Bombykal, a Sex Pheromone of the Sphinx Moth *Manduca sexta*

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Dedicated to Prof. Dr. Hans Brockmann *on the Occasion of His 75th Birthday*

Pheromones, Sex Attractant, *Manduca sexta*

The first sex pheromone of a Sphinx moth has been isolated from *Manduca sexta* (Lepidoptera: Sphingidae) with the aid of an electroantennogram assay. It is attractive to males in a field test where its activity might be augmented by a second component in female extracts. Chemical tests and chromatographic properties identify the pheromone as a C_{16}-aldehyde with a pair of conjugated double bonds. *(E, Z)*-10,12-hexadecadienal, ("bombykal") was identical with the natural product. The comparison includes chromatography on three different capillary columns and determination of specific activities in the electroantennogram test.

The search for the sex attractant of the sphingid moth *Manduca sexta* (Joh.) (tobacco hornworm) has been long and tedious: work on its isolation has been in progress in this laboratory since 1964. One of us (N. A.) described the mating behaviour of the moth already in 1955 [1] and reported in 1962 that extracts of female abdominal tips (last two or three abdominal segments) are attractive to males [2]. We were unable to develop a behavioral assay with captive moths and, until recently, relied on a field test to monitor progress in the purification of the sex attractant. The tests were performed in tobacco fields in the vicinity of the USDA Experimental Station in Florence, South Carolina. The flight season of *Manduca sexta* there lasts only from June to August. Consequently, with time lost for shipping samples and with all the uncertainties of a field test, yearly progress was slow. Until 1973, the sex attractant was prepared exclusively from moths raised from larvae collected in tobacco fields [3]. Since extracts of females sacrificed at other times appeared to have low activity, moths were collected between 11 p. m. and 4 a. m. at which time their abdominal tips were clipped and stored in ether. For complete extraction the tips were homogenized in ether after addition of sodium sulfate. Removal of the solvent in a rotory evaporator at room temperature left a yellow, biologically active oil. The bulk of this material was reduced by low temperature precipitation: one part crude oil in 10 parts of ether were chilled on dry ice, 10 parts of cold acetone were added and the crystalline precipitate finally filtered off on a Büchner funnel. The sex attractant was recovered in the supernatant with 10 to 20% of the mass of the starting material. It was purified further by chromatography on a 100 cm Sephadex LH-20 column in benzene-acetone 1:1. Nonanal- and acetone 2,4-dinitrophenylhydrazone served as internal standards: the fraction from the point where the former to the point where the latter start to emerge from the columns was collected and saved. The same two reference compounds guided the subsequent purification by thin layer chromatography on silica gel in benzene – ethyl acetate (19:1) where they have R_f-values of 0.7 and 0.5, respectively. The biologically active material was recovered from the white zone between the two yellow bands. The mass of the preparation is at this point reduced by a factor larger than 10,000.

All fractions were field tested with specially constructed wire screen traps [4]. While the assay is by no means quantitative, we find no evidence that the biological activity of our samples is diminished during the purification process described so far. From gas chromatographic separations on *e. g.* XE-60 columns the attractant could also be recovered intact if all compounds with retention times between those of methyl myristate and methyl stearate

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were collected. Subdividing this fraction further led to confusing results. Single fractions in contrast to recombined samples did not show high attractancy in the field test. An essential pheromone emerges from XE-60 columns between methyl palmitate and methyl stearate (which were routinely added as internal standards). There are indications for a second component of the sex attractant with a smaller retention time, similar to that of methyl myristate (Table I). However, at this time we cannot say with certainty whether this second pheromone really exists. The field assay of samples with gas chromatographic purity may be complicated by parameters related to the presence of impurities, for instance chemical stability or evaporation rate. Owing to the logistics of the field test and to its severe limitations in regard to quantitative evaluation of data, we were unable to resolve this problem during several years of testing. The purest, biologically active samples showed upon analytical gas chromatography on packed columns no detectable peaks in the areas of interest: the concentration of the pheromone or pheromones must be less than 1 ng per female equivalent. Routine analytical methods indicated that the sex attractant of *M. sexta* is inert to alkaline saponification and mild acid treatment, but that it is inactivated by bromine, catalytical hydrogenation, and reduction with lithium aluminum hydride.

The closure of the USDA Experimental Station in Florence in 1973 abolished our arrangement for field testing and eliminated our source of field collected hornworm larvae. All subsequent work on the identification of the *M. sexta* pheromone is based on its activity on isolated male antennae. Preliminary experiments with the electroantennogram (EAG) technique have been described [5]. Extracts of female moths contain a compound which elicits a response specifically in male antennae and which in all chromatographic and chemical properties is identical with the pheromone of *M. sexta* described above. A second EAG active compound akin to the suspected second component of the sex attractant could not be detected. The EAG active pheromone can be efficiently collected from a gas chromatograph in Pasteur pipets loosely filled with glass wool. The pipets are used directly for the EAG test, but may also be kept under refrigeration for several days without noticably losing activity. This procedure efficiently eliminates from pheromone samples residues of solvents and reagents which have a tendency to interfere with the EAG assay. In consequence, we were able to perform most chemical tests and checks for chromatographic behaviour with samples containing only 1 to 10 female equivalents.

The earlier chemical experiments were repeated and confirmed. The R_f-value of the pheromone in the TLC-system excludes the presence of hydroxy and epoxide groups but would be consistent for a monofunctional ester, aldehyde, or ketone. The presence of an ester linkage is excluded since the pheromone is not destroyed by alkaline saponification. The question whether the pheromone is a ketone or an aldehyde was answered through formation of the bisulfite addition product with a (modified) procedure described by Nesbitt *et al.* [6]. In contrast to reference ketones, the *M. sexta* pheromone formed the bisulfite addition product from which it could be regenerated by addition of sodium carbonate.

Table I. Attraction of *M. sexta* pheromone in the field test. P273, a fraction from a TLC separation, was resolved by GC on a 180 cm 10% XE-60 column. Methyl myristate, methyl palmitate and methyl stearate had been added and were collected in traps chilled with dry ice: T2, T4, and T6. T3 and T5 contained the material emerging between the reference compounds. The traps were rinsed with ether; each combination of traps was tested repeatedly at a level of 20 female equivalents. The major component of the sex attractant has been collected in T5; an augmenting factor might be found in T2.

Preparation	Males caught per test
P273	44 / 26 / 10
T2+3+4+5+6	17 / 5 / 7 / 12
T 3+4+5+6	6 / 0 / 3 / 1
T2+ 4+5+6	22 / 0 / 7 / 17
T2+3+ 5+6	7 / 0 / 9 / 10
T2+3+4+ 6	1 / 0 / 0 / 0
T2+3+4+5	8 / 0 / 9 / 9

Table II. Response of male and female *M. sexta* antennae to (*E*, *Z*)-10,12-hexadecadienal. The samples were applied to the inner glass surface of Pasteur pipettes. a). The response to air has been subtracted from all other data.

Preparation	EAG response [mV] ♂	♀	♂	♀
(*Z*)-8-dodecenyl acetate, 10 µg	1.6	0.6	1.2	0.7
(*E*, *Z*)-10,12-hexadecadienal, 0.1 ng	0.8	0.0	0.6	0.0
(*E*, *Z*)-10,12-hexadecadienal, 0.33 ng	1.9	0.0	1.3	0.0
(*E*, *Z*)-10,12-hexadecadienal, 1.0 ng	2.7	0.0	2.1	0.1
(air)[a]	(0.5)	(0.3)	(0.4)	(0.3)

Important information about the degree of unsaturation was derived from chromatographic data. From an OV-1 column the pheromone emerges before methyl palmitate, but from a Carbowax 20M column about halfway between methyl palmitate and methyl stearate. Such a shift in retention time is compatible with a polyunsaturated C_{16}-aldehyde. On the other hand, on silica gel impregnated with silver nitrate the pheromone migrates between oleyl and stearyl aldehyde. In conjunction, the results indicate the presence of one pair of conjugated double bonds somewhere in the molecule. Consequently, the pheromone of *M. sexta* has been identified as a hexadecadienal with conjugated double bonds. (The chromatographic properties of conjugated monounsaturated aldehydes could not be predicted with certainty: (*Z*) as well as (*E*)-2-hexadecenal were excluded from further consideration by direct comparison with authentic compounds.)

To test our conclusions, we oxidized [7] a sample of bombykol [8] to the corresponding aldehyde. (*E*, *Z*)-10,12-hexadecadienal was within the limits of our data chromatographically identical with the natural pheromone. In the EAG assay it proved to be more active by orders of magnitude than any other compound tested before; the response is specific for males (Table II). The activities of the (*Z*, *E*)- and the (*E*, *E*)-isomers are approximately one and three orders of magnitude lower. If (*E*, *Z*)-10,12-hexadecadienal were indeed the sex pheromone of the tobacco hornworm it could be calculated that active preparations should contain between 0.1 and 1 ng per female equivalent. Inspired by this calculation, we collected 300 female equivalents of an active preparation in succession first from a Carbowax 20M column, then from an OV-1 column, taking care to cut the pheromone zone very narrowly. The recovered material was active in the EAG test and showed upon chromatography on capillary columns a major peak with the retention time of (*E*, *Z*)-10,12-hexadecadienal (Fig. 1). Addition of internal standards to this preparation allowed to measure relative retention times with extreme accuracy: the natural pheromone was indistinguishable from the synthetic product on three different 50 m glass capillary columns: DEGS, SE-30, and Carbowax 20M. Its quantity, 0.14 ng per female equivalent, agrees with the results of EAG-tests.

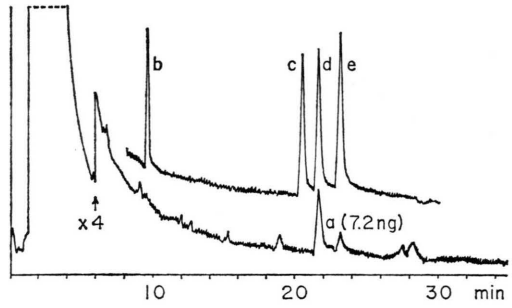

Fig. 1. Gas chromatographic analysis of purified *M. sexta* pheromone on a 50 m WCOT DEGS capillary glass column at 160 °C. The preparation, 50 female equivalents, contained 7.2 ng of the pheromone (a). The insert shows the positions of b: hexadecanal, c: (*Z*, *E*)-, d: (*E*, *Z*)-, and e: (*E*, *E*)-10,12-hexadecadienal in this system.

We submit that (*E*, *Z*)-10,12-hexadecadienal is a sex pheromone of female *M. sexta*, being aware of the fact that the structure has not been established by unequivocal chemical proof. While it is theoretically possible that a C_{16}-aldehyde with a different arrangement of conjugated double bonds has on all three capillary columns the same retention time as the (*E*, *Z*)-10,12-isomer, it would also have to possess the same specific activity for male antennae. We consider the chance for such a coincidence remote. Because of the difficulties in isolating larger quantities of the natural pheromone, the question will most likely not be studied by further analysis, but by a systematic survey of synthetic hexadecadienals. It remains to be seen whether the sex attractant of *M. sexta* is (*E*, *Z*)-10,12-hexadecadienal alone or whether it is composed of several components.

In order to prepare reference aldehydes we asked colleagues earlier this year for samples of unsaturated C_{16}-alcohols, especially for isomers of bombykol. We learned that nineteen years after the identification of bombykol a second pheromone of *Bombyx mori* had been discovered and that an account of this work was in print [9]. The pheromone was the aldehyde corresponding to bombykol and accordingly named bombykal: it is identical with the sex pheromone of *M. sexta*.

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