (Carlson et al., 1981, 1984). The presence of a sex stimulant pheromone has been reported in *G. palpalis palpalis* (Rob-Des) (Offor et al., 1981). Synthetic 15,19-dimethyltritriacontane was stimulatory to male *G. austeni* (Newstead) (Huyton et al., 1980). Alkanes that are predominant in males are usually also present in females of the same species, (Huyton et al., 1980; Nelson and Carlson, 1986; Nelson et al., 1988), but sexual dimorphism is observed in higher chain-length alkanes that are present only in females.

The gas chromatographic (GC) analysis of cuticular hydrocarbons of several species of male *Glossina*, including *G. m. morsitans*, *G. austeni*, *G. fuscipes fuscipes* (Newstead), and *G. p. palpalis*, showed that sex-specific alkenes were present in each species of fly (Carlson and Langley, 1986). Laboratory-reared *G. m. morsitans* contained a major 33-carbon backbone alkene, whereas virgin females had only traces. However, mated females contained appreciable quantities of this compound for several days after copulation. Identical alkenes were found in males of *G. m. centralis* (Machado) and *G. m. submorsitans* (Newstead). *G. austeni* males produced a 31-carbon backbone alkene, whereas *G. p. gambiensis* (Vanderplank) and *G. p. palpalis* males produced a major 26-carbon backbone alkene. The compounds were also found in wild females. Gas chromatography showed that relatively large amounts of these alkenes are transferred from males to females, apparently upon contact (Carlson and Langley, 1986).

The concept that male-produced compounds affect conspecific male behavior has been extended to other insects that have female-produced sex pheromones. *Drosophila melanogaster* (L.) males produce *cis*-vaccenyl acetate (CVA) that is transferred to the female and functions to repel males in subse-

quent mating attempts (Jallon et al., 1981). Recently Scott et al., (1988) found that mutual pheromone exchange in *D. melanogaster* decreases the sexual attractiveness of these flies, due to (*Z*)-7-tricosene, a 23-carbon alkene, and not CVA. The male stable fly *Stomoxys calcitrans* (L.) possesses a large quantity of a triply unsaturated alkene, (*Z,Z*)-1,7,13-pentacosatriene (Muhammed et al., 1975, Sonnet et al., 1977), which has been synthesized (Sonnet, 1979, Carlson and Mackley, 1985). It is transferred in significant quantities to females upon mating (Carlson and Mackley, 1985). The reason for such a transfer is not known, but the alkene is recovered by extraction of old mated females (Muhammed et al., 1975). In insects other than diptera, studies of the cockroach *Nauphoeta cinerea* showed that an unsaturated ester, octadecyl (*Z*)-9-tetracosenoate, inhibits the wing-raising behavior released by contact with the stimulatory, wing-raising hydrocarbon fraction from conspecific females (Fukui and Takahashi, 1983). A similar phenomenon was reported in the tobacco budworm moth (Jacobson et al., 1984).

The only reported methyl alkenes are three 2-methyl alkenes from male house crickets, including 2-methyl-24-hexatriacontene and minor amounts of the 22- and 26- isomers (Warthen and Uebel, 1980). These structures were determined by ozonolysis and GC retention indices to establish the presence of branched 23-, 25-, and 27-carbon aldehydes. GC-MS was not helpful in determining which aldehydes were isomethyl-branched.

We studied the effect of male-produced alkene upon the sexual responses of male tsetse flies to show that it is primarily responsible for the antiaphrodisiac effect seen in *G. m. morsitans* males. Natural alkenes and synthetic alkanes and alkenes were assayed against male *G. m. morsitans*, establishing dose–response relationships to show the abstinon effect. We also describe the chemical structures of these unusual alkenes transferred between sexes in four species of tsetse flies.

METHODS AND MATERIALS

Biological Materials. Tsetse flies were obtained as pupae from the Tsetse Research Laboratory, University of Bristol. Adults were sexed on the first day, maintained at 26°C, and fed on guinea pigs. Flies used for the experiments were 5–10 days old. Treatments of extracts and samples onto subject decoys were as previously described (Schlein et al., 1981a). Freshly killed females with amputated legs and wings were pin-mounted on corks and used as decoys with or without test materials, which were applied to each in 50-μl *n*-hexane aliquots using a microsyringe. Other females were washed with *n*-hexane three times following amputation to remove the cuticular lipids. The bodies then were dried in an air stream and each was dosed with 20 μl hexane solution containing

4 μg synthetic morsilure (15,19,23-trimethylheptatriacontane) and different quantities of test materials. Test materials were: *G. m. morsitans* alkenes from males, *G. m. morsitans* and *G. pallidipes* alkanes, and vaseline, which consists of purified hydrocarbons (Merck Index, 1976). Treated decoy females were dried in an air stream for 30 min. Males that had been fed 24 hr earlier were each placed in a corked glass tube 7 × 2.5 cm diameter and allowed 1 hr acclimatization. For testing, the cork of each tube was replaced by one bearing a mounted female, and males were brought into contact with the decoy three times. The behavior of the male was scored "nil" for no response, stage I for a short arrest in the male's movements on the decoy female, stage II for correct positioning of the male, either with or without flexing of the genitalia, and stage III for an attempt at copulation.

Chemical Separations and Identifications. Extracts were made of frozen laboratory flies or wild flies by washing with hexane or diethyl ether solvent for 1 hr at the rate of 1 ml/fly (Carlson et al., 1978, 1984). The hydrocarbon fraction was obtained by chromatography on silica gel and was further fractionated by chromatography on silver nitrate-impregnated silica gel. Samples of alkenes were collected by preparative argentation TLC to ensure purity (Carlson et al., 1978).

Analyses by fused silica gas chromatography (FSGC) were conducted using a Varian 3700 GC with a 15-m × 0.32-mm-ID FS column of DB-1 via a split/splitless injector, temperature programmed at 150°C to 300°C at 12°/min and with H_2 carrier gas. Alkane standards were used to determine retention indices.

GC–mass spectrometry (GC-MS) utilized a Finnigan model 4000 fitted with a 15-m × 0.32-mm-ID FSGC column (DB-1, J & W Co., Rancho Cordoba, California) that was temperature programmed as above for GC, and an INCOS data system. Alternatively, a Hewlett-Packard model 5988A GC-MS was used, fitted with a 30-m × 0.32-mm-id FSGC column of DB-1, temperature programmed as above. Alkenes were derivatized using dimethyl disulfide (DMDS) and analyzed by GC-MS (Dunkelblum et al., 1985, Carlson et al., 1989).

Peak ratios for a small but consistently appearing alkane (KI 3265) were obtained by dividing the quantity found in each fly by that of another alkane (KI 3355). Similarly treated were the KI 3755 and 3775 alkanes, using the same samples used in Carlson and Langley (1986) in order to evaluate transfer of cuticular alkanes between flies.

RESULTS

Isolation and Characterization of Alkenes. Alkanes from male *G. m. morsitans* included about equal quantities of 2-methyltriacontane (KI 3065) and 11,15-dimethyltritriacontane (KI 3355). The latter was present at 3.3 μg/male

(range 1–4 μg), which comprised an average of 22% of the extracted hydrocarbon (range 13–28%). This quantitation is consistent with Nelson and Carlson (1986), in which alkanes were identified by GC-MS. A minor KI 3155 compound was homologous, as was one of the two homologs at KI 3555. Hydrogenation of the corresponding KI 3355 alkene gave an alkane, 11,15-dimethyltritriacontane, that produced major fragment ions at m/z 168, 169, 239, 280, and 351 (Carlson and Langley, 1986), but did not locate the double-bond position.

Here, the alkene fraction (MA) containing the major male alkene (MMA KI 3355) was isolated as previously described (Carlson and Langley, 1986). MMA was present at 1–2 μg/male (Figure 1), with much smaller quantities of homologs. Milligram quantities were obtained from extracts of large collections of each sex of *G. m. morsitans* and checked for the absence of appreciable quantities of female-produced alkenes (Figure 1) before bioassay. After development with hexane–20% benzene, the alkenes were collected from the region of a silver nitrate silica gel TLC plate consistent with (*Z*)-alkene standards (*Rf* = 0.5). No evidence was seen for (*E*)-alkenes (*Rf* = 0.65) by TLC or GC. Alkenes were collected from pooled samples of males of the other species and purified by the same procedures, with the resulting composition essentially the same as in the previous study (Carlson and Langley, 1986).

The EI mass spectra of the minor KI 3155 alkene homolog from *G. m. submorsitans* showed fragment ions at m/z 167, 250–251, and 462 (M) that were consistent with the structure 19,23-dimethylhentriacont-1-ene. The major KI 3355 alkene from both *G. m. submorsitans* and *G. m. morsitans* showed fragment ions at m/z 167 (C_{12}), 278–279 ($C_{20:1}$), 349 ($C_{25:1}$), and 490 (M), indicating a structure consistent with 19,23-dimethyltritriacont-1-ene (Figure 2). The minor ions at m/z 181 and 295 are consistent with "apparent β-cleavage fragmentation" internal to methyl branch points that was described for EI-MS of dimethyl-branched long-chain alcohols and their acetate esters (Nelson et al., submitted). This β-cleavage is apparently suppressed in saturated methyl-branched alkanes that normally undergo α-cleavage but is observed in unsaturated compounds, here causing the appearance of additional fragment ions that are otherwise difficult to explain, and also causing loss of 1–3 amu from diagnostic fragment ions. However, the homologous alkenes at KI 3155 and KI 3555 were consistent with the presence of the double bond on the longer end of this structure and with alkanes produced by a previous hydrogenation of similar alkene fractions.

The EI mass spectra of the major KI 2720 alkene from *G. p. palpalis* showed fragments at m/z 139 (C_{10}), 209 (C_{15}), 222/223 ($C_{16:1}$), and 406 (M), indicating a structure consistent with 4,8,12-trimethylhexacos-25-ene (Figure 3). Additional ions at m/z 154, 224, 238, and 308 could be ascribed to β-cleavage as described above. The minor alkene at KI 2640 showed fragments

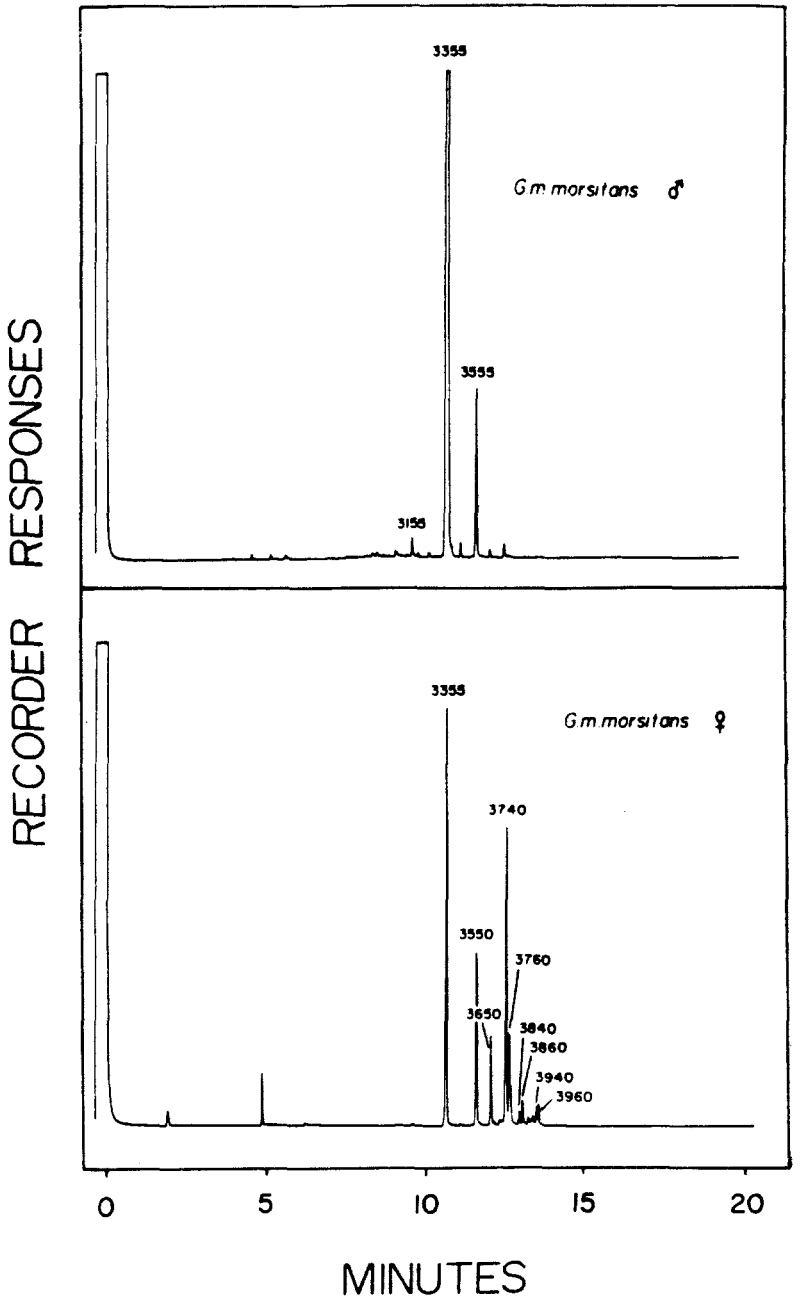

FIG. 1. Capillary gas chromatogram of alkenes from male and female *G. m. morsitans*.

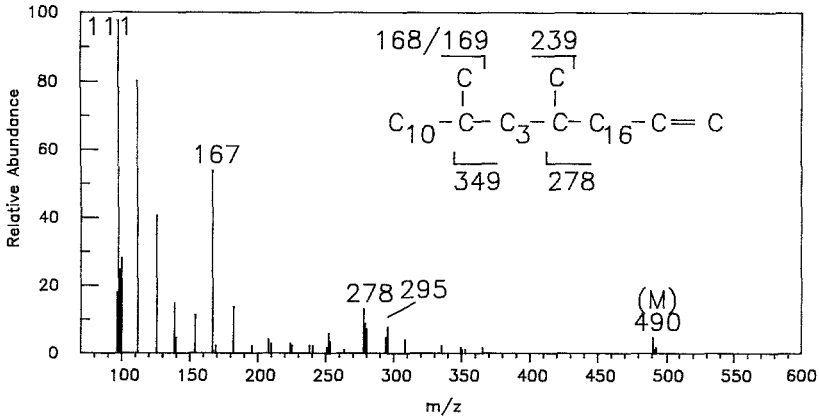

FIG. 2. Electron-impact GC-MS of major alkene from *G. m. morsitans* males.

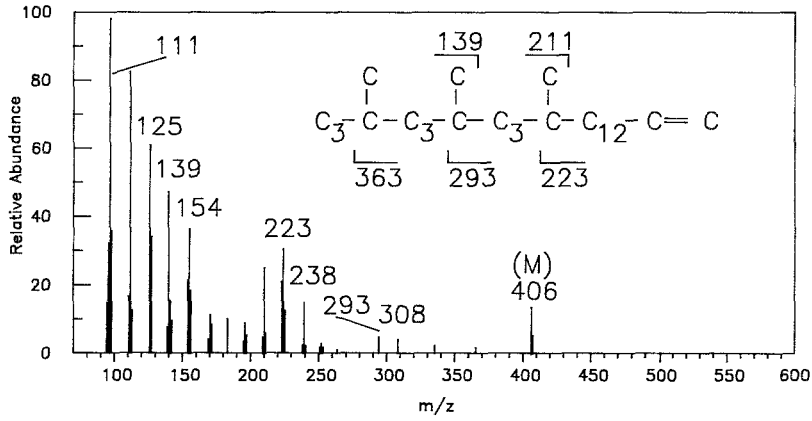

FIG. 3. Electron-impact GC-MS of major alkene from *G. p. palpalis* males.

at m/z 125 (C_9), 223 ($C_{16:1}$), and 392 (M), indicating a structure consistent with 3,7,11-trimethylpentacos-24-ene. Thus, the methyl branchings are analogous with the corresponding trimethylalkanes from these species (Nelson et al., 1988).

DMDS Location of Double-Bond Position in Alkenes from Males. The alkene fraction from *G. m. morsitans* males was derivatized with DMDS. The resulting major adduct eluted at KI 4000, indicating that an equimolar amount of DMDS had been added to the major male alkene KI 3355. Electron-impact GC-MS did not show a molecular ion for the monoadduct at m/z 582 or M-47 (m/z 537) (Figure 4). However, the adduct showed one major fragment ion at

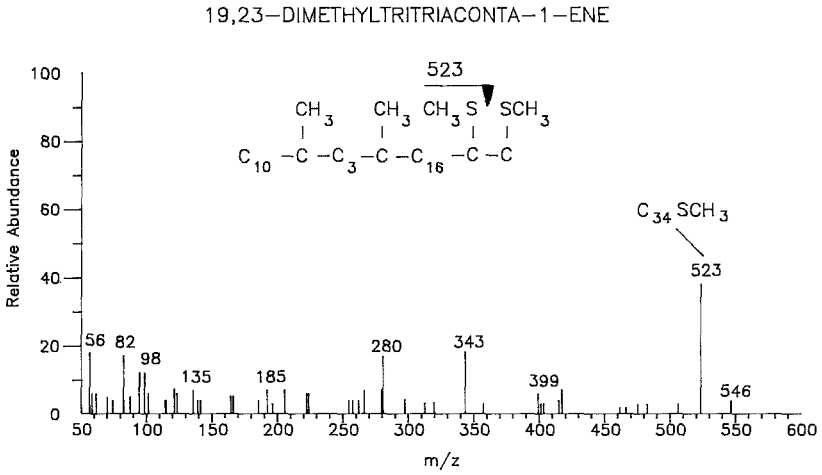

FIG. 4. Electron-impact GC-MS of major male alkene dimethyl disulfide adduct from *G. m. morsitans* males.

m/z 523 that was consistent with a structure of $C_{34}SCH_3$ in both species. This was consistent with addition of DMDS to a terminal position of unsaturation. The mass spectra presented fragment ions for $C_{18}SCH_3$ (*m/z* 297) and $C_{18}(SCH_3)_2$ (*m/z* 343), indicating fragmentation at a methyl branch located 19 carbons from the unsaturated end of the molecule. This is consistent with an alkene of structure 19,23-dimethyltritriacont-1-ene, in which the double bond is on the long alkyl chain. The fragments are inconsistent with the isomer 11,15-dimethyltritriacont-1-ene, for which cleavage at a methyl branch cannot be drawn to give an 18-carbon fragment, as the assignments do not fit.

The alkene fractions from *G. p. palpalis* and *G. f. fuscipes* males contained a major alkene at KI 2720, with minor quantities of an alkene, apparently homologous, at KI 2640 as reported by Carlson and Langley (1986). Derivatization with DMDS yielded one major adduct that eluted at KI 3300, indicating that an equimolar amount of DMDS had been added. Electron-impact GC-MS showed a molecular ion for the monoadduct (M+, *m/z* 500) in *G. p. palpalis*, and M-47 (*m/z* 453) was seen in both species (Figure 5). Both alkene adducts showed one major fragment ion at *m/z* 439 that was consistent with a structure of $C_{28}SCH_3$ in both species. This was consistent with addition of DMDS to a terminal position of unsaturation on the long alkyl chain. These mass spectra did not present any fragment ions suggesting fragmentation at methyl branches.

High-resolution proton NMR spectra were recorded on original alkene fractions from males of these species. The samples contained microgram quantities of the major alkene and minor amounts of other compounds as shown in Carlson and Langley, 1986. Chemical shifts of two allylic hydrogens (α-meth-

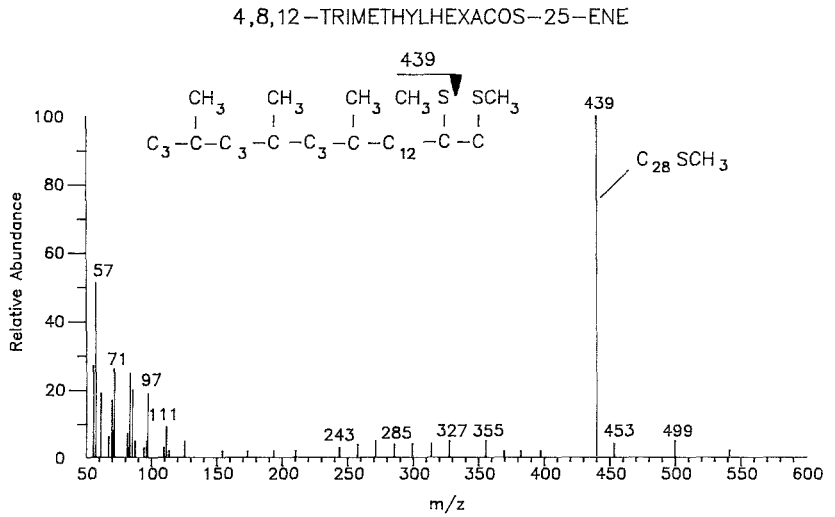

FIG. 5. Electron-impact GC-MS of major male alkene dimethyl disulfide adduct from *G. p. palpalis* males.

ylenes) were seen in all three samples, as were two homoallylic hydrogens (β-methylenes). Thus, the position of unsaturation could not be located on the short, two-carbon alkyl chain of the *palpalis* or *fuscipes* alkenes (A. Allerhand, unpublished data).

Bioassays. A clear dose–response relationship was obtained to natural MA treated onto unwashed but legless and wingless female decoys. Increasing dosages resulted in a clear antiaphrodisiac effect, with loss of all three progressive stages of sexual response, and concomitant increase of nil responses (Table 1). Stage III responses declined steadily to 24% with 1 μg MA, declined still more to 19% with 4 μg of MA, and were eliminated with 10 μg of MA, with nil responses increasing to 91%. In contrast, nearly obligatory stage III responses (93, 91, and 96%) were obtained to wingless, legless, untreated female controls; solvent-treated controls; and those treated with 5 μg vaseline, respectively. Even 25 μg of vaseline did not abolish male response, although 15% of males ignored decoys.

Similar dose–response relationships were obtained using hexane-washed female decoys that were treated with a uniform quantity (4 μg) of synthetic female pheromone (morsilure) mixed with increasing quantities of MA. A linear decline in response was observed with increasing quantities of MA, until a mixture of 1 : 1 proportion abolished all but 3% of stage III responses, or 85% of total male responses (Table 2). Similarly, *G. morsitans* alkanes at 4 μg and hydrocarbons of *G. pallidipes* at 8 μg [2 male equivalents (ME)] also had an

TABLE 1. RESPONSE OF *G. morsitans* MALES TO FRESHLY KILLED FEMALES FOLLOWING APPLICATION OF MALE ALKENES (MA) OR VASELINE DISSOLVED IN 50 μl HEXANE

Substance applied	No. of males	Degree of response (%)[a]			
		III	II	I	0
None	60	93.3	0	0	6.6
Hexane only	60	91.7	0	1.7	6.7
MA					
0.25 μg	69	59.4	8.7	7.2	24.6
0.5 μg	71	38.0	9.9	24.0	28.2
1 μg	82	24.4	7.3	19.5	48.8
2 μg	74	21.6	6.8	17.6	54.1
4 μg	73	19.2	5.8	11.0	64.4
10 μg	66	0	0	9.1	90.9
Vaseline					
5 μg	60	96.7	0	0	3.3
25 μg	60	76.7	3.3	5.0	15.1

[a] 0 = no response; I = short arrestment in male movement on decoy; II = correct positioning of male, with or without flexing of genitalia; III = copulatory attempt.

inhibitory effect on male courting behavior and reduced stage III responses to 4.7% and 12.1%, respectively (2 ME) (Table 2). There was, however, a difference in the stage in which the inhibition was elicited by MA or alkanes. The *G. morsitans* MA and alkanes differed in their effects on stages II and III of the courting behavior. This is evident from the frequency of responses at these stages in pairs of series where the proportion of nonresponses and stage I responses did not differ statistically (Fisher exact text, Table 2, group 3 vs. 6, group 4 vs. 7, group 5 vs. 8). Differences in the proportions of stage II responses were highly significant between series 3 (10.0%) and series 6 (25.6%), also between series 4 (8.3%) and series 7 (22.5%), and differences were significant between series 5 (3.2%) and series 8 (11.6%). Similarly there was a highly significant difference in courting cessation at stage III between series 3 (52.9%) and 6 (20.9%) and between series 4 (24.0%) and 7 (9.2%). The number of these responses in series 5 (2 flies) and 8 (4 flies) was too low for statistical analysis. Series of tests were compared where the overall effect of the inhibition was similar, regardless of the various stages of response.

Thus, with the alkenes, very few reactions stopped at stage II and most males completed the course of mating. In contrast, the largest group among the flies exposed to alkanes ceased courting at stage II. The effect of male hydrocarbons was enhanced when aliquots of both chemical classes were applied together. Abolishment of courting at stages II and III by 0.5 μg MA plus 1 μg

TABLE 2. RESPONSE OF *G. morsitans* MALES TO FRESHLY KILLED, HEXANE-WASHED FEMALES TREATED WITH MORSILURE ALONE OR MIXED WITH HYDROCARBON FROM MALES: *G. morsitans* ALKENES (MA) AND ALKANES OR *G. pallidipes* HYDROCARBONS

Test series	Male substance plus 4 μg morsilure	No. of males	Degree of response (%)[a]			
			III	II	I	0
1	none	60	78.7	6.6	8.3	6.6
2	MA					
	0.25 μg	70	62.9	8.6	10.0	18.6
3	0.5 μg	70	52.9	10.0	8.6	28.6
4	1 μg	60	24.0	8.3	8.3	58.3
5	4 μg	62	3.2	3.2	8.1	85.5
6	*G. morsitans* 2 μg alkanes	32	20.9	25.6	16.3	37.2
7	*G. morsitans* 4 μg alkanes	56	9.2	22.5	11.2	57.2
8	*G. morsitans* 8 μg alkanes	64	4.7	11.6	9.3	74.4
9	MA					
	0.5 μg + 1 μg alkanes	44	1.6	8.1	19.4	71.0
10	1 μg + 2 μg alkanes	41	0	0	18.0	82.0
11	*G. pallidipes* 8 μg hydrocbn.	39	11.8	8.8	22.1	57.4
12	Dotriacosane 4 μg	40	20	25	25	20
13	Tetracontane 4 μg	40	13	25	25	40

[a] Same legend as Table 1. Results of Fisher exact test:

Group MA vs. *G. morsitans* sum of all doses:

deg. response	0 — $P < 0.5$	NS
	I — $P < 0.09$	NS
	II — $P < 7.2 \times 10^{-5}$	highly sig.
	III — $P < 2.7 \times 10^{-6}$	highly sig.

Group 3 vs 6

deg. response	0 — $P < 0.13$	NS
	I — $P < 0.08$	NS
	II — $P < 6.4 \times 10^{-3}$	highly sig.
	III — $P < 1.7 \times 10^{-3}$	highly sig.

Group 4 vs 7

deg. response	0 — $P < 0.44$	NS
	I — $P < 0.28$	NS
	II — $P < 0.01$	sig.
	III — $P < 3.59 \times 10^{-3}$	sig.

Group 5 vs 8

deg. response	0 — $P < 0.05$	NS
	I — $P < 0.30$	NS
	II — $P < 0.03$	sig.
	III — $P < 0.33$	NS

alkanes was similar to that of 4 μg alkenes and greater than that of 4 μg alkanes (Table 2).

Application to decoys of *n*-hydrocarbons at 4 μg with 4 μg morsilure also reduced male response. Of 40 repetitions with dotriacontane, the results for stages 0, I, II, and III were 12, 10, 10, and 8, respectively, and with tetracosane were 16, 10, 9, and 5, respectively (Table 2).

Transfer of Alkanes. Extracts of mated and unmated flies (5–10 days old) were analyzed by GC after Carlson and Langley (1986). Chemical analyses showed that quantities of alkanes were also transferred from males to females (Figure 6). Both KI 2965 and KI 3355 were more prominent in males than females, and both increased in females after mating, while KI 3065 and 3265 remained constant in all flies. In a pooled sample of female flies, the peak ratios R1 for KI 3355/KI 3265 were nearly double in mated females (Table 3). Similarly, small amounts of female-produced sex pheromone, KI 3755 and 3775, were found on males that were held for a short time (4 hr) with conspecific females, as the ratios R2 and R3 also increased (Figure 7, Table 3).

DISCUSSION

Dipteran sex pheromone receptors were described as pairs of shallow convex structures on both the inner and outer surfaces of the upper tibiae (Schlein et al., 1981b). However, Langley et al. (1987) describe active contact chemoreceptors as setae located on the tibiae and tarsomeres that possess straight longitudinal ribbing with pores and are confined primarily to the first two pairs of legs. All males have these setae (770), with nearly equal numbers on each leg, although females have more than half as many.

By isolating, identifying, and quantifying a compound responsible for "abstinon" behavior in tsetse males, we have made significant advances in understanding the nature of this phenomenon. Regardless of the current disagreement over the types and location of sex pheromone receptors and/or abstinon receptors, this information should allow further attack upon the type and location of receptors. Perhaps more importantly, we can then study the still obscure mechanism of reception for hydrocarbon pheromones.

It appears that in the higher diptera, production of sex stimulant pheromone is under gonadotrophic control, that is, its production is coincident with ovarian maturation and unavoidably continues with age. Thus, in several species there are indications of an antiaphrodisiac that would serve to lessen mating attempts by males upon mated females, as well as protecting males from excessive homosexual assault.

While somewhat variable, the average quantity of natural sex stimulant pheromone on a female is 9.4 μg (Carlson et al., 1978). When applied alone,

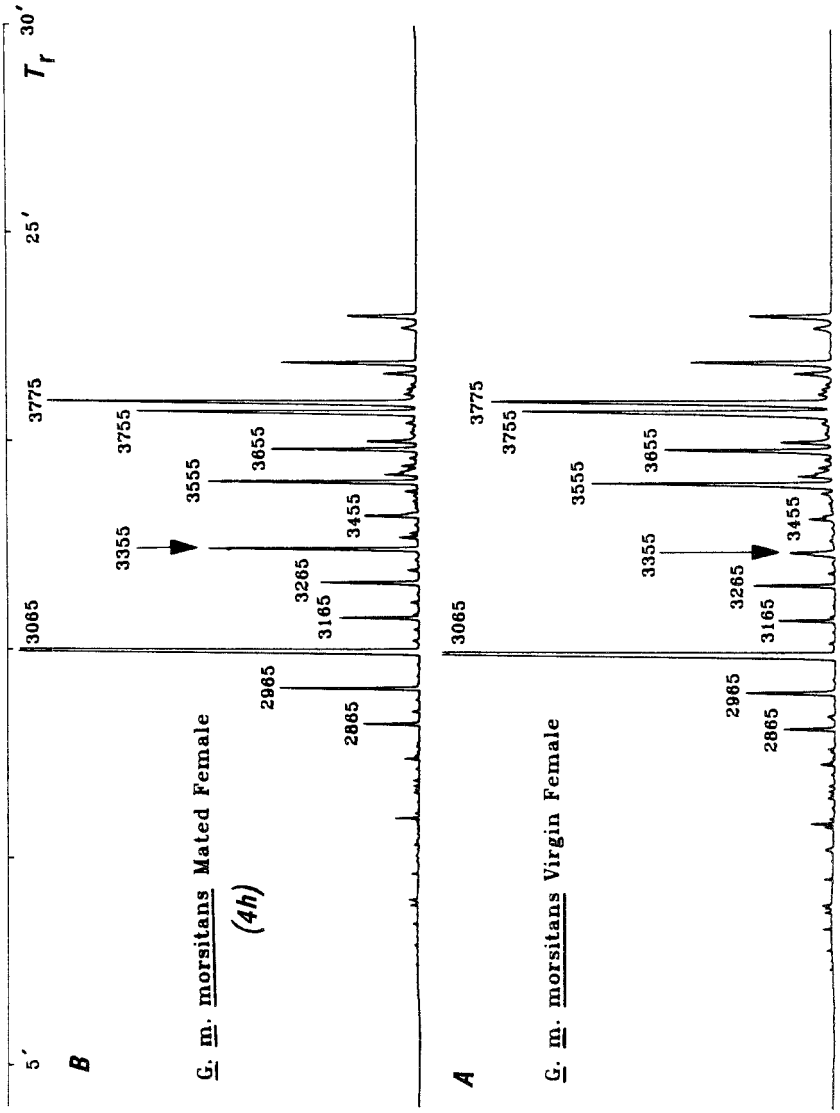

FIG. 6. Capillary GC of alkanes from *G. m. morsitans* females: (A) virgin; (B) mated.

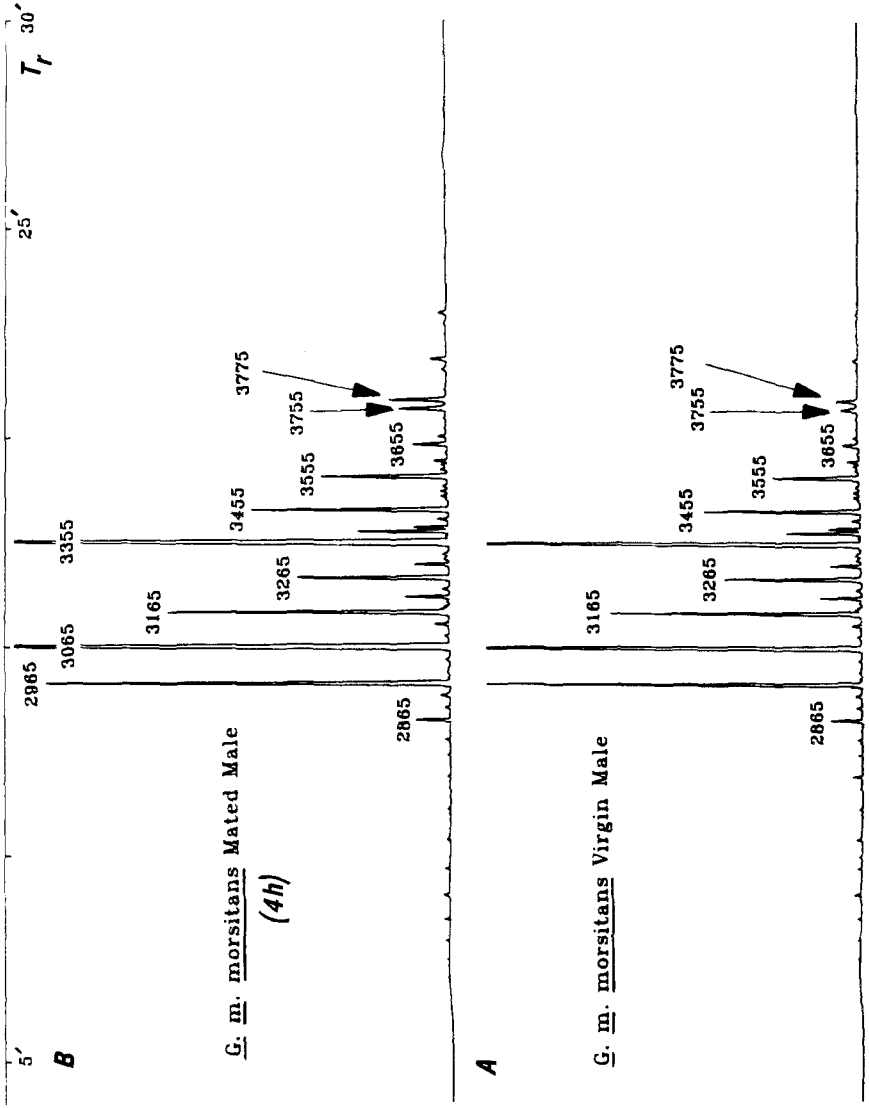

FIG. 7. Capillary GC of alkanes from *G. m. morsitans* males: (A) virgin; (B) mated.

TABLE 3. PEAK RATIOS OF ALKANES PRESENT ON CUTICLE OF *G.m. morsitans* MALES OR FEMALES HELD WITH OPPOSITE SEX

Ratios of alkanes	No. of insects	Females		Males	
		Virgin	Mated	Virgin	Mated
R1 = 3355/3265	10	0.9	2.1	4.6	5.2
R2 = 3755/3265	10	7.1	3.9	0.2	0.3
R3 = 3775/3265	10	7.8	5.2	0.2	0.4

4 μg of synthetic morsilure elicited copulatory responses in more than 90% of the males (Table 2), which is consistent with many previous tests.

Synergism is indicated between male-produced alkanes and alkenes: While it is obvious here that MMA are transferred, transfer of alkanes is less obvious. Nelson and Carlson (1986) show that the saturated analog of MMA (11,15-dimethyltritriacontane, KI 3355) is male-produced and not present in virgin females. Therefore, we rechecked samples of mated females for the male-produced KI 3355 alkane. It appears that small amounts of male alkanes are, in fact, transferred between the sexes with alkenes, as female-to-male transfer can be observed. It is known that male *G. m. morsitans* can be held in cages of conspecific females, then returned to cages of male flies, only to undergo prolonged attacks by sexually stimulated male flies (P.A. Langley, unpublished observations). Chemical analysis shows a small amount of female-produced sex pheromone in extracts of such males. Apparently, not much material is needed to evoke sexual stimulation in males, especially if it has been applied by physical contact rather than by a rather crude application in solvent. If surface contact is enough to transfer biologically active female material onto seti or other outer body parts of males, by analogy, not much material should be necessary to evoke an abstinon-type response in attacking males. A very small amount of male alkene may be sufficient to release this response. We believe that we have observed this effect. As mentioned in Carlson and Langley (1986), MMA or other alkanes are not present in seminal fluid but seem to be cuticular in nature. It appears that both male alkenes and alkanes are deposited on the cuticle of females.

The present tests demonstrated that male *G. morsitans* hydrocarbons include abstinons, which can inhibit male stimulation by female pheromones. Males have 1.3–4 μg of MA (Carlson and Langley, 1986), which was enough to demonstrate dose–response activity when applied to females, and inhibited all but 9% of stage I behavior at a quantity of 10 μg (Table 1). A similar effect was obtained when MA was applied to washed females in a mixture of 4 μg

together with 4 μg of morsilure. Tests with male alkanes similarly showed dose–response activity and inhibited most male response when used at 8 μg together with 4 μg of morsilure on hexane-washed females (Table 2). The alkenes and alkanes functioned synergistically and a combination of 1.5 or 3 μg of both was as effective as higher quantities of each of these compounds alone. The observed degree of synergistic effect is similar to the 96% inhibition of male response by the application of unfractionated extract containing 0.25 male equivalent, reported by Schlein et al. (1981a). The effect of male abstinons in the natural situation must be much stronger since they function without antagonists, and their unequivocal message is the one that inhibits courting. The perception of the female cue by the male must also be very specific to prevent courting of different species in the biotope. Thus, it is not surprising that (synthetic) linear hydrocarbons, which do not occur except as very minor components in tsetse flies of any species, were somewhat effective in reducing male sexual responses.

The classification of the response to alkenes and alkanes indicated differences in their effect. The quantities of MA and alkenes tested in the experiments were not the same, although the quantities of test materials can be compared most readily when their quantities are the same. In this situation, dissimilar quantities of test materials were used because the activity was similar, since the final stages of courting behavior were usually eliminated when responses were minimal. Conversely, initial and intermediate stages of behavior would not be represented adequately in tests with high doses of inhibitor. The alkanes of male *G. morsitans* produced alternating degrees of courting inhibition at stages II and III in series where their total effect was similar. The proportion of no responses in series 3, 4, 5 (192 flies), and also in series 6, 7, 8 (270 flies) was 56.3% The response to MA in the first group of these series amounted to 7.3% at stage II and 28.1% at stage III. The relative proportion of responses at these stages was reversed when alkenes were used and in the second group of experiments. The percentage of stage II responses was 20.0% and stage III was 11.5%. The increase in cessation at stage II in the presence of alkanes involved, in many cases, an unusual behavior expressed by prolonged grasping of the females without moving the genitalia. This behavior was not observed in other tests, including those carried out with *G. pallidipes* male alkanes. In view of these differences, it is suggested that the species-specific male alkenes and alkanes exert a different type of inhibition. The alkenes seem to block mostly the initial cue for courting, and copulation is completed once their effect is overcome, while the alkanes exert their effect in the initial stage, and in addition they mainly inhibit the engagement of the genitalia.

In this context, it is interesting to note that each of the three synthetic candidate pheromones treated against *G. morsitans* males in the first report (Carlson et al., 1978) appeared to elicit male reaction mostly to one specific

stage. For example, following the application of 10 μg of each to shoelace decoys, the respective responses at stages I, II, and III were, to 15,19-dimethylheptatriacontane (compound 1), 4%, 44%, and 8%; to the least active 17,21-dimethylheptatriacontane (compound 2), 16%, 0%, and 0%; to morsilure (compound 3), 4%, 36%, and 40%. Responses to application of a larger quantity of 40 μg of morsilure for stages I, II, and III were 0%, 16%, and 80%. Note that there were errors in numbering compounds 1 and 2 at two locations in this reference; in the center of p. 471 in the text and in Figure 1, compound 2 should refer to the 15,19-dimethyl isomer, and compound 1 to the least-active symmetrical 17,21-dimethyl isomer, as above, and elsewhere (Huyton et al., 1980).

It is tempting to suggest that each of the natural pheromones can initiate at least some courting stimulus, and afterwards each is mostly responsible for one step of the male's sexual behavior. Correspondingly, males possess a set of inhibitory compounds that are clearly transferred to females during courtship. Further studies with fractionated hydrocarbons are needed to clarify the actual role of the different compounds in *G. morsitans* courting.

It is interesting that MMA is structurally related to 15,19-dimethyltritriacontane, which was found to be a candidate pheromone in *G. austeni* and also had excellent sex stimulant activity against *G. morsitans*. Thus we present the first evidence that the unsaturated analog of a sex stimulant is a potent antipheromone.

The decline in responses to decoys treated with *n*-dotriacontane and *n*-tetracosane showed that a physical masking effect is partially responsible for the reduced activity. This effect is difficult to quantitate, but cannot be disregarded in evaluating behavioral effects of natural alkanes.

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