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ARTICLE

Agonists and Antagonists of Antennal Responses of Gypsy Moth (*Lymantria dispar*) to the Pheromone (+)-Disparlure and Other Odorants

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Insects use the sense of smell to guide many behaviors that are important for their survival. The gypsy moth uses a pheromone to bring females and males together over long distances. Male moth antennae are equipped with innervated sensory hairs that selectively respond to pheromone components and other odors. Host plant odors, in particular, are detected by moths and sometimes cause an enhancement of the antennal and behavioral responses of the moths to their pheromone. Inspired by naturally occurring agonists and antagonists of insect pheromone responses, we have screened, by electroantennogram (EAG) recordings, a collection of compound sets and of individual compounds. We have detected interference of some compounds with the EAG responses of male gypsy moth antennae to the pheromone. We describe three activities: (1) short-term inhibition or enhancement of mixed compound + pheromone plumes, (2) long-term inhibition of pure pheromone plumes following a mixed compound + pheromone plume, and (3) inhibition of the recovery phase of mixed compound + pheromone plumes. Long-term inhibition was robust, decayed within 30 s, and correlated with the inhibition of recovery; for both activities clear structure−activity patterns were detected. The commercial repellent *N,N*-diethyltoluamide (DEET) was included for comparison. The most active and reproducible short-term inhibitor was a mixture of 1-allyl-2,4-dimethoxybenzene and 2-allyl-1,3-dimethoxybenzene. The most active long-term inhibitors were a set of 1-alkoxy-4-propoxybenzenes, DEET, and 1-ethoxy-4-propoxybenzene. DEET was more specific in the olfactory responses it inhibited than 1-ethoxy-4-propoxybenzene, and DEET did not inhibit recovery, whereas 1-ethoxy-4-propoxybenzene did. Target sites for the three activities are discussed.

KEYWORDS: *Lymantria dispar*; dialkoxybenzene; olfaction; antenna; insect; agonist; antagonist

INTRODUCTION

The gypsy moth, *Lymantria dispar*, is a pest of hardwood trees in Europe, Asia, and North America. The moth begins its life as egg masses deposited by flightless females, near the place where the females emerged and were mated. Larvae hatch and feed on the leaves of the tree where they eclosed (*1*). During epidemics gypsy moth larvae have been known to completely defoliate large areas of forest (*2*). The larvae pupate and enter diapause until the next summer, when they emerge as adults. At this time, the female moths produce a sex attractant pheromone, which they disperse into the air. Male moths search for females many kilometers upwind, using the pheromone plumes to orient (*1*, *2*).

The structure of this pheromone was determined to be *cis*-(7,8)-epoxy-2-methyloctadecane (disparlure), by isolation of the compound from $\sim 10^5$ female gypsy moths (*3*, *4*). Further research, in which the enantiomers of disparlure were tested against the antennae of male gypsy moths (*5*−*8*) and in field trapping experiments (*8*−*10*). revealed that (+)-disparlure, *cis*-(7*R*,8*S*)-7,8-epoxy-2-methyloctadecane (+)**-1**, is the main active component of the sex attractant pheromone of *L. dispar* (**Scheme 1**). The enantiomer, (−)**-1**, has been identified as a major component of the pheromone of the nun moth, a closely related species (*5*, *6*). This enantiomer is not attractive by itself to either species, but prevents upwind flight behavior in the gypsy moth, when presented with (+)**-1**. The nun moth also uses (+)**-1** as a component in its attractant pheromone, and enantiomer (−)**-1** neither attracts nor inhibits the nun moth (*5*, *6*). This discrimination between blends of enantiomeric and other components has been proposed as one mechanism for species differentiation (*5*, *6*, *11*, *12*).

The moths perceive the pheromone through sensory hairs, *sensilla trichodea*, on their feather-like antennae (*13*). Electrophysiological studies with male gypsy moth antennae have revealed that the gypsy moth has innervated sensory hairs that respond only to (+)**-1** or only to (−)**-1** (*7*). This means that the moth detects both enantiomers of **1**, distinguishes them, and integrates the information in the brain. A practical consequence of this enantiomer discrimination is that the number of moths

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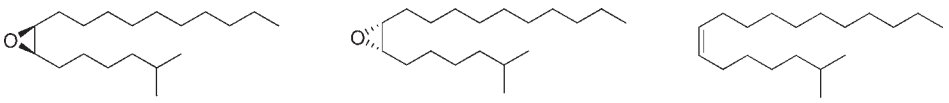
Scheme 1. Gypsy Moth Sex Attractant Pheromone, (+)-**1**, and Two Behavioral Antagonists



(+)-disparlure, **(+)-1** **(-)-1** dispar alkene

caught in pheromone-baited traps is highest with (+)-**1** of high enantiomeric purity (≥98% ee) (*8*). Thus, the pheromone plays a central role in the reproduction of this moth species, and eavesdropping into this pheromone communication has been used in attempts to control the moth.

Pheromone-based control methods that have been tested for the gypsy moth fall into three classes: (1) saturation of the air with pheromone to mask the females and cause mating disruption, (2) trapping large numbers of males into strategically placed traps, and (3) trapping samples of males in monitoring traps and spraying the appropriate area with an insecticide (*2*, *14*). Of these three methods, the third one is widely used to pre-empt outbreaks (*14*). The second approach (mass trapping) has had only limited success because the areas in which mass trapping is necessary, to have a significant impact, are very large. The first approach (mating disruption) carries the risk that large numbers of moths will be attracted to the treated area by the applied pheromone from nearby nontreated zones (*14*). For the gypsy moth, mating disruption is complicated by the hydrophobicity of the pheromone, which makes formulation and biodegradation difficult (*14*), and by the high cost of (+)-**1**. To implement mass trapping or disruption schemes in heavily infested areas, it is thus of interest to develop compounds that are not perceived by themselves but that either enhance or interfere with the perception of the naturally emitted pheromone plumes. These compounds should not be toxic or bioaccumulate. In nature, host plant odors have been known to synergize with pheromone responses (*15*−*19*) and nonhost plant odors sometimes antagonize pheromone responses (*20*). Natural (*5*) or synthetic pheromone mimics can also antagonize the response of an insect to its pheromone (*21*, *22*).

Here we describe the screening of pheromone agonists and antagonists, using the gypsy moth as a model species. We have investigated compounds that are synthetic mimics of phenol-derived odorants (**Scheme 2**) (*23*). Phenolic compounds are found in wood smoke with known insect repellent properties, making them candidates for laboratory screening. We have screened a collection of individual compounds and sets of two to five compounds with two substituents, prepared previously (*23*). We employed electroantennograms (EAG) as screening method. The activities we detected in the EAG assays were short-term and long-term inhibition or enhancement of the antennal response to pheromone (+)-**1**. Short-term inhibition or enhancement was seen with mixed (+)-**1**/compound stimuli, and long-term inhibition of pure (+)-**1** stimuli was seen after administration of mixed (+)-**1**/compound stimuli.

The activities of sets of compounds with one substituent constant and the other varied were compared with the activities of individual compounds with both substituents equal. Comparing structure−activity patterns, we were able to find active candidates and focus on these. DEET, a commercial insect repellent (*24*), was included for comparison. The development of pheromone olfaction agonists and antagonists is important for practical applications as well as for understanding the insect olfactory system. For example, agonists and antagonists could be assessed for disorienting mate-seeking male gypsy moths. EAG response of insects to their pheromone has been correlated to upwind flight behavior (*5*, *6*, *9*, *12*, *17*, *21*, *25*). Agonists would reduce the amount of expensive (+)-**1** required, and antagonists would render (+)-**1** unnecessary.

MATERIALS AND METHODS

Compounds. The synthesis of compounds tested is described in a previous paper (*23*). The compounds can be grouped into five classes. One class of compound was monoalkoxyphenols (**2** series), the second class was dialkoxybenzenes (**3** series), the third class was monoalkoxy allyl phenols (**4** series), the fourth class was dialkoxyallylbenzenes (**5** series), and the fifth class was eugenol and alkyleugenols.

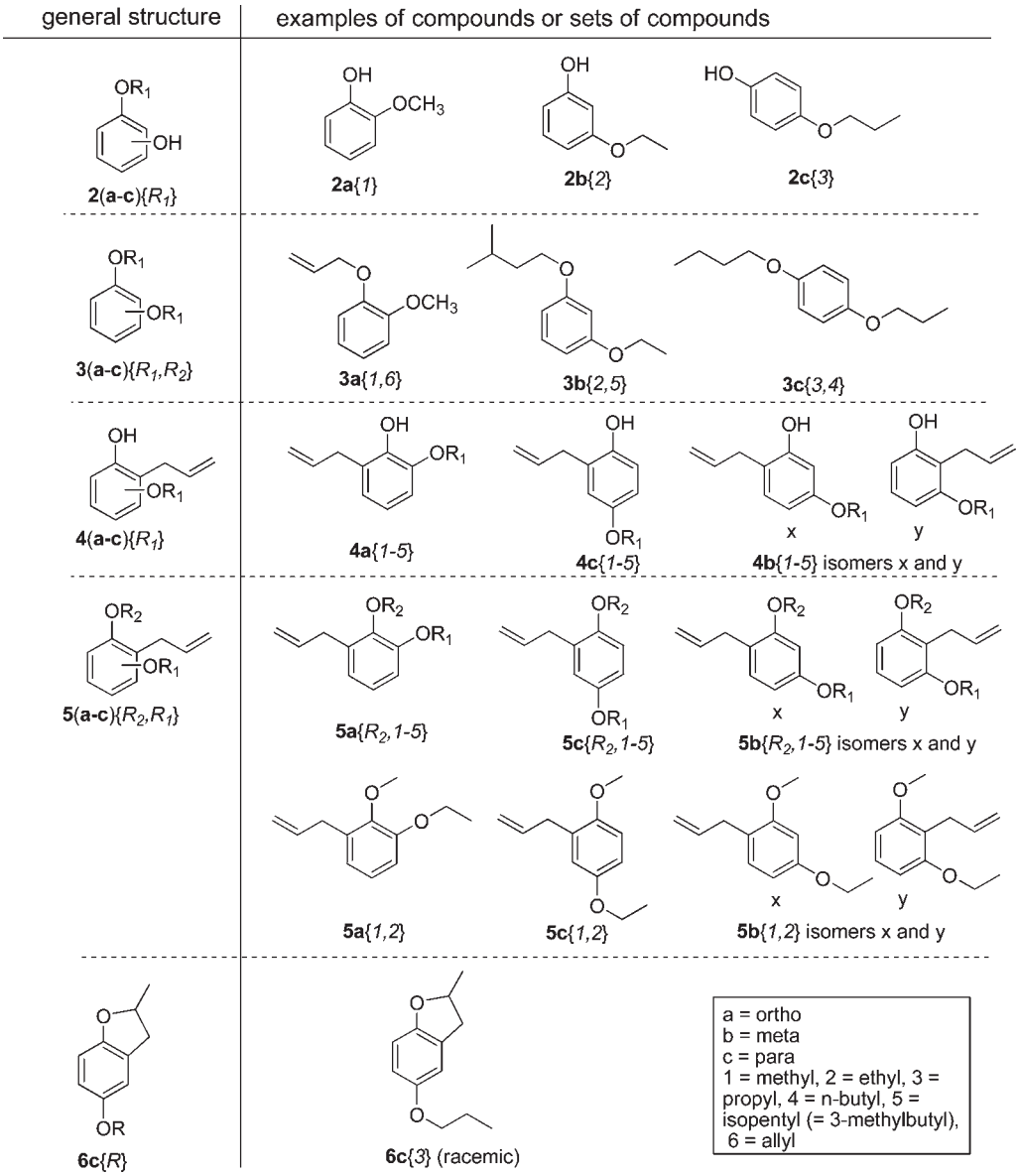
Briefly, compounds were prepared from catechol (**a**), resorcinol (**b**), or dihydroquinone (**c**) by alkylations and Claisen rearrangements. The compounds derived from catechol are labeled "**a**", the ones from resorcinol "**b**", and the ones from dihydroquinone "**c**". Groups were varied within a set of six substituents: methyl *1*, ethyl *2*, propyl *3*, butyl *4*, isopentyl *5* and allyl *6* (**Scheme 2**). Larger substituents were not explored, because we needed the compounds to be sufficiently volatile for the electroantennogram assays. Compounds and small sets of compounds ("minilibraries") are named in the same manner as in the synthesis reference (*23*).

Disparlure was from the following sources: (+)-disparlure (Aldrich, 95%, $[\alpha]_D^{20}$ +0.8 (*c* 1.0, $CHCl_3$), (−)-disparlure (gift from Dr. J. M. Chong, University of Waterloo, ON, Canada to G. and R. Gries in 1996, >98% ee), racemic disparlure, and dispar alkene (synthesized in E.P.'s group, >98% pure cis and Z isomer, respectively), eugenol (vacuum distilled (*23*)), 1-hexanol (Sigma-Aldrich, used as received).

Insects. Male moth pupae were obtained from the Insect Production Service at the Canadain Forest Service's Great Lakes Forestry Centre in Sault Ste. Marie, Ontario (www.insect.glfc.cfs.nrcan.gc.ca) (We are grateful to John Dedes for rearing the pupae.) Prior to 2006, their insect rearing facility had a nondiapausing colony of gypsy moths, from which they reared pupae. When the colony collapsed, they switched to frozen egg masses from infested zones in eastern North America. Regardless of the egg source, larvae were reared on sterile insect media (prepared in-house) and allowed to pupate, and male pupae were shipped in cardboard containers. Pupae were placed individually in small, round Ziploc containers (diameter ~ 7 cm, height ~ 4 cm) with a moist piece of filter paper (Whatman) and a ~1 cm diameter ventilation opening, covered with mosquito netting, in the lid. Pupae were kept at 18−20 °C during light (15 h) and at 16−18 °C during dark (9 h) periods.

Electroantennograms. Compounds were assayed by electroantennogram (EAG) experiments. EAG traces were recorded as follows: a fully dislodged antenna from a male gypsy moth was mounted on the end of a reference electrode [a glass capillary with a silver wire, filled with buffered saline (5 mM NaH_2PO_4, 10 mM Na_2SO_4, 4.5 mM $KHCO_3$, 18 mM $MgCl_2$, 4 mM $CaCl_2$, 6 mM KCl, pH adjusted to 6.8 using 5 mM Na_2HPO_4)]. The tip of the antenna was severed with microscissors and placed in the recording electrode, which was mounted on a micromanipulator. A constant stream of air, which had been purified (charcoal filter), was blown over the antenna at a rate of 300 mL/min. Test samples were delivered through a stimulus delivery tube with a side opening ~2 cm from the distal opening. The proximal end of the delivery tube was connected to a controlled stimulus delivery apparatus (stimulus controller CS-O5, Syntech Research and Equipment, Hilversum, The Netherlands). The distal end of the delivery tube pointed at the antenna (~1 cm from the antenna). The side opening was connected to a cartridge fashioned from a Pasteur pipet, which contained a small filter paper (Whatman no. 1) with the stimulus. These pipet cartridges could be prepared a few days in advance and stored at −80 °C wrapped in aluminum foil and in sealable plastic bags. Sample cartridges were mounted once they had reached room temperature. The stimulus delivery apparatus delivered a second stream of air (a puff) through the Pasteur pipet at a velocity of 600 mL/min, at a specified point in time, for 0.3 s. Three types of experiments were conducted with this setup.

Scheme 2. Compounds Screened in This Study and the Numbering System Used in the Description of the Synthesis of the Compounds[a]



[a] Sets of compounds consisted of two to five compounds with the first group (R_1) variable between five choices (methyl = 1, ethyl = 2, propyl = 3, *n*-butyl = 4, and isopentyl = 5) and the second group attached (R_2) constant. Group R_2 could be choices 1−5 or allyl = 6. Eugenols were also tested here for three reasons: (1) eugenol has insect repellent properties, (2) eugenol occurs in oak (*26*), a preferred host of *L. dispar* (*1*,*2*), and (3) the eugenol substitution pattern was not accessible through the Claisen chemistry we used to generate the **4** and **5** series of compounds (*23*).

Experiment 1. This experiment explored the agonistic or antagonistic activity of the synthetic aromatic compounds (**Scheme 2**) with the gypsy moth pheromone (+)-**1**. The pheromone was kept constant at 100 ng/cartridge, and six puffs were recorded for each replicate: (i) clean air, (ii) pure (+)-**1** (100 ng on the cartridge), (iii) (+)-**1** (100 ng) and the compound (1 μg on the cartridge, mixed with the pheromone), (iv) (+)-**1** (100 ng) and the compound (10 μg), (v) (+)-**1** (100 ng) and the compound (100 μg), and (vi) pure (+)-**1** (100 ng). The following four parameters were measured with this experiment, using the various phases of the EAG signal (**Figure 1**):

(a) net depolarizations, $d_{(net)}$ (in mV), of puffs ii−vi, $d_{(sample)}$, corrected for the depolarization with clean air, $d_{(air)}$

$$d_{(net)} = d_{(sample)} - d_{(air)} \quad (1)$$

(b) percentage short-term inhibition for each compound ($STI_{(compound)}$) of puffs iii−v, relative to the first pure pheromone puff (ii)

$$STI_{(compound)} = 100 \times (d_{(net)(ii)} - d_{(net)(compound)})/d_{(net)(ii)} \quad (2)$$

(c) percentage long-term inhibition for each compound ($LTI_{(compound)}$) of the pure pheromone puff that followed the last mixed puff (vi) relative to the first pure pheromone puff (ii)

$$LTI_{(compound)} = 100 \times (d_{(net)(ii)} - d_{(net)(vi)})/d_{(net)(ii)} \quad (3)$$

(d) percentage inhibition of the recovery period ($RI_{(compound)}$) of the mixed puff with the highest dose of the compound (v) (eq **6**). The height of the recovery (hyperpolarization), $r_{(puff)}$ above the baseline is usually ~20% of the total deviation of the signal from the baseline ($r_{(puff)} + d_{(puff)}$, e.g., **Figure 1**). For many of the mixed puffs, the proportional height of the recovery was either greater or lesser, depending on the compound. The proportional height of the recovery for the first pure (+)-**1** puff (ii) is

$$R_{(puff\ ii)} = 100 \times r_{(ii)}/[r_{(ii)} + d_{(ii)}] \quad (4)$$

and the proportional height of the highest dose mixed puff (v) is

$$R_{(puff\ v)} = 100 \times r_{(v)}/[r_{(v)} + d_{(v)}] \quad (5)$$

The relative change in the recovery is

$$RI_{(compound)} = 100 \times [R_{(puff\ ii)} - R_{(puff\ v)}]/R_{(puff\ ii)} \quad (6)$$

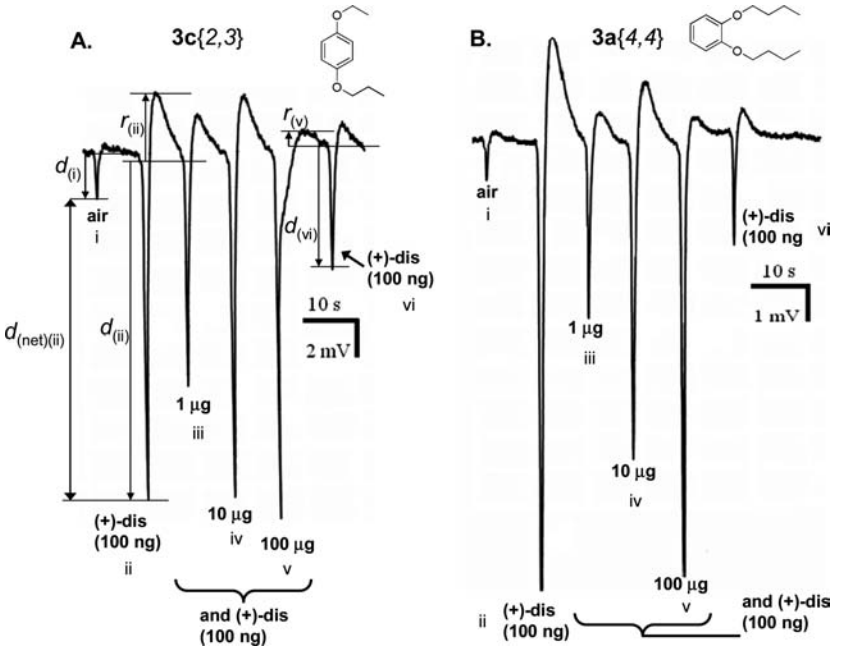


Figure 1. EAG assay for the compounds tested in experiment 1 (see text): (**A**) typical trace for compound **3c**{*2,3*}; (**B**) typical trace for compound **3a**{*4,4*}. The puffs are numbered i−vi (see Materials and Methods) and were (i) air, (ii) pure pheromone (+)-disparlure, (+)-**1** (100 ng), (iii−v) disparlure mixed with the test compound, the latter at three different doses, or (vi) pure (+)-**1**. Depolarizations from the resting potential are labeled d; hyperpolarizations seen during the recovery phase after a puff are labeled r. Subscripts refer to the puff number; calculations done are shown under Materials and Methods.

Experiment 2. The decay of the long-term inhibitory effect was investigated with the strongest long-term inhibitor, set **3c**{*3,1−5*}, and the strongest, most consistent short-term inhibitor, set **5b**{*1,1*}. One hundred micrograms of the inhibitor was premixed with different doses of (+)-**1**, and the following seven puffs were aimed at the antenna: (i) clean air, (ii) pure (+)-**1** at the overall dose being tested (10, 50, 100, 500, or 1000 ng), (iii) the mixed puff with (+)-**1** (test dose) and (100 μg of) **5b**{*1,1*}, (iv) the mixed puff with (+)-**1** (test dose) and (100 μg of) **3c**{*3,1−5*}, (v−vii) pure (+)-**1** at the dose being tested, administered sequentially, always allowing the antenna to recover to baseline before a new puff. This recovery usually took ~5−7 s. The short-term inhibition $STI_{(compound,\ dose)}$ was calculated for each mixed plume. The long-term inhibition $LTI_{(dose,\ time)}$ was calculated for each dose and for each of the three puffs following the mixed pheromone−inhibitor plume.

Experiment 3. The objective of this experiment was to determine whether the long-term or short-term inhibitors have any effect on the pheromone signal if puffed in pure form and well separated from the pheromone and whether these compounds elicit net EAG signals by themselves. This experiment was also done with sets **3c**{*3,1−5*} and **5b**{*1,1*}, tested at 1, 10, and 100 μg, and with (+)-**1** cartridges containing 100 ng of the pheromone. The following stimuli were given: (i) clean air, (ii) (+)-**1** (100 ng), (iii) **5b**{*1,1*} (variable dose) or **3c**{*3,1−5*} (variable dose), and (iv-vi) (+)-**1** (100 ng). Again, the latter puffs were given to test for any time decay of repeated disparlure signals following the pure inhibitor stimulus.

Experiment 4. (1) The first objective of this experiment was to determine whether the strongest long-term inhibitors (antagonists, see Results, **3c**{*2,3*} and DEET) could also alter the EAG responses to odorants other than (+)-**1**. These included (−)-**1**, racemic **1**, dispar alkene (the alkene corresponding to the carbon framework of **1**), methyleugenol (a characteristic oak wood odorant), and 1-hexanol (a green leaf volatile, shown previously to be detected by gypsy moth antennae). For the pure odorant puffs, (-)-**1** and racemic **1** were administered at 100 ng/cartridge, the alkene, methyleugenol, was administered at 1 μg/cartridge, and 1-hexanol was administered at 100 μg/cartridge. The antagonists were both administered at 100 μg/cartridge, mixed with the appropriate dose of the odorant. The puff order was (i) air, (ii) pure odorant, (iii) odorant + antagonist, and (iv) pure odorant. All puffs were corrected for the air response as in eq **1**. STI values were obtained from the depolarization in response to puff ii relative to the depolarization seen with puff i. LTI values were obtained by comparing the depolarization of puff iv relative to that of puff ii.

(2) The second objective of this experiment was to determine whether the oak volatiles, methyleugenol and 1-hexanol, can enhance the antennal response to (+)-**1** and whether the mixed pheromone/plant odorant stimulus can be inhibited by compound **3c**{*2,3*} or DEET. The puff order was (i) air, (ii) pure (+)-**1** (100 ng), (iii) (+)-**1** (100 ng) and plant odorant (1 μg for methyleugenol and 100 μg for 1-hexanol), (iv) (+)-**1** and plant odorant as in (iii) + antagonist (100 μg), and (v) same as in (iii). Depolarizations were corrected for the air response as in eq **1**, and the short-term effects were calculated for puff iii relative to puff ii and for puff iv relative to puff ii. Long-term effects were calculated for puff v relative to puff iii (for LTI of the mixed pheromone/plant odorant plume) and for puff v relative to puff ii (to determine whether long-term effects of the plant odorant and of the antagonist cancel).

Structure−Activity Modeling of Pure Compounds onto a Three-Point Site. The compounds chosen for this exercise were pure compounds (not "minilibraries") and DEET. DEET was the reference compound, as it was among the compounds with highest LTI and a molecular target site for this compound has been proposed (*28*). Compounds were drawn using ChemDraw (CambridgeSoft, 2007, version 11.0) and copied into Chem 3D (CambridgeSoft, 2007, version 11.0). Each compound was minimized first using molecular mechanics and the MM2 force field, then using a semiempirical method via the MOPAC interface (method = PM3; wave function = closed shell; optimizer = EF; solvent = H_2O). All minimizations were run to an rms of ≤0.1. Compounds were then compared to the minimum DEET conformer by superposition. The comparison is based on the hypothesis that an active compound will bind to a cognate site only if the recognition groups on the compound are predisposed in the correct orientation at or close to a stable conformation of the compound. The more closely the recognition groups are predisposed to the active site in a stable conformer, the more active the compound. Conversely, the less stable the conformer of a compound that matches the active site, the less active the compound. Details of the site and data are shown in the Supporting Information.

RESULTS

The compounds we tested gave weak or no olfactory responses by themselves in electroantennogram (EAG) experiments (Supporting Information). The EAG trace reflects the change in the potential across the antenna when an air puff with an

odorant is passed over the antenna. To understand the agonistic or antagonistic effect of the compounds on pheromone responses by male moth antennae, four types of EAG experiments were conducted. First, the response of the antenna elicited by a stimulus of pure (+)**-1** was compared to the responses elicited by blends of (+)**-1** with the test compound (Materials and Methods, **Figure 1**). The mixed plumes often gave a significantly different response, compared to the pure (+)**-1** stimuli. This effect was termed short-term inhibition (STI). A pure (+)**-1** stimulus, given after the mixed stimuli, was sometimes significantly inhibited, compared to the initial pure (+)**-1** stimulus, and this was termed long-term inhibition (LTI). Second, the time decay and dose responses for LTI activities were studied. Third, the strongest inhibitors were tested for their ability to cause LTI by themselves. Fourth, the strongest long-term inhibitors were tested (1) against other host plant and pheromone odorants and (2) against mixtures of (+)**-1** and host plant odorants.

Experiment 1: Short- and Long-Term Inhibitors of Pheromone Signaling. The complete set of STI and LTI, obtained in experiment 1, is shown in Tables S1−S3 of the Supporting Information.

STI showed structure−activity patterns for some compounds or sets (Tables S1−S3 and Figure S1 of the Supporting Information), but for many compounds it varied between different batches of moths. The strongest, most robust short-term inhibitor was set **5b**{*1,1*} (a mixture of 1-allyl-2,4-dimethoxybenzene and 2-allyl-1,3-dimethoxybenzene). The eugenols showed consistent negative STI values, signifying that they enhanced the antennal responses to the pheromone.

Long-term inhibition (LTI) showed robust structure−activity patterns that could be reproduced from year to year and between different sources and lots of moths. The alkoxyphenols showed less activity than the dialkoxybenzenes (**Figure 2A−D**). For *o*-alkoxyphenols the LTI increased from methyl to propyl and then decreased for butyl and isopentyl. The dialkoxybenzenes with $R_1 = R_2$ gave higher LTI and showed structure−activity patterns (**Figure 2B**): ortho compounds showed increasing LTI with increasing group size from methyl to butyl, and a loss of activity for isopentyl; meta compounds had highest activity for midsized groups; and para compounds had moderate and variable activity. The dialkoxybenzene sets (**Figure 2C**) showed moderate activity for all ortho and meta sets, with the methyl sets being highest. The para sets showed a structure−activity pattern: the propyl set had the highest LTI activity. Individual compounds from the propyl set tested (**Figure 2D**) and compound **3c**{*2,3*} (1-ethoxy-4-propoxybenzene) were the strongest long-term inhibitors. The meta allyl series of compounds (**3b**{*n,6*}) was also tested (**Figure 2E**), and **3b**{*3,6*} (1-allyloxy-3-propoxybenzene) was the most active long-term inhibitor.

The ortho and para 1-allyloxy, 2- or 4-alkoxybenzene sets (**3a**{*6,1−5*} and **3c**{*6,1−5*}) were subjected to thermal Claisen rearrangement to provide sets **4a**{*1−5*} and **4c**{*1−5*}, respectively. These sets of phenols were divided and alkylated to give six new sets of compounds: **5a**{*1, 1−5*} to **5a**{*6,1−5*} and **5c**{*1,1−5*} to **5c**{*6,1−5*}. In the meta case, the 1-allyloxy-3-alkoxybenzene precursors were kept in smaller groups (methyl by itself, ethyl and propyl, butyl and isopentyl) (**Figure 2H**), because each precursor could form two products (*23*). These Claisen rearranged products or the eugenols showed little LTI compared to the most active dialkoxybenzenes. Among the **5a** compounds, the ones with a midsized second alkyl group (R_2) were most active (**Figure 2G**), and among the **5b** compounds the ones with the methyl group were most active (**Figure 2H**).

The strongest long-term inhibitors were DEET and set **3c**{*3,1−5*} (1-alkoxy-4-propoxybenzene), of which compound **3c**{*2,3*} (1-ethoxy-4-propoxybenzene) was the most active.

Experiment 2. Decay of the Long-Term Inhibitory Effect and Dose Responses. The LTI was strongest 10 s after the mixed antagonist/pheromone stimulus, and the inhibition decayed to ≤20% within 30 s (**Figure 3A**) The same decay pattern was seen for pheromone doses of 10−1000 ng. This indicates that the antenna can fully recover from the LTI caused by a mixed antagonist/pheromone plume. The highest LTI was seen for the lowest competing doses of pheromone (+)**-1**, but even at very high doses of pheromone (1000 ng), there still was significant LTI (**Figure 3B**). This dose−response pattern suggests that both the inhibitor and the pheromone are necessary, in a particular ratio, to cause LTI.

The dose response of male gypsy moth antennae to pure pheromone (+)**-1** is shown in **Figure 3C**. The LTI dose responses with respect to (+)**-1** and antagonists **5b**{*1,1*} or **3c**{*3,1−5*} are shown in **Figure 3D**. As noted previously, set **5b**{*1,1*} (30 ± 7% LTI) was a less effective long-term inhibitor than set **3c**{*3,1−5*} (71 ± 13% LTI). This was apparent in the dose response of the first pure pheromone puff after the mixed antagonist/pheromone plume. For example, at 100 ng of pure pheromone the depolarization was at its maximal value (∼20 mV on average), but after the mixed plume the depolarization for 100 ng of pheromone was only 10 mV for **5b**{*1,1*} and 7 mV for **3c**{*3,1−5*}. The difference between the two antagonists became magnified at higher doses (**Figure 3D**). This suggests that LTI results from allosteric effects, caused by the antagonist and the pheromone in a particular ratio.

Experiment 3. Long-Term Inhibition after Pure Antagonist Plumes. Sets **5b**{*1,1*} and **3c**{*3,1−5*} showed no significant depolarization by themselves, indicating that these compounds do not function as odorants (Supporting Information). Pure pheromone (+)**-1** puffs (given at 10 s intervals, starting 10 s after the antagonist puff) showed no significant LTI for either set or for the mixture of **5b**{*1,1*} and **3c**{*3,1−5*} (Figure S2 of the Supporting Information). There was no significant change in the depolarization of the puffs that followed the antagonist, compared to the puff preceding the antagonist. This shows that the antenna does not exhaust itself with repeated puffs at 10 s intervals and that the LTI seen in experiments 1 and 2 is due to the mixed pheromone/antagonist plumes and not due to antennal exhaustion. The data obtained in this experiment also indicate that LTI activity requires the exposure of the antenna to a mixed plume, consisting of the odorant being inhibited (pheromone (+)**-1** in this case) and the antagonist. The long-term inhibitors are not odorants themselves, but interfere with the pheromone response in a mixed plume, leading to a reversible long-term effect. A possible mechanism for this is discussed later.

Experiment 4. Long-Term Inhibition of Host Plant Odor Responses. To determine whether the long-term inhibitory effects of **3c**{*2,3*} and DEET are specific to (+)**-1** or also apply to other odorants, compound **3c**{*2,3*} and DEET were tested further with other odorants: (−)**-1**, racemic **1**, dispar alkene, methyleugenol, and 1-hexanol. Compound **3c**{*2,3*} and DEET differed in their short-term and long-term effects on the other odorants tested (**Table 1**). With (−)**-1** or racemic **1**, **3c**{*2,3*} had a highly variable short-term enhancing effect, whereas DEET had a more consistent effect. Compound **3c**{*2,3*} had a moderate LTI activity, and DEET was not active. With dispar alkene, both **3c**{*2,3*} and DEET showed similar short-term enhancements and LTI activities. Methyleugenol gave the same responses as clean air, yet the weak signal was enhanced short-term by both **3c**{*2,3*} and DEET, an activity not seen with the test compounds by themselves. In terms of LTI, only **3c**{*2,3*} was active, giving peaks that were smaller on average than the response to air. Leaf volatile 1-hexanol is a weak odorant whose response was not significantly affected short-term by either antagonist. There was weak LTI

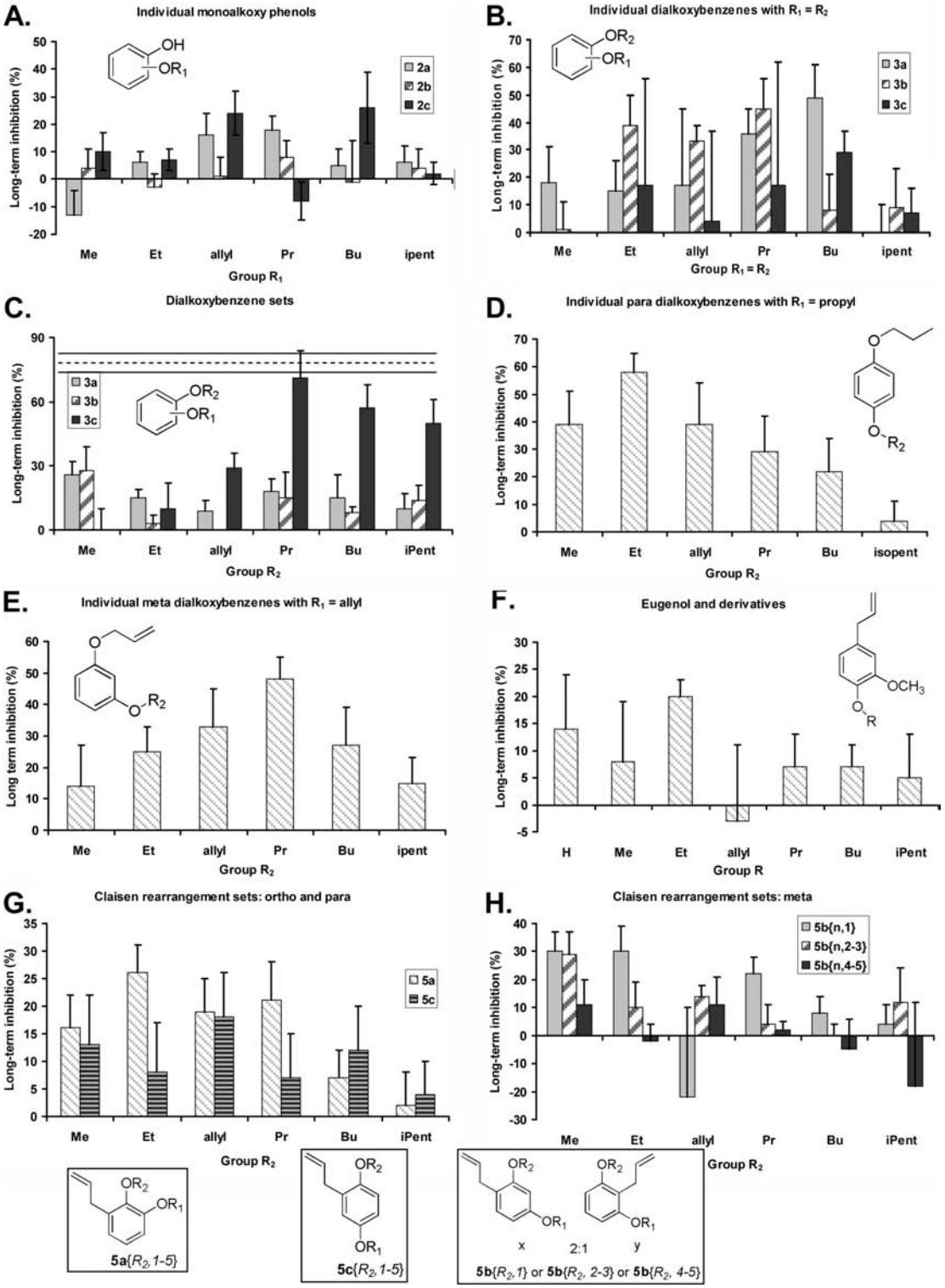


Figure 2. SAR for long-term inhibition (LTI) of EAG responses toward (+)**-1**: (**A**) alkoxyphenols; (**B**) dialkoxybenzenes with equal substituents; (**C**) dialkoxybenzene sets; (**D**) individual *o*-dialkoxybenzenes (**3c**{*3,n*},1-propoxy-4-alkoxybenzenes) [the dashed line indicates the LTI value obtained for DEET (±SE, shown with the solid lines)]; (**E**) individual *m*-dialkoxybenzenes (**3b**{*n,6*},1-allyloxy-3-alkoxybenzenes); (**F**) eugenol and derivatives; (**G**) allyl dialkoxybenzene sets obtained from Claisen rearrangement of 1-allyloxy-2-alkoxybenzenes and 1-allyloxy-4-alkoxybenzenes, followed by alkylation of the new phenol group (**Scheme 1**); (**H**) allyl dialkoxybenzene sets obtained from Claisen rearrangement of 1-allyloxy-3-alkoxybenzenes (**Scheme 2**). In **Figure 1**, the depolarization for puff vi (second pure (+)-1 puff) is much smaller than for puff ii (first pure (+)**-1** puff). The percentage difference between these two puffs is the LTI (see Materials and Methods for calculations). Positive values denote inhibition, negative ones enhancement.

against 1-hexanol by **3c**{*2,3*} but not by DEET. Compared to DEET, compound **3c**{*2,3*} was the more broadly tuned long-term inhibitor. This broader inhibition of olfaction suggests that compound **3c**{*2,3*} targets a component of several different populations of sensory hairs (sensilla) on the antenna.

Because insect pheromone responses are altered by host plant odors and both methyleugenol and 1-hexanol are odorants of oak, we compared the ability of compound **3c**{*2,3*} and DEET to alter the response of the male moth antennae to mixtures of pheromone and the plant odorant (**Table 2**). Methyleugenol, at 10

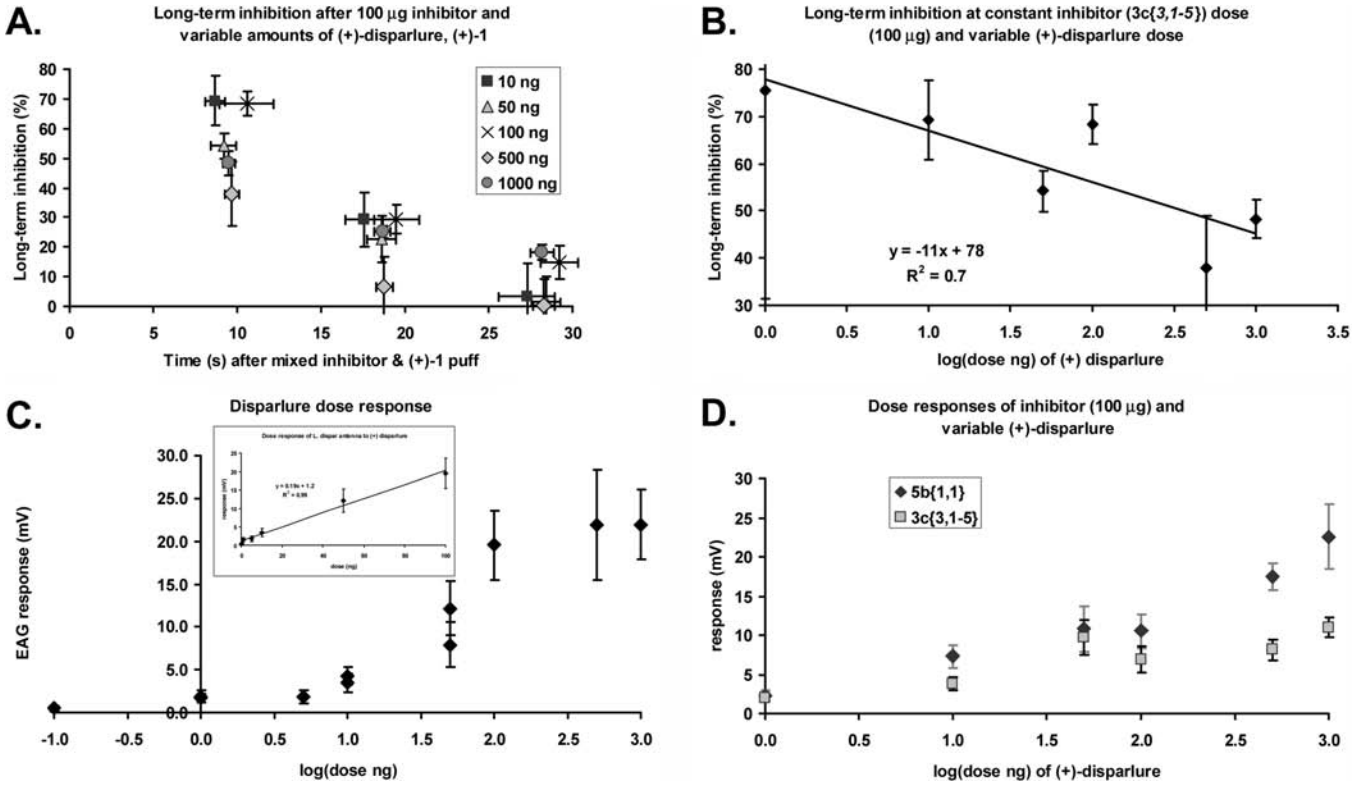


Figure 3. (**A**) Dose and time decay properties of the long-term inhibition (LTI) activity by set **3c**{*3,1*−*5*}: LTI dependence on the delay time of the pure (+)**-1** puffs (v−vii, experiment 2) after the mixed (+)**-1**/inhibitor puff. The amount of (+)**-1** was varied from 10 ng to 1 μg, and the amount of inhibitor was constant at 100 μg. (**B**) Dose response of LTI with respect to variable (+)**-1** responses and time responses for the strongest long-term inhibition of EAG responses toward (+)**-1**. (**C**) Dose response of pure pheromone (+)**-1**. (**D**) Dose response of (+)**-1** mixed with 100 mg of either compound **5b**{*1,1*} (the strongest short-term inhibitor) or set **3c**{*3,1*−*5*} (the strongest long-term inhibitor).

times excess relative to (+)**-1**, enhanced the response to pheromone weakly in a few cases, consistent with the previous data with three different methyleugenol doses (see Table S3 of the Supporting Information). Interestingly, the ternary mixture of (+)**-1**, methyleugenol, and the antagonist gave a significantly enhanced short-term response, relative to the (+)**-1**/plant odorant mixture. Also, there was no significant LTI for either antagonist on the (+)**-1**/plant odorant mixture or on a pure (+)**-1** puff following the mixed puff. A similar picture was obtained with 1-hexanol, except that DEET was weakly long-term enhancing, whereas **3c**{*2,3*} had no LTI activity. The similarities in activity between DEET and **3c**{*2,3*} suggest that they may act on a similar target site, but the differences in activity also suggest that there are additional modes of action that differ between DEET and **3c**{*2,3*}.

Correlation between Recovery from the Mixed Antagonist/Pheromone Plume and LTI from Experiment 1. The recovery phase (hyperpolarization) of a pheromone stimulus is proportional to the magnitude of the preceding depolarization (**Figure 1**). Analysis of the correlation between the inhibition of the recovery phase, RI (see Materials and Methods), and the LTI revealed a strong positive correlation between these two parameters for all of the compounds (Supporting Information Figure S3). Groups of compounds with similar structures also showed correlation between LTI and RI, for example, the ortho compounds (Figure S3-B of the Supporting Information, $R^2 = 0.9$). This suggests that the recovery phase from the antagonist/pheromone plume can determine the ability of the antenna to respond to a pure pheromone plume: the shallower the hyperpolarization of the mixed plume, the stronger the inhibition of a following pure pheromone plume. This indicates that the dialkoxybenzene long-term inhibitors can interfere with recovery processes in the antenna (see below). In contrast, DEET did not show significant RI activity ($9 \pm 9\%$, $N = 6$), but had strong LTI activity. This suggests that the dialkoxybenzenes cause LTI via at least two modes: one similar to DEET and a second mode that affects RI and is not affected by DEET.

Correlation of LTI in Adult Male Gypsy Moths with Larval Feeding Deterrence in *Trichoplusia ni*, the Cabbage Looper, and in Gypsy Moth. Most of the compounds that were screened in this work have also been assessed against *T. ni* as either feeding deterrents or oviposition deterrents (Akhtar, Isman, Plettner, unpublished data). There was a positive correlation between LTI of pheromone stimulation in gypsy moth and feeding deterrence in cabbage looper larvae for a set of 39 active compounds/sets ($R^2 = 0.66$). Preliminary tests with gypsy moth larvae also suggest a correlation between LTI of pheromone stimulation in adult males and feeding deterrence of the larvae for some of the active compounds. This suggests that the antagonists identified in that group of compounds target a component of the olfactory system that is conserved between these two species of Lepidoptera and between different developmental stages.

Modeling of Long-Term Inhibitors onto a Three-Point Site. The single-compound antagonists with the strongest LTI activity were modeled onto a three-point recognition site for DEET or DDT (Figure S4-A of the Supporting Information), because DEET and DDT target sites are known, and both compounds cause deterrence of insects by interference with olfactory cues (*27*, *28*). Also, DEET was one of the best LTI-causing compounds in this study (Table S1 of the Supporting Information; **Figure 2C**), so it was used as a reference compound. We assumed that three odotopes, arranged around a benzene ring, exist for the repellent activity of

DEET or DDT: (1) a hydrocarbon chain end (CH_3) or group of equivalent size (Cl in DDT), (2) a polar moiety (amide carbonyl group in DEET or CCl_3 in DDT), and (3) a hydrocarbon chain end (CH_3 group) or π-system (aryl or vinyl) near the polar moiety. These three odotopes are arranged in DEET as follows: (1)−(2) 6.1 Å; (2)−(3) 3.6 Å; and (3)−(1) 6.0 Å. For the compounds described here, we assumed that one of the oxygen atoms serves as the polar group (Supporting Information Figure S4-A). For each compound modeled, the most stable conformer was compared to the three-point site with respect to the position of the three odotopes. If the conformer did not fit into the site, then individual bonds were rotated, until the distance between the odotope was close. The distances were then constrained, and the compound was minimized again. The heat of formation of the conformer constrained to the three-point site was compared to the heat of formation of the most stable conformer.

There was a negative correlation between the log of enthalpy penalty (the enthalpy a particular compound had to invest to fit into the three-point site relative to its minimum conformation) and the LTI (Supporting Information Figure S4-B,C). This suggests that, to cause LTI through the same target site as DEET, a compound needs to fit into the three-point site at or close to its minimum conformation. To have a stable conformation that fits the site, the alkyl groups of the dialkoxybenzenes needed to be within a particular size range for a particular geometry. For example, methyl was too small overall; ethyl was too small for ortho and meta, but reasonable for para; one propyl group along with a smaller group was appropriate for para; butyl was appropriate for ortho but too large for meta and para; isopentyl was too large overall.

Table 1. Interaction of Several Pheromone and Plant Odorants Relevant to the Gypsy Moth with DEET or Compound **3c**{*2,3*} in the EAG

odorant	antagonist	*N*	short-term activity[b,d] (%)	long-term activity[c,d] (%)
(−)-disparlure	**3c**{*2,3*}	5	−1226 ± 1056	43 ± 12
	DEET	5	−322 ± 66	−10 ± 29
racemic disparlure	**3c**{*2,3*}	6	−154 ± 45	69 ± 5
	DEET	6	−468 ± 231	−126 ± 133
dispar alkene[a]	**3c**{*2,3*}	4	−244 ± 15	39 ± 12
	DEET	4	−955 ± 647	52 ± 22
methyleugenol	**3c**{*2,3*}	4	−1158 ± 834	46 ± 24
	DEET	4	−598 ± 506	8 ± 8
1-hexanol	**3c**{*2,3*}	4	−34 ± 28	29 ± 18
	DEET	4	6 ± 30	7 ± 18

[a] This compound is (*Z*) 2-methyloctadec-7-ene. [b] This refers to the short-term inhibition (positive) or enhancement (negative) of the mixed odorant/antagonist puff, relative to the first pure odorant puff. [c] This refers to the long-term inhibition (positive) or enhancement (negative) of the pure odorant puff that followed the mixed odorant/antagonist puff, relative to the first pure odorant puff (see Materials and Methods). [d] Mean ± SE of *N* replicates.

DISCUSSION

Peripheral (on the Antenna) versus Central (in the Brain) Odorant Agonism and Antagonism. Short-term (blend) effects in insect olfaction have been described before, for various naturally occurring and synthetic blends of odorants. For example, host plant odors are able to modulate both the EAG (*15*, *17*, *18*, *25*, *29*−*34*) and behavioral responses of insects to their pheromone (*15*−*20*, *25*, *31*, *33*, *35*, *36*). Synthetic compounds can also alter the peripheral responses of insects. For example, trifluoromethyl ketone mimics of pheromones interfere with the EAG responses (*21*, *22*, *37*, *38*) and with the behavior of the insects toward their pheromone (*21*, *22*, *38*). Another example is DEET: it caused either a decrease or an enhancement in the response to food odors of selected olfactory neurons in *Drosophila* (*28*). Peripheral enhancements of pheromone responses by plant volatiles appear to be interpreted as higher pheromone doses in the brain. For example, synergistic pheromone and leaf volatile mixtures elicited enhanced responses by selected projection neurons in the macroglomerular complex in silk moths, similar to higher doses of pheromone (*39*). In the same study, the moths showed greater behavioral sensitivity to the pheromone/plant volatile mixtures than to the pheromone alone (*39*).

Direct effects of agonists or antagonists on the EAG are mediated peripherally, through the antenna. In contrast, known synergy/antagonism between pheromone components is mediated not at the antennal level but in the brain. On the antenna, different neurons (housed in distinct populations of sensilla) respond to various pheromone components highly selectively (see, e.g., ref *40*). The information corresponding to individual pheromone components is relayed from the primary sensory neurons on the antenna to the macroglomerular complex (*41*) and to higher brain centers. Because primary neurons respond in a dose-dependent manner, even pheromone blend ratios can be interpreted by specialized interneurons in the brain (*42*). Such pheromone component mixtures can also elicit enhancement or inhibition of a characteristic behavior triggered by the pheromone. An example is the pheromone synergy and antagonism in the gypsy moth/nun moth presented in the introduction. Social insects are another example, in which synergy and antagonism between large multicomponent pheromone blends control the social fabric of the colony (*43*, *44*).

Potential Target Sites for Peripherally Active Compounds. Sensilla trichodea are responsible for pheromone detection. These are single-walled sensilla with pores, innervated by two or three sensory neurons (*13*). The neurons project a dendrite into the hollow space of the hair, the sensillar lymph space (**Figure 4**), and their axon projects to the macroglomerular complex. Three accessory cells support the neurons physiologically. First, the thecogen cell surrounds the neuronal cell body and the inner dendritic segment. Tight junctions between the membranes of the neuron and the thecogen create a diffusion barrier for many solutes and isolate the sensillar lymph space (*45*). Second, the thecogen cell is surrounded by the tricogen cell, and this is surrounded by the third cell, the tormogen. The tricogen and

Table 2. Effect of Plant Odors and DEET or Compound **3c**{*2,3*} on the EAG Responses of Male Gypsy Moth Antennae to Pheromone (+)-**1**[a]

plant synergist	antagonist	short-term effect with the plant synergist[d]	short-term effect with plant synergist + antagonist	long-term effect on the mixed (+)-**1**/plant odor plumes	long-term effect on (+)-**1** response
methyleugenol[b]	**3c**{*2,3*}	−39 ± 37 (9)	−125 ± 95 (4)	11 ± 47 (4)	12 ± 33 (4)
	DEET		−210 ± 120 (5)	30 ± 27 (5)	27 ± 29 (5)
1-hexanol[c]	**3c**{*2,3*}	−19 ± 24 (10)	−99 ± 60 (5)	5 ± 24 (5)	20 ± 18 (5)
	DEET		−339 ± 206 (5)	−24 ± 19 (5)	−53 ± 48 (5)

[a] Pheromone (+)-**1** was applied at 100 ng/cartridge. [b] Applied at 1 μg/cartridge. [c] Applied at 100 μg/cartridge. [d] Number of replicates given in parentheses; means ± SE.

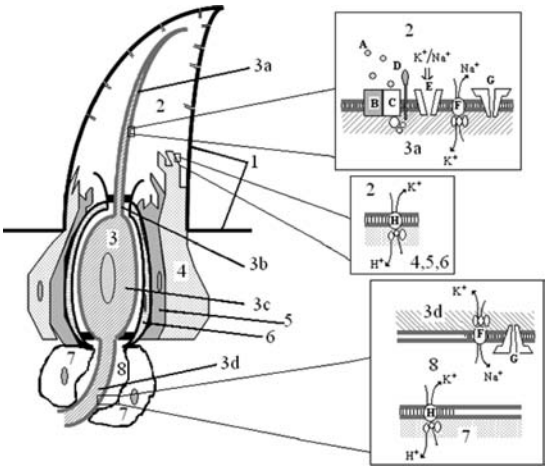


Figure 4. Schematic diagram of a *sensillum trichodeum*: 1, cuticle (the cuticular wall of the sensillum has pores that end in pore canals); 2, sensillar lymph space [this compartment contains a solution rich in K^+ and pheromone-binding protein (PBP, A in the top expansion window)]; 3, sensory neuron (3a, outer dendritic segment; 3b, inner dendritic segment; 3c, cell body; 3d, proximal axon segment); 4−6, accessory cells (4, tormogen; 5, tricogen; 6, thecogen) [thecogen, 6, envelopes the cell body and the inner dendritic segment; the space between the cell body, 3c, and the thecogen cell, 6, is separated from spaces 2 and 8 by tight junctions that are diffusion barriers for ions and other solutes (black bars)]; 7, glial cells (these cells envelope the axon); 8, perineurial space [this is found only between the proximal part of the axon and the glia; the expansion windows show known molecular components, relevant to this study, from the outer dendrite and sensillar lymph (top), the apical membrane of the accessory cells (middle), and the axonal/glial membranes (bottom)]; A, PBP; B, conserved receptor; C, olfactory receptor; D, sensory neuron membrane protein; E, ligand-activated monovalent cation channel; F, sodium/potassium ATPase; G, voltage-gated ion channel; H, V-type proton/potassium ATPase.

tormogen have highly folded membranes on their apical sides, facing the sensillar lymph space. These cells secrete pheromone-binding proteins (PBPs) and K^+ ions. The ion secretion is mediated by a vacuolar-type H^+/K^+-ATPase, found only on the apical membranes of the three support cells (*46*). The K^+ ions appear to be important in the maintenance of the resting receptor neuron potential, partly through a Na^+/K^+-ATPase located on the neuronal plasma membrane (*45*).

The outer dendritic membrane is the location of the signal transduction pathway that interacts with the pheromone and causes the slow depolarization seen in EAG traces. The main components that interact with odorants are specialized odorant receptors (OR), each type being expressed in different populations of neurons, housed in different populations of sensilla (*47*−*51*). The insect ORs are seven-transmembrane G-protein coupled proteins; a member of the G_q family has been mapped to insect sensory neuron dendritic membranes on the intracellular side (*52*) and has been shown to play a role in insect olfactory transduction (*53*, *54*). One protein that resembles an OR, but is topologically reversed, is expressed in all olfactory sensilla and appears to be a chaperone and dimerization partner for the odorant-selective OR (*55*). This "conserved receptor" (CR) also appears to form or interact with a monovalent cation channel, upon odorant activation of the OR. This might be the conduit for the initial slow depolarization of the sensory neuron (*56*, *57*).

Once the dendrite has been depolarized, voltage-gated sodium channels generate action potentials. Sodium channels have been detected in cultured insect neurons on the outer dendritic segment and the axon (*58*). Whether other voltage-gated channels are involved in the sensillar responses to odorants in insects is not known, but likely: in lobsters several IP_3, one IP_4, and one cAMP-activated channel have been identified (*59*). Once the pheromone stimulus has ended, the neuron ceases to depolarize and spike, and the recovery process begins with an arrestin, which has been mapped to sensilla of insects (*60*). The resting potential can then regenerate via the Na^+/K^+-ATPase and the support cell vacuolar ATPase (*45*). The spiking onset and termination to background levels is also controlled by two perireceptor proteins that bind odorants and that have opposing effects on neuronal spiking: the PBP and the sensory-neuron membrane protein (SNMP). The SNMP appears to inhibit background spiking (*61*, *62*), and the PBP appears to stimulate it (*63*). When PBP is missing from a sensillum, the neuron shows no background spiking and is unresponsive, and when SNMP is missing, the neuron is permanently activated. Neither mutant responds to the pheromone, and a double mutant appears like a SNMP mutant, suggesting that SNMP is upstream of the PBP in the control of the CR (*61*, *62*) and participates in signal termination (*62*). Genetic experiments with fluorescent proteins have suggested that SNMP, CR, and OR interact in close proximity (*55*).

Short-term agonists or antagonists may target the OR. Agonists perhaps stabilize an active form of the OR, and antagonists either compete with the activating odorant or stabilize an inactive form. Similar blend effects have been observed with vertebrate receptors. For example, the response of one mouse OR that responds to eugenol was significantly inhibited when mixtures of eugenol and methylisoeugenol or isosafrole were given in the stimulus. The effect was short-term: responses to pure eugenol following the mixed stimulus were normal (*64*). In a second example, the mouse octanal receptor was specifically short-term inhibited by citral, which did not activate the OR by itself (*65*). In a third example, the human sperm OR response to burgeonal was inhibited by the addition of undecanal (*66*).

Long-term inhibitors may target a similar site as DEET, for which one site has been mapped within this cascade to the CR: mosquitoes lacking the CR did not respond to DEET behaviorally. In addition, DEET appears to either inhibit or enhance the response of selected ORs to their cognate odorants (*28*). Because DEET is a strong long-term inhibitor in this study, it is reasonable to assume that the long-term inhibitors target a similar site as DEET. This is also suggested by our modeling of the compounds onto a three-point contact site that fits DEET or DDT. It is interesting that long-term inhibition is seen only when a mixed inhibitor/pheromone plume precedes the pure pheromone stimulus that is inhibited. This observation is consistent with observations made for DEET with *Drosophila*: DEET affected the responses to food odorants only when it was applied simultaneously with the odorants (*28*). It is also important to note that the long-term inhibition in this study decays within ca. 30 s (**Figure 3A**) and that the inhibitor by itself, even when given alternating with pheromone every 10 s, does not cause long-term inhibition of pheromone signals. One interpretation of these three observations is that the three-point site for the inhibitor is at a protein/protein interface that transiently arises when pheromone activates the system. This site could be between the CR/OR or CR/SNMP pairs, and this needs to be investigated further.

Unlike DEET, many of the long-term inhibitors from our collection of dialkoxybenzenes also caused inhibition of the hyperpolarization of the mixed puff preceding the pure pheromone test stimulus. This suggests that these compounds may bind to a second site that is involved in the recovery phase. One

candidate for this second site is the Na^+/K^+-ATPase. If this enzyme is inhibited, then the potentials across the dendritic membrane will not return to the optimal level, and the EAG depolarization of a closely following signal will be less because the ion gradients are smaller. DDT also affects olfactory responses in insects, and it is known that DDT targets the mitochondrial ATPase and a Ca^{2+}-ATPase, but can also weakly inhibit the Na^+/K^+-ATPase (*67*). It is unlikely that our compounds inhibit the mitochondrial or vacuolar ATPases, because they do not show acute toxicity toward moths (*68*). A second candidate for the site of action for the recovery inhibitors is the voltage-gated sodium channel. Insecticides that cause paralysis (knockdown) and sensory disruption, such as DDT, are known to bind to the insect voltage-gated Na^+ channel (*69*). Our inhibitors did not cause paralysis in the moths, which suggests that the voltage-gated Na^+ channel is an unlikely target site. However, odorant-activated channels could be affected by the inhibitor + pheromone mixtures. Finally, the compounds that inhibit the recovery phase of EAG traces from mixed stimuli might interfere with the recovery function of SNMP. The SNMP has been shown to regulate the decay of action potential frequency in sensilla, after a stimulus, to the background frequency (*62*). This recovery process may require the close interactions that have been demonstrated between SNMP, CR, and OR (*55*). Our observation that compound **3c**{*2,3*} is inhibitory against other odorants and other developmental stages of moths is consistent with the hypothesis that it targets conserved components of the peripheral olfactory system.

Potential Practical Application of the Agonists and Antagonists. Some of the compounds from this study have been field tested, but more tests are required to develop a practical application. Potential applications could include enhanced attraction of insects to traps, masking of the pheromone emitted by the females, or deterrence of the insects from certain areas. Attraction could be used for monitoring or, if attraction is sufficiently strong, sufficient traps are deployed, and the population density is suitable, mass trapping of males. Masking of the natural pheromone plumes and deterrence could be used to protect valuable stands of trees or to prevent an outbreak in certain areas.

Conclusion. We describe the activity of synthetic aromatic compounds that modulate the electrophysiological responses of male gypsy moth antennae to the pheromone. Compounds were identified that cause short-term enhancements of inhibition of olfactory responses. Other compounds caused inhibition of the recovery phase of mixed pheromone + compound plumes and, finally, several compounds caused the inhibition of pure pheromone stimuli that followed the mixed plume. The best long-term inhibitor was 1-ethoxy-4-propoxybenzene. On the basis of the structure−activity relationships we propose that this long-term inhibitor acts on a conserved component of the moth's peripheral olfactory system.

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Supporting Information Available: Tables S1−S6, Figures S1−S4, and experimental results. This material is available free of charge via the Internet at http://pubs.acs.org.

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