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Oxaspiropentane Derivatives as Effective Sex Pheromone Analogues in the Gypsy Moth: Electrophysiological and Behavioral Evidence

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Abstract

A number of oxaspiropentane derivatives (OXPs) were tested as potential (+)-disparlure analogues, with the aim of identifying any possible interaction of these compounds, be it additive, synergetic, or inhibitory, with the pheromone response in the male gypsy moth *Lymantria dispar*. As assessed by male electroantennograms, 2 OXPs, 2-decyl-1-oxaspiro[2.2]pentane (OXP-01) and 4-(1-oxaspiro[2.2]pent-2-yl)butan-1-ol (OXP-04), were found to be effective. OXP-01 had no stimulatory effect but strongly decreased the response to (+)-disparlure in a blend in a 1:1 ratio. By contrast, OXP-04 proved to be more stimulating than (+)-disparlure and also had an additive effect in the blend. Single-cell recordings from the sensilla trichoidea showed the activity of 2 cells, one of which responded to (+)-disparlure. OXP-01 reduced the stimulating effectiveness of pheromone by silencing the pheromone-responding unit when the 2 compounds were presented in blend, whereas OXP-04 mimicked the pheromone response, evidenced by exciting the pheromone-responding neuron when tested alone. Behavioral observations are in agreement with electrophysiological results.

Key words: (+)-disparlure, electroantennograms, insect, *Lymantria dispar*, single-cell recordings

Introduction

Female moths produce and release sex pheromones for upwind attraction of conspecific males for mating. The key to such a long-distance communication is the high selectivity and sensitivity of the olfactory system toward particular pheromone blend components or toward antagonists from related species. Previous studies have shown that stereochemical features play an important role in the molecular recognition of pheromone components (Mustaparta 1997; for a review, see Kaissling 2004). The main sex pheromone component of the gypsy moth *Lymantria dispar*, a widespread forest pest that causes severe foliage losses during outbreaks in Europe, Asia, and North America (Montgomery and Wallner 1988), is (+)-(7*R*,8*S*)-epoxy-2-methyloctadecane (Cardé et al. 1977; Plimmer et al. 1977; Hansen 1984), also called "(+)-disparlure." The male olfactory system recognizes pheromone structure and concentration by means of specialized sensory hairs, the sensilla trichoidea, localized on the antennal branches. Each sensillum houses 2 olfactory neurons responding to (+)- and (−)-disparlure, respectively (Hansen 1984). The latter is a major component of the nun moth *Lymantria monacha* pheromone blend (Hansen 1984) and strongly antagonizes the attractiveness of (+)-disparlure in the male gypsy moth, although it is neither attractive nor repellent by itself (Gries et al. 1996). Occasionally, impulses from a third cell can be seen, but these are related neither to synthetic disparlure nor to pheromone gland extracts of female moths (Hansen 1984).

Because the chemical structure of (+)-disparlure has been fully elucidated, the use of synthetic analogues as putative agonists, antagonists, or synergists of pheromone responses has been considered. Studies with a number of synthetic epoxides related to disparlure clearly demonstrated that a few key features of the molecule are critical for biological activity. For instance, the long and the short alkyl chains of the pheromone, as well as the cis configuration and the presence of a 2-methyl substituent, are all critical factors (Schneider et al. 1977). Chirality is also an essential property of the pheromone molecule in eliciting behavioral activity (Cardé et al. 1977).

Moreover, several studies have shown the importance of the epoxide group because its relocation results in a substantial loss of attractiveness (Adler et al. 1972; Bierl et al. 1972; Sarmiento et al. 1972; Schneider et al. 1974; Dickens et al. 1997; Inkster et al. 2005).

The aim of this work was to screen a variety of pheromone derivatives to identify stronger attractants than the natural pheromone to bait traps that provide simple, specific tools for monitoring insect pests and also be used to control insect population by mating disruption. Moreover, this study may contribute to understand the binding and releasing mechanisms between odorant molecules and binding proteins or receptor proteins.

To this end, we focused our attention on a number of oxaspiropentane derivatives (OXPs) as potential (+)-disparlure analogues, with the aim of identifying any possible interaction of these compounds, be it additive, synergetic, or inhibitory, with pheromone response in the male gypsy moth *L. dispar*. OXPs were selected as they represent a class of potential pheromone analogues, the activity of which was never tested before. Moreover, this class of compounds was chosen to verify how the simplest cyclic radical (cyclopropyl) inserted next to the epoxy ring—thus forming an oxaspiropentane—in substitution of various other sections of the aliphatic regions of the pheromone molecule can modify its biological effects.

Seven compounds differing by functional group with respect to (+)-disparlure were synthesized and preliminarily screened both alone and blended with the pheromone on the male gypsy moth olfactory apparatus by way of an electroantennogram (EAG) bioassay. Two of them, 2-decyl-1-oxaspiro[2.2]pentane (OXP-01) and 4-(1-oxaspiro[2.2]pent-2-yl)butan-1-ol (OXP-04), found to be effective on male antennal responses, were consequently tested in depth by means of the single-cell recording (SCR) technique as well as behavioral (field) trials. The present study is the first to candidate OXPs as effective sex pheromone analogues for the gypsy moth.

Materials and methods

Insects

All electrophysiological experiments were performed on 2- or 3-day-old gypsy moth adult males (day 1 being the day of emergence), obtained as late instar larvae from the Stazione Sperimentale del Sughero (Tempio Pausania, Italy).

Larvae were reared in 240-ml plastic cups (8–10 per cup) containing approximately 10 ml of a modified wheat germ diet, kept in an environmental growth incubator (24–25 °C, 70% relative humidity, 16:8 h light:dark photoperiodic regime), and checked daily until adult emergence. Males and females were kept separate to avoid any exposure of males to female sex pheromone.

Odor delivery system

An air-stimulus control unit (model CS-55, Syntech, Hilversum, The Netherlands) was used for air and odor delivery with a constant flow rate (1500 ml/min) of charcoal-filtered and humidified air continuously blown over the antennal preparation through the open end of a glass tube (8 mm in diameter, 15 cm long), positioned 15 mm from the antenna. During odor stimulation, the stimulus-bearing airflow (500 ml/min) was switched for 2 s to a Pasteur pipette (15 cm long) containing a pleated filter paper strip impregnated with the stimulus, inserted about 3 mm into a small hole in the wall of the steel tube. Stimuli were presented in a randomized sequence, separated by interstimulus intervals of at least 2 min. Each stimulus was tested more than once to verify the reproducibility of the responses. The air containing the stimulus was removed from the experimental arena by means of a suction pump operating at a flow rate slightly higher than that of stimulation.

Stimuli

The OXPs may be considered (+)-disparlure analogues with a cyclopropyl group replacing one of the 2 aliphatic chains, while the epoxy ring is conserved.

Seven compounds differing by functional group with respect to (+)-disparlure were synthesized (Table 1) and tested both alone and blended with the pheromone. All OXPs were prepared following the general procedure reported for the synthesis of OXP-01 (Bernard et al. 2003; Bernard, Floris, et al. 2004; Bernard, Frongia, et al. 2004; Table 1 and Scheme 1). Each molecule was first dissolved (100 μg/ml) in dichloromethane (CH_2Cl_2) and then a 50-μl volume of solution was pipetted onto the pleated strip of filter paper (80 × 5 mm), to yield a final dosage of 5 μg for each compound effectively loaded on filter paper at the highest dosage tested. The CH_2Cl_2 was evaporated before the experiments started. To obtain the other (lower) concentrations, decadic dilutions from the former were prepared and 50 μl of each solution was pipetted onto the filter paper strip with the same procedure, in order to obtain 0.5, 0.05, or 0.005 μg of compound loaded on filter paper.

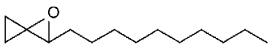
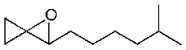
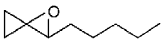
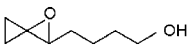
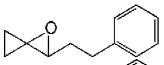
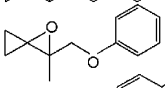
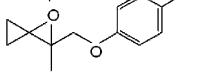
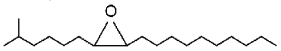
Hereafter, all compounds will be indicated with the abbreviations in Table 1 and stimulus dosages expressed as μg of molecule loaded per filter paper.

Preliminary experiments indicated that CH_2Cl_2 is not stimulating by itself. In each experiment, before each stimulation the response to air was tested and its value subtracted from the EAG value obtained in response to the test stimulation that ensued. When not in use, stimuli were stored at −20 °C.

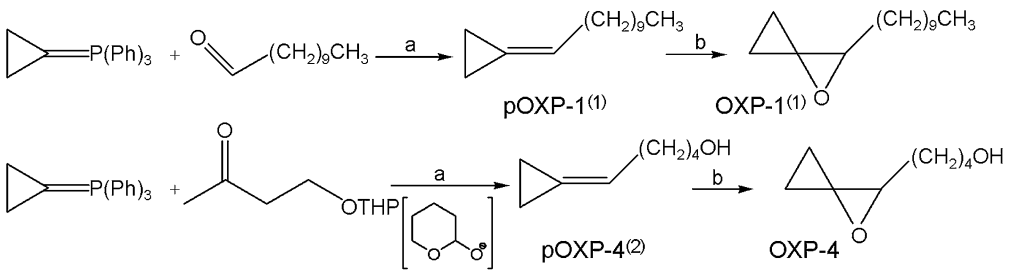
The stimulation protocols were as follow:

- Dose–response curves were obtained, in EAG and SCRs, for (+)-disparlure (Sigma-Aldrich, Italy, code 510769, 95% pure), OXP-01, and OXP-04, in the dosage range 5–0.05 μg. The OXPs were also tested added to an equal dosage (ratio 1:1) of (+)-disparlure, and their responses were compared with those of pure pheromone.
- SCRs were also obtained in response to 0.5 μg (+)-disparlure blended with 0.005, 0.05, 0.5, or 5 μg of the analogue OXP-04, respectively (mixture ratio: 1:0.01, 1:0.1, 1:1, and 1:10), or 0.5 μg of (+)-disparlure + 0.005 μg of OXP-04.

Table 1 List of OXPs tested in this study as compared with the natural pheromone (+)-disparlure

Nomenclature	Structure	Abbreviation
2-decyl-1-oxaspiro[2.2]pentane		OXP-01
2-(5-methylhexyl)-1-oxaspiro[2.2]pentane		OXP-02
2-pentyl-1-oxaspiro[2.2]pentane		OXP-03
4-(1-oxaspiro[2.2]pent-2-yl)butan-l-ol		OXP-04
2-(2-phenylethyl)-1-oxaspiro[2.2]pentane[a]		OXP-05
2-methyl-2-(phenoxymethyl)-1-oxaspiro[2.2]pentane[a]		OXP-06
2-[(4-methoxyphenoxy)methyl]-2-methyl-1-oxaspiro[2.2]pentane[a]		OXP-07
(+)-(7*R*,8*S*)-epoxy-2-methyloctadecane[(+)-disparlure]		(+)-D

[a]Bernard, Floris, et al. (2004).



a) NaH(60% in oil dispersion) (4.0 equiv.), THF, TDA-1 (0.1 equiv.), 60 °C, 10 h 85% yield
b) *m*-CPBA (1.0 equiv.), CH_2Cl_2, 0 °C, 1-4 h, 45% yield
(1) Bernard, Frongia, et al. 2004
(2) Bernard et al. 2003

Scheme 1 Synthesis of OXP-01 and OXP-04.

Following electrophysiological screening, the 2 analogues of choice, OXP-01 and OXP-04, were used during behavioral trials at their 2 most effective dosages (5 and 0.5 µg, respectively). To test their overall biological activity, these 2 synthetic analogues were both used as racemate.

Electrophysiology

Synthetic pheromone and OXPs were tested on male gypsy moth antennae by means of both EAG and SCR techniques.

The excised antennae, one per moth, were positioned in such a way as to expose the largest surface to the stimulus-bearing airstream. A glass micropipette (20 µm in average tip diameter) filled with moth saline solution (NaCl 12 mM, KCl 6.4 mM, $MgCl_2$ 12 mM, $CaCl_2$ 1 mM, glucose 354 mM, KOH 9.6 mM, final pH 6.59; Kaissling 1995) was inserted into the base of the antennal shaft and acted as the reference electrode in both EAGs and SCRs. The connection to the amplifier input was established with Ag/AgCl wires immersed in moth saline. In the EAG experiments, the recording electrode was a similar micropipette brought into contact with the distal end of the antenna, from which 1 to 2 segments were excised. In SCRs, the tips of long sensilla trichoidea, known to respond to sex pheromone (Hansen 1984), were clipped off using sharpened forceps. The recording electrode (internal diameter < 3 µm), filled with moth saline, was slipped over one sensillum.

All signals were recorded with a high input impedance (10^{15} Ω) electrometer (WPI Duo 773), band-pass filtered (DC to 1 KHz for EAGs and 0.1–3 KHz for SCRs), digitized by means of an Axon Digidata 1200B A/D converter (10 000 Hz), and stored on PC for further analyses.

The absolute EAG amplitudes during the 2-s stimulation period were calculated by means of Axoscope 8.1 software. In SCR experiments, spike firing frequencies in the first second of the response were sorted and analyzed with SAPID Tools software (Smith et al. 1990) on the basis of shape and amplitude. The time course of response frequency of cell A to (+)-disparlure, OXP-04, and their 1:1 blend was analyzed, during the 1-s stimulation period, at time bins of 100 ms.

All differences in EAG and SCR values with respect to (+)-disparlure, clean air, and/or spontaneous activity were calculated by means of Student's *t*-test with a 95% confidence level.

Regression lines were calculated for the time courses (decay time) of cell A spike frequency in response to (+)-disparlure, OXP-04, and their blend, and the lines were then cross compared with the test of parallelism and coincidence (Zar 1984).

Behavior

Field tests were carried out in the localities of Tempio Pausania and Gesturi (Sardinia, Italy), in 2 cork oak forests, during the peak of the gypsy moth flight season (June–July) in 2004 and 2005. "Delta"-type traps baited with a pleated strip of filter paper (80 × 5 mm) impregnated with the different stimuli were suspended from trees, 1.5 m above ground level and spaced 55–60 m from each other in a randomized order. Two different subsets of experiments, one per analogue, were performed. For each subset, traps (6 replicates per compound) were baited with the analogue, with the (+)-disparlure or the blend in ratio 1:1. Equal numbers of nonbaited traps (blanks) were used as controls. As mentioned before, following the indications of the EAG and SCR bioassays, the subset with OXP-01 was used at 5 μg of compound loaded per filter paper, whereas that with OXP-04 at 0.5 μg. Traps were checked 2–3 h after setting them, and the number of caught males was recorded.

All differences in behavioral trials with respect to (+)-disparlure and blank controls were calculated by means of Student's *t*-test with a 95% confidence level.

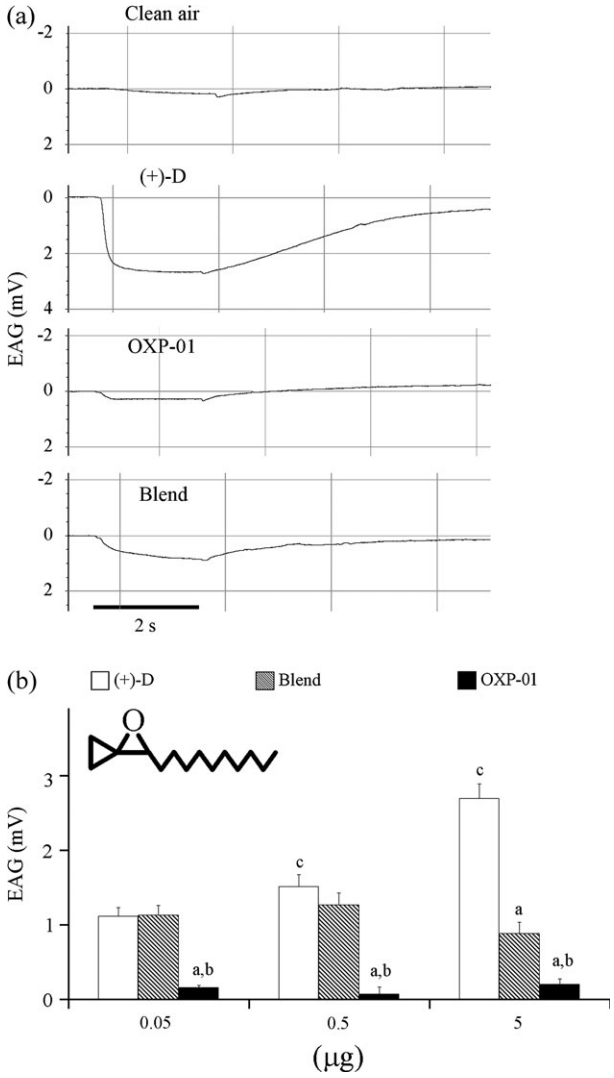


Figure 1 **(a)** Sample EAG recordings from male antennal preparations following stimulation with sex pheromone (+)-disparlure ((+)-D), OXP-01, and their 1:1 blend at 5 μg. Black bar indicates the stimulation time. **(b)** EAG mean amplitude values ± SE elicited by stimulation with (+)-D, OXP-01, and their 1:1 blend in the range 0.05–5 μg. Legend of significant differences ($P \leq 0.05$): a = from (+)-D, b = between OXP-01 and the blend, c = from preceding dosage of same stimulus. Recordings from 20 antennae.

Results

EAG responses

As assessed by EAG responsiveness of gypsy moth male antennal preparations to the tested OXPs (Table 1, Scheme 1), only the 2 compounds, OXP-01 and OXP-04, were found to be effective. The other OXPs derivatives tested elicited no significant responses both alone and blended with the pheromone and were subsequently discarded in further analyses.

Figure 1 shows that OXP-01, as compared with (+)-disparlure, evoked by itself a negligible effect on male antennal receptors at any tested dosage. At the highest one (5 μg), it caused a strong decrease in the response to the blend (0.89 ± 0.15 mV) with respect to (+)-disparlure (2.70 ± 0.19 mV), whereas no reduction in the stimulating effectiveness was detected at the 2 lowest dosages (0.5 and 0.05 μg).

Conversely, as shown in Figure 2, OXP-04 elicited by itself a response from male antennal preparations and, at 0.5 μg, it was higher (2.48 ± 0.18 mV) than that of the corresponding (+)-disparlure (1.73 ± 0.25 mV). As for the blend, at 0.05 and 0.5 μg, it evoked a stronger response (1.86 ± 0.14 and 2.47 ± 0.14 mV) with respect to (+)-disparlure (1.40 ± 0.16 and 1.73 ± 0.25 mV). At 5 μg, the responses to the blend and (+)-disparlure did not differ.

The corresponding alkylidene cyclopropane precursors of OXP-01 and of OXP-04 (pOXP-1 and pOXP-4, respectively;

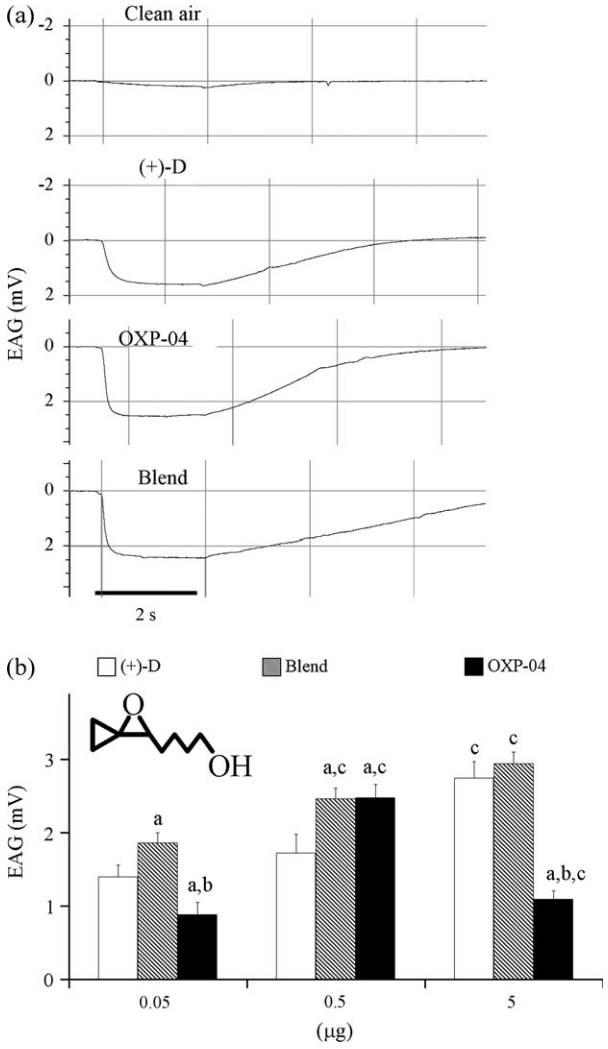


Figure 2 **(a)** Sample EAG recordings from male antennal preparations following stimulation with sex pheromone (+)-disparlure ((+)-D), OXP-04, and their 1:1 blend at 0.5 μg. Black bar indicates the stimulation time. **(b)** EAG mean amplitude values ± SE elicited by stimulation with (+)-D, OXP-04 and their 1:1 blend in the range 0.05–5 μg. Legend of significant differences ($P \leq 0.05$): a = from (+)-D, b = between OXP-04 and the blend, c = from preceding dosage of same stimulus. Recordings from 20 antennae.

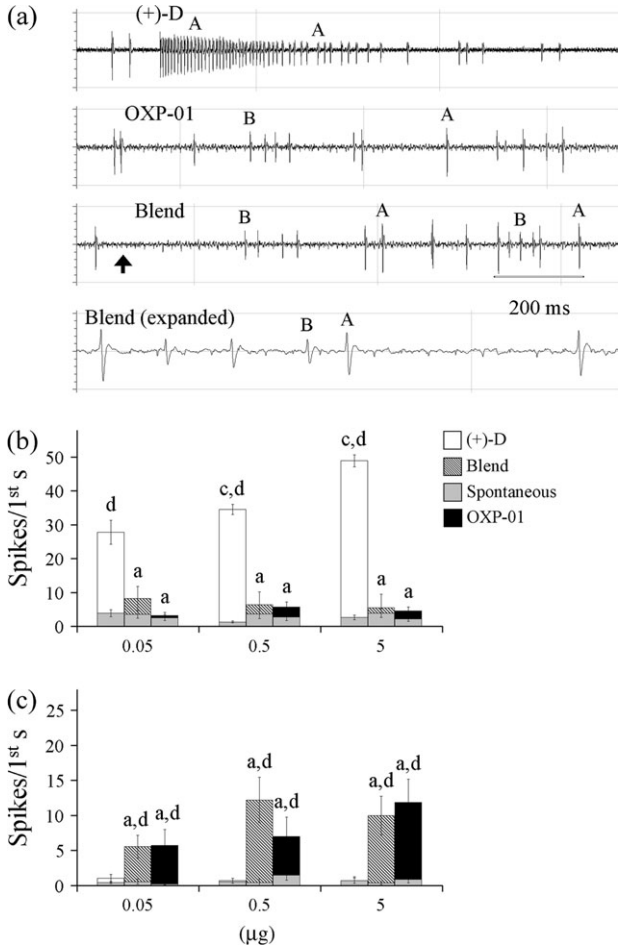


Figure 3 **(a)** Sample SCRs following stimulation of long sensilla trichoidea of male antennae with sex pheromone (+)-disparlure ((+)-D), OXP-01, and their 1:1 blend at 5 μg. Section underscored (200 ms) is expanded in lowermost trace. Black arrow indicates stimulation onset. A and B = cells A and B, respectively. Spike frequency mean values ± SE elicited by stimulation of cell A **(b)** and cell B **(c)** with (+)-D, OXP-01, and their 1:1 blend in the range 0.05–5 μg, superimposed on respective spontaneous activity. Legend of significant differences ($P \leq 0.05$): a = from (+)-D, b = between OXP-01 and the blend (no cases), c = from preceding dosage of same stimulus, d = from respective spontaneous activity. Recordings from one sensillum for each of the 20–22 antennal preparations (specimens) tested.

Scheme 1) were also tested, but neither elicited any response (data not shown).

Single-cell responses

On the basis of amplitude and shape, 2 different classes of action potentials could clearly be recorded from each sensillum and were assigned to 2 different olfactory receptor neurons (ORNs), hereafter referred to as cells A and B. Cell A normally fired at a higher frequency than B.

Cell A, which fires the larger amplitude spike, responded to the pheromone (+)-disparlure with a dose-dependent increase in firing frequency, as shown in Figure 3. When blended with OXP-01, however, the response was suppressed at all tested dosages (Figure 3a,b). OXP-01 alone had no effect on cell A. The other analogue tested, OXP-04, elicited an excitatory response from cell A (Figure 4a,b). In a combination with the pheromone, it elicited a similar response to that of the pheromone alone, but for the middle dosage tested (0.5 μg). The activity of cell A in response to OXP-04 at 0.05 and 0.5 μg did not differ from that of the pheromone, whereas it was lower at 5 μg. Regardless of tested compounds and dosages, the spike firing frequency elicited from cell A was significantly different from the spontaneous activity.

Cell B, which fires the smaller amplitude spike, showed no response to the pheromone at any tested dosages. However, its responses significantly differed from spontaneous activity

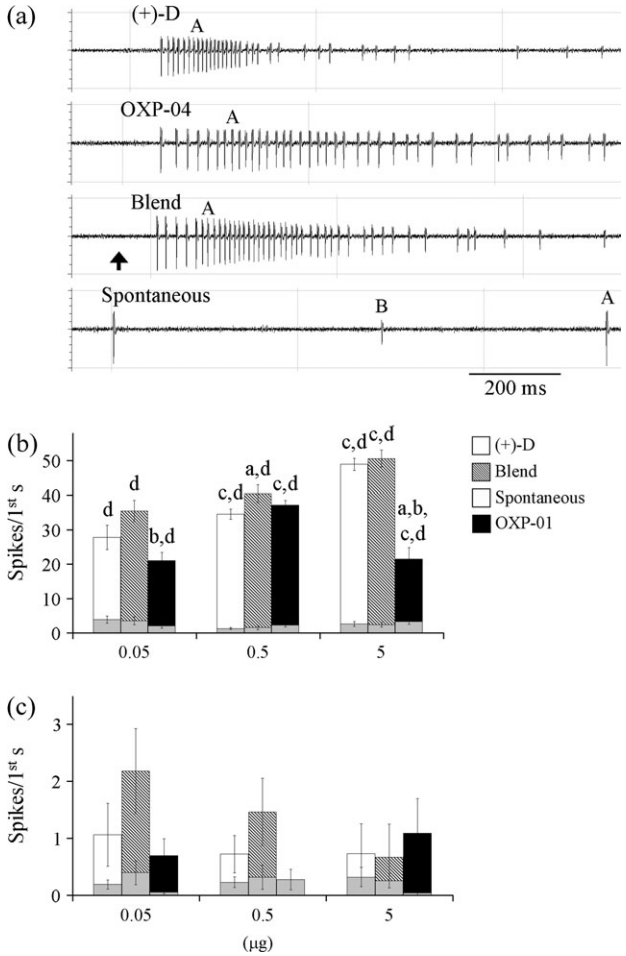


Figure 4 **(a)** Sample SCRs following stimulation of long sensilla trichoidea of male antennae with sex pheromone (+)-disparlure ((+)-D), OXP-04, and their 1:1 blend at 0.5 µg. Black arrow indicates stimulation onset. A and B = cells A and B, respectively. Spike frequency mean values ± SE elicited by stimulation of cell A **(b)** and cell B **(c)** with (+)-D, OXP-04, and their 1:1 blend in the range 0.05–5 µg, superimposed on respective spontaneous activity. Legend of significant differences ($P \leq 0.05$): a = from (+)-D, b = between OXP-04 and the blend, c = from preceding dosage of same stimulus, d = from respective spontaneous activity. Recordings from one sensillum for each of the 20–22 antennal preparations (specimens) tested.

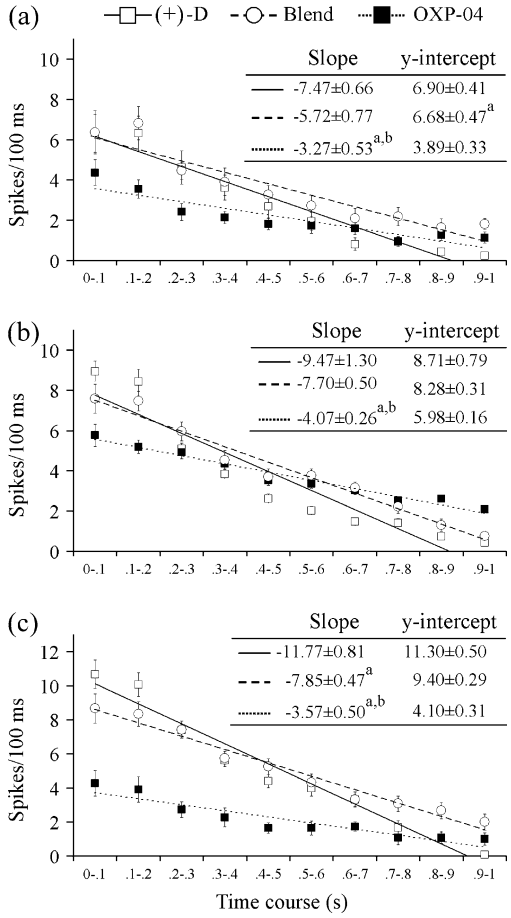


Figure 5 Plots of spike frequency versus time of cell A from SCRs following stimulation of long sensilla trichoidea of male antennae with (+)-disparlure ((+)-D), OXP-04, and their 1:1 blend at 0.05 µg **(a)**, 0.5 µg **(b)**, and 5 µg **(c)**. Time bins = 100 ms. Linear regression lines are superimposed, and their slopes and *y* intercepts are given in the inset. Legend of significant differences ($P \leq 0.05$): a = from (+)-D, b = between OXP-04 and the blend. Recordings from one sensillum for each of the 20–22 antennal preparations (specimens) tested.

for all dosages of the OXP-01, either alone or in blend with the pheromone (Figure 3a,c). A mixture of OXP-01 and the pheromone acted as the analogue alone (Figure 3c).

OXP-04 neither alone nor in combination with the pheromone had any effect on cell B (Figure 4a,c), which therefore did not respond differently from the spontaneous activity.

Linear regression analysis was performed for spike firing frequency time courses of cell A in response to pheromone, OXP-04, and their 1:1 blends (Figure 5). Slopes and *y*-intercepts values of the regression lines were cross compared and show that the pheromone response decayed faster than that of OXP-04 at all dosages because their slope values were significantly different. As for the blend, the slopes were different from those of pheromone only at 5 µg (Figure 5c), whereas at 0.5 µg, they were coincident (neither slopes nor *y* intercepts differ from each other; Figure 5b) and parallel at 0.05 µg (*y* intercepts only differ; Figure 5a).

Effects of various mixtures of the analogue OXP-04 + (+)-disparlure on the response of cell A

Histogram in Figure 6a shows the response of the cell A of long sensilla trichoidea (mean spike frequency values ± standard error [SE] in the first second of the response) following stimulation with 0.5 µg of (+)-disparlure blended with 0.005, 0.05, 0.5, or 5 µg of the analogue OXP-04 (mixture ratio: 1:0.01, 1:0.1, 1:1, and 1:10).

As compared with the response to 0.5 µg of (+)-disparlure (dashed line), the addition of OXP-04 significantly increased the response of cell A regardless of the analogue dosage, but the responses to the mixtures did not vary from one another.

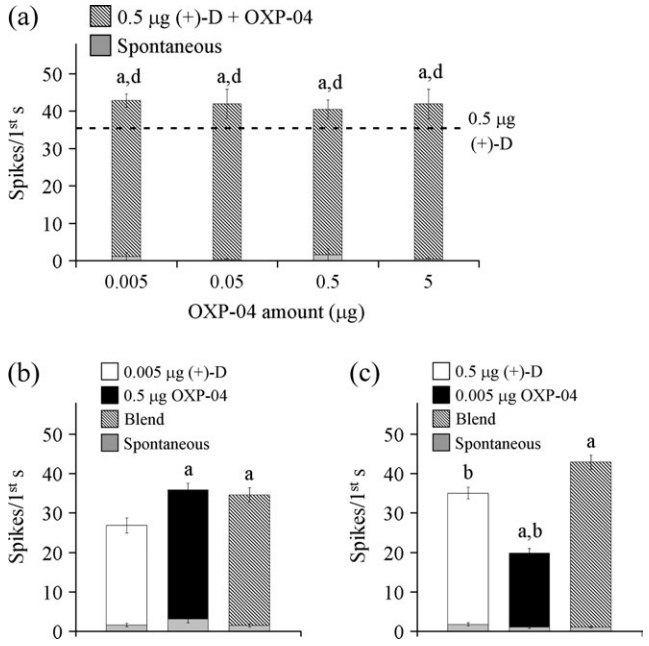


Figure 6 **(a)** Spike frequency histograms following stimulation of cell A with (+)-disparlure ((+)-D) alone (dashed line) and blended with OXP-04 at the different blend ratios 1:0.01, 1:0.1, 1:1, 1:10. **(b)**, **(c)** Spike frequency histograms following stimulation of cell A with (+)-disparlure ((+)-D), OXP-04, and their 1:1 blend. Spike frequency mean values are superimposed on respective spontaneous activity. Legend of significant differences ($P \leq 0.05$): a = from (+)-D, b = from blend, d = from spontaneous activity. Recordings from one sensillum for each of the 20 **(a)** or 24–25 **(b)** and **(c)** antennal preparations (specimens) tested.

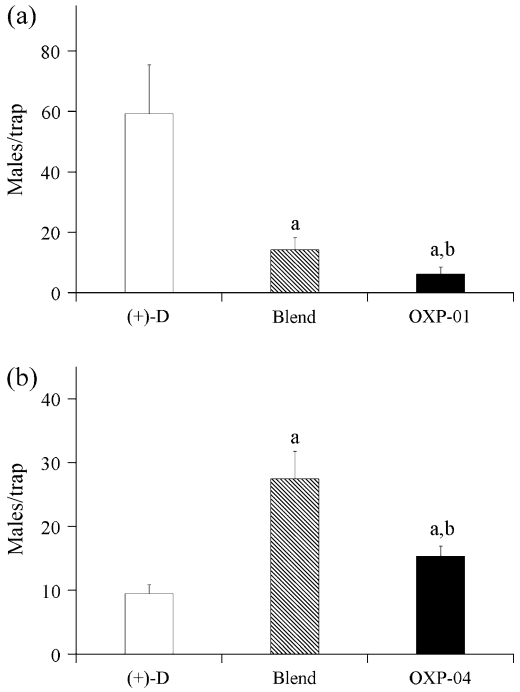


Figure 7 Mean numbers ± SE of trapped males with **(a)** (+)-disparlure ((+)-D), OXP-01, and their 1:1 blend at the dosage of 5 µg and **(b)** (+)-D, OXP-04, and their 1:1 blend at the dosage of 0.5 µg. Legend of significant differences ($P \leq 0.05$): a = from (+)-D, b = between OXP-01 or OXP-04 and the blend. Captures from 6 replicates per compound.

To further elucidate the effects of mixtures, the cross dosage of 0.005 µg for (+)-disparlure + 0.5 µg for OXP-04 was tested.

As shown in Figure 6b, the spike frequency in response to the mixture containing 0.005 µg of (+)-disparlure + 0.5 µg of OXP-04 was not significantly different from that of the analogue alone, but is higher than that to the pheromone. On the other hand, as shown in Figure 6c, the response evoked by 0.5 µg of pheromone + 0.005 µg of the analogue was higher than that to the pheromone and the analogue.

Behavior

In Figure 7a,b are shown the mean values ± SE of trapped males with the 2 OXPs (OXP-01 and OXP-04) tested, respectively, at 5 and 0.5 µg, on the basis of their effectiveness assessed by electrophysiological experiments, as compared with (+)-disparlure at same dosages. Trapping trials showed that males were attracted to the pure pheromone (59.33 ± 16.22), whereas OXP-01 was very little attractive (6.31 ± 2.29). Furthermore, OXP-01 significantly decreased the attractiveness of 5 µg of (+)-disparlure when the 2 compounds were presented as a blend (Figure 7a).

The other derivative, OXP-04, revealed a stronger degree of attractiveness than that evoked by the corresponding dosage of pheromone (Figure 7b). Also, the blend of the 2 substances attracted significantly more males than the (+)-disparlure.

Number of insects captured with blank (nonbaited) traps was negligible (0.83 ± 0.31 males per trap, data not shown).

Discussion and conclusions

The gypsy moth sex pheromone (+)-disparlure is a molecule made of 3 different functional portions: 2 aliphatic chains of different length and 1 epoxide ring. This molecule is recognized as a sex attractant by the highly specific male olfactory system. In the present work, we have focused our attention on a number of OXPs as (+)-disparlure analogues, with the aim of measuring their biological potency compared with natural pheromone. We also studied their interaction, be it additive, synergetic, or inhibitory, with the pheromone response. These analogues basically differ from (+)-disparlure by the presence of a cyclopropyl group in the place of either aliphatic chain, while retaining the epoxide ring, which is strictly necessary for pheromone recognition, as assessed by various studies (Adler et al. 1972; Bierl et al. 1972; Sarmiento et al. 1972; Schneider et al. 1974; Dickens et al. 1997; Inkster et al. 2005). The OXPs presented here were synthesized and preliminarily tested on the male olfactory apparatus by way of an EAG bioassay and 2 of them, OXP-01 and OXP-04, were found to be particularly effective,

although in opposite ways. In fact, OXP-01 had no appreciable stimulatory effectiveness by itself on male antennal receptors but strongly decreased the EAG response to (+)-disparlure when presented at the highest dosage as a 1:1 mixture. In other words, the substitution of the sole short aliphatic chain with a cyclopropyl group to obtain OXP-01 (the long aliphatic one remaining intact) led to a compound with no efficacy but somewhat capable of counteracting the stimulating effectiveness of natural pheromone.

Substitution of the long chain with a cyclopropyl group produced an analogue (OXP-02) that had no effect either by itself or in mixture with the pheromone.

The following modifications of the short chain produced various kinds of effects. Removal of the 2 terminal methyl groups from the short chain led to a compound (OXP-03) with no appreciable activity. By subsequent addition of a hydroxyl group at the end of the chain (in position 2), we obtained OXP-04, which proved to be more stimulating than (+)-disparlure at 0.5 μg and also had an additive effect in the blend at the 2 lower dosages.

Other substitutions on the short chain (OXP-05, OXP-06, OXP-07) that enlarged the overall size of the molecule, such as insertion of phenyl groups and/or oxy groups, produced ineffective molecules. Similar results were obtained by Inkster et al. (2005) after substitutions on the short chain in the pheromone.

In agreement with the numerous reports in literature that indicate the epoxy ring as a functional key group for the pheromone molecule to be active (Adler et al. 1972; Bierl et al. 1972; Sarmiento et al. 1972; Schneider et al. 1974; Dickens et al. 1997), alkylidene cyclopropane precursors of OXP-01 and OXP-04 (pOXP-1 and pOXP-4, respectively; Scheme 1) were also ineffective.

To gain a better understanding of the mechanisms by which the 2 analogues OXP-01 and OXP-04 interact with the male olfactory system, their effectiveness was then evaluated by means of the SCR technique on the long sensilla trichoidea, the sensory structures specialized in pheromone detection (Hansen 1984). We recorded spike activity from 2 different ORNs in each sensillum, indicated, respectively, as cells A and B on the basis of their spike amplitude. In our experiments, they were activated in a highly selective way, depending on the tested stimulus: cell A responded best to the pheromone at all tested dosages, whereas cell B was virtually silent in response to it. These data are in good agreement with those previously reported by Hansen (1984), which showed that sensilla trichoidea contain 2 different odor receptor cells, one highly specialized for (+)-disparlure and the other for (−)-disparlure. In our experiments, OXP-01, pure or blended with (+)-disparlure, evoked no response from cell A, which we consider the pheromone cell, but stimulated the cell B, the same cell activated by the (−)-disparlure.

These data clearly confirm the lack of stimulatory effect of OXP-01 by itself observed in EAG experiments and suggest that the inhibitory effect in the trapping experiments on the attraction to disparlure exerted by this analogue in the blend might be achieved by silencing the response of cell A to (+)-disparlure, in addition to increasing the activity of cell B.

Conversely, in single-cell experiments, OXP-04—pure or blended—evoked a spike frequency response from cell A similar to that elicited by the pheromone, also in agreement with the effect observed in EAG experiments, even though the stimulatory pathway could be different given the faster decay time of the spike frequency of cell A in response to (+)-disparlure than to the analogue.

Although the relationship between EAG and behavior is generally not straightforward, as it is influenced by the complexity of the integration process at the central nervous system level, our field observations support the electrophysiological data of the laboratory. In fact, OXP-01 produced a negligible attraction on male gypsy moths, whereas it strongly decreased the attractiveness of (+)-disparlure when blended with it. In this respect, its behavioral effect resembles that previously reported by Gries et al. (1996) for (−)-disparlure, which strongly antagonizes the attractiveness of (+)-disparlure in the male gypsy moth, but is neither attractive nor repellent by itself.

At 0.5 μg, OXP-04 and the blend alike were more attractive than a corresponding dosage of pheromone. Moreover, the spike responses of single sensilla to mixtures of pheromone and OXP-04, assessed at various dosages ratios (Figure 6a), are significantly higher than both the pheromone and the analogue. Both behavioral and electrophysiological results suggest a synergetic effect when the 2 compounds are presented simultaneously.

At present, we cannot hypothesize at which level OXPs implement their effects: pheromone-binding proteins (PBPs) or receptor membrane level or a combination of both. PBPs are known to transport pheromone molecules through the sensillar lymph to the dendritic membrane (Vogt et al. 1999), where they activate a G-protein–coupled transduction mechanism leading to the receptor potential (Krieger and Breer 1999). In the gypsy moth, 2 different PBPs have been reported, each having a higher affinity for one of the 2 different enantiomers of disparlure: PBP1 for (−)-disparlure and PBP2 for (+)-disparlure (Plettner et al. 2000).

Honson et al. (2003) suggested that: "PBPs bind a variety of ligands that are structurally related to pheromone." They also observed binding synergy between different ligands when these are presented simultaneously. Finally, the binding between ligands and PBPs depends on the ligand concentration. They concluded that "through this mechanism, the PBP may provide an automatic stimulus attenuation: at low doses, the protein acts as a carrier, and at high doses, it acts as a carrier for a portion of the material and sequesters the excess." It is conceivable that a similar mechanism may underlie our observations with the 2 analogues alone or blended with the pheromone.

Further investigations are needed to elucidate these aspects, in particular more detailed studies on the

structure–activity relationship of both OXP-01 and OXP-04 as concerns chirality, because we cannot exclude that they may have different effects as individual enantiomers.

In conclusion, both electrophysiological and behavioral results concurrently support the hypothesis that in the gypsy moth *L. dispar*, 2 pheromone analogues from OXPs, OXP-01 and OXP-04, may affect the olfactory response to pheromone. In addition to increasing the activity of cell B, the OXP-01 derivative strongly reduces the stimulating effectiveness of (+)-disparlure by silencing the cell best responding to pheromone when the 2 compounds are presented in blend; the OXP-04 compound mimics the pheromone response by interacting with the same receptor neuron, both by itself and blended with pheromone. This study may contribute to understand the binding and releasing mechanisms between odorant molecules and binding proteins or receptor proteins.

Thus, the former may be used in pest-control strategies by disrupting mate-searching behavior in gypsy moths, whereas the latter could be used to bait traps in substitution for (+)-disparlure.

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