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(−)-Germacrene D: Masking Substance of Attractants for the Cerambycid Beetle, *Monochamus alternatus* (HOPE)[1]

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Oxygenated terpenes separated from healthy *Pinus densiflora* wood are attractive to the cerambycid beetle, *Monochamus alternatus*, while the healthy pine is unattractive to it. Thus, the attractiveness of the pines appears to be masked by an unknown substance occurring in the healthy pine. A key compound, (−)-germacrene D, was isolated from the steam distillate of healthy pine leaves by reduced-pressure distillation, HPLC and TLC. In an olfactometer chamber, the oxygenated terpenes elicited flight response in the female beetle, but (−)-germacrene D in combined use diminished the attractiveness. The inhibitory activity was also confirmed in a linear tube olfactometer. The locomotory response of the female was released by a mixture of (+)-juniperol and (+)-pimaral—attractants isolated from the healthy pine. However, the female ceased her locomotory movements when (−)-germacrene D was added to the attractant odor. Therefore, (−)-germacrene D functioned as a masking substance, i.e., an agent inhibiting the locomotory movements toward the attractant source.

Key words: *Monochamus alternatus*, *Pinus densiflora*, masking substance, (−)-germacrene D, attractants

INTRODUCTION

The cerambycid beetle, *Monochamus alternatus* (HOPE), is a vector of the pine wood nematode, *Bursaphelenchus xylophilus* (STEINER et BUHRER) NICKLE (MORIMOTO and IWASAKI, 1972; MAMIYA and ENDA, 1972). The combination of both species has caused severe mortality of *Pinus densiflora* (SIEB. et ZUCC.) and other pines. Paraquat (herbicide)-induced lightwood (oleoresin-soaked wood) in pines is highly attractive to the beetle (YAMASAKI and SUZUKI, 1982). The component of lightwood that is attractive to the female was identified as a synergistic mixture of the sesquiterpene alcohol (+)-juniperol and the diterpene aldehyde (+)-pimaral (SAKAI and YAMASAKI, 1990). The monoterpene alcohol (+)-*cis*-3-pinen-2-ol, characteristic of lightwood, enhances male attraction by the mixture (SAKAI and YAMASAKI, 1991). (+)-Juniperol and (+)-pimaral were also isolated from the healthy pine (SAKAI and YAMASAKI, 1990). Moreover, oviposition stimulants for the beetle occur in the inner bark of pine (YAMASAKI et al., 1989). However, the beetle does not select healthy pines as preferable sites for oviposition. Therefore it is suggested that healthy pine tissues, unlike lightwood, may emit an unknown substance that is responsible for the negative selection by the beetle as an oviposition site. This communication deals with a novel type of masking activity of a sesquiterpene hydrocarbon isolated from healthy *P. densiflora*.

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MATERIALS AND METHODS

Instruments. Optical rotation was measured with a JASCO J-20 polarimeter, and specific rotation ($[\alpha]_D$) was calculated. A high-resolution mass (HR-EIMS) spectrum was taken with a VG AutoSpecQ mass spectrometer at an ionizing voltage of 70 eV. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded with a JEOL JNM-A 400 spectrometer. The carbon types were determined by NMR distortionless enhancement by polarization transfer (DEPT). ^{13}C-^{1}H shift correlation spectroscopy (COSY)-, ^{1}H-^{1}H COSY- and ^{1}H-detected multiple-bond connectivity (HMBC) spectra were also recorded with the spectrometer. All NMR spectra were taken at 20°C using deuterochloroform solutions with TMS as an internal standard.

Extraction and fractionation of essential oils. Essential oils were obtained by steam distillation from freshly crushed trunks and leaves of five healthy 11-year-old *P. densiflora*, i.e., 84.3 g and 320.5 g of essential oils, respectively. A portion of the trunk essential oil was subjected to low-temperature liquid chromatography (YAMASAKI et al., 1986) to separate a fraction of the oxygenated terpenes (diethyl ether eluate) from the other terpene hydrocarbons (*n*-pentane eluate). Terpene hydrocarbons were also separated from the leaf essential oil. In addition, essential oil was also obtained from paraquat-induced lightwood (SAKAI and YAMASAKI, 1991) in the xylem of a 15-year-old *P. densiflora.*

Isolation of (−)-germacrene D. The leaf essential oil (200.1 g) was distilled up to 45.5°C at 0.5 mmHg. A residual oil was obtained at a yield of 15%. This residue was dissolved in acetonitrile, and water was added to obtain an 85% aqueous acetonitrile solution containing 2.9 vol% residue. Aliquots (0.8 ml each) of this solution were applied to a LiChrosorb RP-18 column for HPLC (7 μm, 10 mm i.d.×250 mm, Merck). The pump was a JASCO 880-PU. Aqueous acetonitrile (85%) with a flow rate of 2.0 ml/min was used as the mobile phase at room temperature. Detection was carried out at 220 nm using a JASCO UVIDEC-100-V UV spectrophotometer. The compound that eluted with a retention time (t_R) of 61.1 min was isolated. Aliquots (0.8 ml each) of a 0.4 vol% solution of the crude compound in 85% aqueous acetonitrile, were again applied to the column. The final purification was achieved by preparative TLC over Kieselgel 60 GF_{254} (Merck) using *n*-pentane as the mobile phase at 10°C.

Colorless oil. $[\alpha]_D^{20}=-218°$ ($c=0.23$ in methanol). Previous $[\alpha]_D^{23}=-240°$ (YOSHIHARA et al., 1969). HR-EIMS: found; 204.1882, calcd. for $C_{15}H_{24}$; 204.1878. DEPT, ^{13}C-^{1}H/^{1}H-^{1}H COSYs and HMBC spectra aided in assigning the ^{1}H and the ^{13}C NMR signals. Previous ^{1}H (MORI et al., 1990) and ^{13}C (RANDRIAMIHARISOA et al., 1986) NMR data supported the identification of the sesquiterpene (−)-germacrene D.

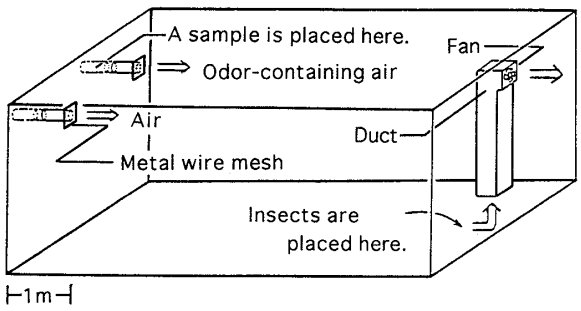

Fig. 1. An olfactometer chamber.

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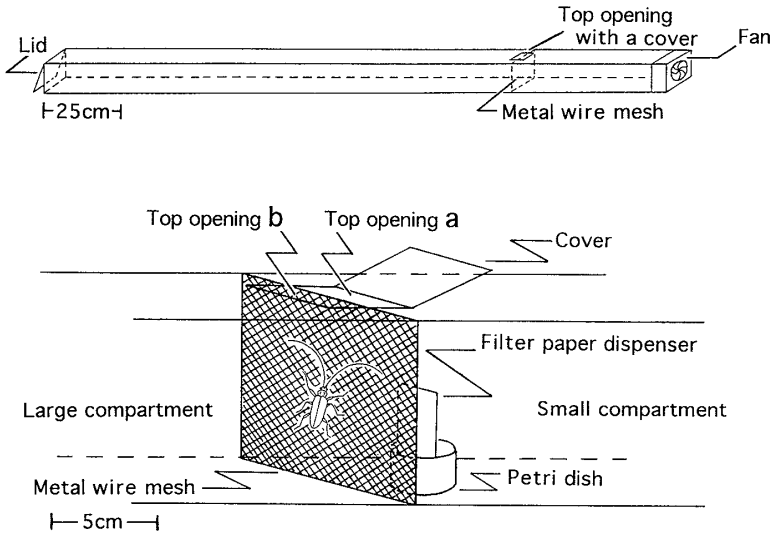

Fig. 2. A linear tube olfactometer: an actograph for the odorous behavior of insects. A filter paper dispenser (9×2.5 cm, No. 1, Advantec Toyo; folded twice) was put into the small compartment through top opening **a** and set into a Petri dish at ca. 3 cm from the wire mesh. Then, a test female was introduced into the large compartment through the other top opening **b** and placed on the mesh. The locomotory behavior and the behavior inhibition elicited by test samples were observed. After the test, the inside of the apparatus was ventilated with a fan.

Isolation of (+)-juniperol and (+)-pimaral. These compounds were isolated from the trunk essential oil by a combination (SAKAI and YAMASAKI, 1990) of reduced-pressure distillation, preparative gas chromatography and TLC. The ^{1}H NMR spectra of the (+)-juniperol and the (+)-pimaral coincided with those of their respective specimens (SAKAI and YAMASAKI, 1990).

Gas chromatographic determination. (−)-Germacrene D, (+)-juniperol and (+)-pimaral were determined by a Shimadzu GC-14A gas chromatograph equipped with an FID and a DB-5 fused-silica capillary column (0.25 mm i.d.×30 m, J & W Scientific). Nitrogen gas with a flow rate of 30 ml/min was used as a carrier. The split ratio was held at 75:1. Injector and detector temperatures were 250°C and 260°C, respectively. The oven temperature was programmed to remain at 50°C for 5 min, then increase at 5°C/min for another 38 min and finally was maintained at 240°C for 17 min.

Insects. Female beetles—both sexes being attracted to paraquat-treated pines (YAMASAKI and SUZUKI, 1982)—were collected at Miki-cho, Kagawa Pref. in July of 1993–1995. They were kept individually in ca. 100 bottles and subjected to behavioral assays during the following two weeks.

Olfactometer chamber. Flight responses of the females to the chemicals were assayed in an olfactometer chamber (6.8×2.6×3.8 m) equipped with odor and air (control) inlets and a ventilation duct (Fig. 1) (SAKAI and YAMASAKI, 1990). Individual sheets (30×30 cm) of metal wire mesh, necessary for attracted females to perch on, were fixed to one opening of each inlet. Elytra of the females were marked with spot(s) of white paint for individual identification (SAKAI and YAMASAKI, 1990). Twenty-five females were randomly selected from the group and placed on the floor below the duct. Air was introduced at 5.1 m^3/min. A filter paper dispenser (5×5 cm, No. 1, Advantec Toyo) was set in a Petri dish inside the odor inlet. Solutions (40 μl each) of a sample in *n*-pentane were applied onto the dispenser

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every 15 min from the outside of the chamber. The numbers of females which landed on the wire mesh sheet on the odor and the control inlets within 1 h were counted. After the first hourly assay, the chamber was ventilated at 12.3 m^3/min for 30 min. A second assay was done after the females were re-positioned at the starting point. Thus, hourly assays of each of four or five samples with the same females were performed at the 30-min ventilation intervals each night. The assay with females randomly selected every night was begun at 20:00.

Linear tube olfactometer. The locomotory behavior of each female and the behavior inhibition elicited by the chemicals, were observed in a 195-cm polymethyl methacrylate tube (Fig. 2) with a square section (9×9 cm) installed in the chamber described above. A ventilation fan and a lid, respectively, were each set into one end of the tube, which was partitioned into a large and a small compartment with a sheet of metal wire mesh 45 cm from the fan. A filter paper dispenser and a female each were introduced into the apparatus through separate top openings (4×2 cm each) at the mesh site so that they were separated by the mesh. If the female remained still within 15 cm from the border mesh for 10 min, a solution (40 μl) of a sample in *n*-pentane was applied onto the dispenser to start observation under still air conditions with all openings closed. The female's locomotory activity was recorded every 30 s for 10 min. After the first test, which started at 20:00, the female and the dispenser were removed, and the apparatus as well as the chamber were ventilated for 20 min. A second test with a female followed. Thus, tests with each of four or five females were performed in each night. The results of tests with 30 females were compiled.

RESULTS

Contents of (−)-germacrene D and attractants

The trunk essential oil (17.3 mg) from healthy pines consisted of terpene hydrocarbons (17.0 mg) and oxygenated terpenes (260 μg). The same amount of leaf essential oil contained 16.2 mg of terpene hydrocarbons. Gas chromatographic determination showed that the trunk and the leaf terpene hydrocarbon fractions (17.0 mg each) contained 160 μg and 490 μg of (−)-germacrene D, respectively, which eluted with a t_R of 28.8 min (relative t_R = 1.07, based on a t_R of longifolene). The oxygenated terpenes (260 μg) in the trunk contained the attractants, i.e., (+)-juniperol (12.8 μg) and (+)-pimaral (18.7 μg), whereas the leaf essential oil lacked both attractants. On the other hand, the essential oil from paraquat-induced lightwood was devoid of (−)-germacrene D, while it (17.3 mg) contained 7.1 μg of (+)-juniperol and 13.3 μg of (+)-pimaral.

Flight response of the female beetle in the olfactometer chamber

As Table 1 shows, no flight response of the female was stimulated by any of the following: (−)-germacrene D, terpene hydrocarbons, *n*-pentane and air only. In most instances, the females remained resting in their positions, except for an occasional unoriented walk or "short-range" (ca. 1.5 m at most) flight, but no female landed on either wire mesh. In contrast, the trunk oxygenated terpene fraction induced flight to its odor inlet, as previously described (SAKAI and YAMASAKI, 1990). However, the flight response was significantly reduced when the odor was combined with either (−)-germacrene D or a terpene hydrocarbon fraction. Such a reduction in flight activity was similarly observed in the trunk essential oil containing (−)-germacrene D and the attractants.

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Table 1. Flight response of female *M. alternatus* to volatiles in an olfactometer chamber

Volatile[a]	No. of females[b] (Mean ± S.E.)	
	Attracted to	
	Odor inlet[c]	Control inlet
Germacrene	0	0
Trunk Hc	0	0
Leaf Hc	0	0
Ox-terpene	8.8 ± 0.8 a	0.4 ± 0.2
Ox-terpene + Germacrene	2.4 ± 0.2 b	0
Ox-terpene + Trunk Hc	2.8 ± 0.4 b	0
Ox-terpene + Leaf Hc	2.2 ± 0.4 b	0
Trunk Eo	2.6 ± 0.2 b	0
n-Pentane	0	0
Air	0	0

[a] Volatiles are abbreviated as follows; Amounts/15 min are shown in parentheses. Germacrene, (−)-germacrene D (160 μg); Trunk Hc, trunk terpene hydrocarbons (17.0 mg); Leaf Hc, leaf terpene hydrocarbons (17.0 mg); Ox-terpene, trunk oxygenated terpenes (260 μg); Trunk Eo, trunk essential oil (17.3 mg). In combination, *n*-pentane solutions (40 μl each) were consecutively supplied onto each of two pieces of filter paper set side by side.

[b] A test with 25 beetles was replicated five times.

[c] Means with different letters are significantly different at the 5% level (one-way ANOVA followed by a TUKEY-KRAMER multiple-comparison test).

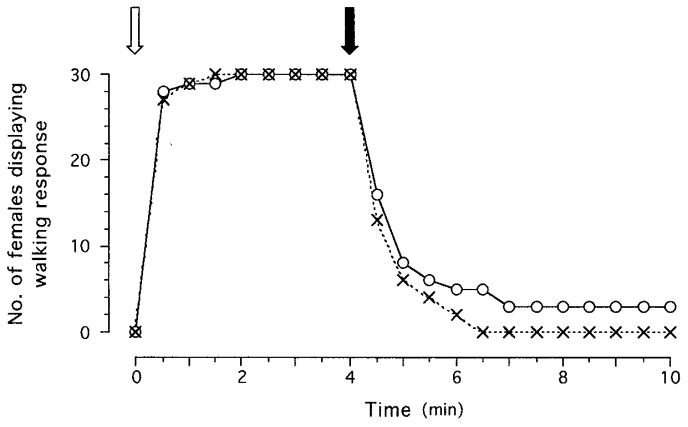

Fig. 3. Induction and inhibition of the locomotory activity of female *M. alternatus*. Activity was stimulated by the application (⇨) of (+)-juniperol and (+)-pimaral (15 μg each), and inhibited by the addition (➡) of (−)-germacrene D (○: 32 μg, ×: 160 μg). $N = 30$.

Behavioral response of the female beetle to (−)-germacrene D in the linear tube olfactometer

The response of a female to (−)-germacrene D was observed in this particular olfactometer. After exposure to the solvent *n*-pentane (40 μl/dispenser) for 4 min, the female was subjected to (−)-germacrene D (32 or 160 μg/40 μl of *n*-pentane) for 6 min. Almost all females, in 30 repetitions, were motionless throughout the test time except that three of them twitched either antenna at the 160-μg dosage, indicating that, like *n*-pentane, (−)-germacrene D did not elicit any locomotory activities at all.

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Behavioral response of the female beetle to the attractants and additional (−)-germacrene D in the linear tube olfactometer

First, the locomotory behavior of the female elicited by the attractants [(+)-juniperol and (+)-pimaral (15 μg each/40 μl of *n*-pentane)] applied onto one of two dispensers set side by side was observed. Four minutes later (−)-germacrene D (32 or 160 μg/40 μl) was added to the other dispenser, and the consequent behavior was observed. The female, initially at rest, raised her antennae and waved them within 30 s after the application of the attractants. She then began walking and were ranging within 15 cm of the border mesh, especially on the mesh. However, the locomotory response was halted by additional exposure to (−)-germacrene D. Figure 3 shows that 14 females, in 30 repetitions, suddenly stopped at that point; 3 min after the addition of (−)-germacrene D (32 μg), only three females were observed roaming slowly on the border mesh. The inhibition of the locomotion was enhanced at the higher dosage (160 μg); all females became motionless within 2.5 min.

DISCUSSION

(−)-Germacrene D alone failed to induce a flight response from the female beetle in the olfactometer chamber (Table 1). Further, no behavioral response of the beetle at rest in the linear tube olfactometer was elicited by this compound. On the other hand, the trunk oxygenated terpenes containing the attractants elicited flight response from the beetle. In combination, (−)-germacrene D, however, diminished the attractiveness of the oxygenated terpenes (Table 1). This inhibitory activity was also confirmed in the tube olfactometer (Fig. 3). Locomotory behavior of the female beetles was induced by the attractants, but they became motionless when (−)-germacrene D was added to the attractant odor. The results show that (−)-germacrene D functioned as a masking substance, i.e., an agent inhibiting the locomotory movements toward the attractant source.

Orientation of the Colorado potato beetle, *Leptinotarsa decemlineata*, to a *Solanum* host odor is thrown into disorder—but not halted—by blending the host odor with a *Lycopersicon* or a *Brassica* nonhost odor (THIERY and VISSER, 1986, 1987). In contrast, (−)-germacrene D acts as a locomotion inhibitor. To our knowledge, such activity is of a novel type in terms of the effect (DETHIER et al., 1960) of chemicals on the insect behavior.

Crypsis is defined as the condition whereby a prey masks signals that it is emitting, so that the predator can not perceive them (EDMUNDS, 1974). Thus far chemical crypsis has been little understood (EDMUNDS, 1974; DETTNER and LIEPERT, 1994). The present experimental designs give a "laboratory model system" of chemical crypsis. It is suggested that healthy *P. densiflora*, unlike paraquat-treated pines, can be cryptic with the aid of the masking substance, (−)-germacrene D, which it emits itself. During oviposition site selection, the cerambycid beetle, *M. alternatus*, may not detect the plant's presence.

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