Evidence for (*E*)-pityol as an aggregation pheromone of *Pityophthorus pubescens* (Coleoptera: Curculionidae: Scolytinae)

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Abstract—We present evidence favoring the use of (*E*)-pityol as an aggregation pheromone in *Pityophthorus pubescens* (Marsham). (*E*)-Pityol was detected in effluvia of male and female *P. pubescens*, and antennae of both sexes responded to (*E*)-(+)-pityol in electroantennogram assays. In two-choice olfactometer tests, males significantly preferred (*E*)-(+)-pityol and (*E*)-(±)-pityol to blank controls at doses of 1, 10, and 100 ng, whereas females only showed a preference for (*E*)-pityol at the 1 ng dose.

Résumé—Dans ce travail, nous présentons la preuve de l'utilisation de (*E*)-pityol comme une phéromone d'agrégation par *Pityophthorus pubescens* (Marsham). (*E*)-Pityol a été détecté dans les volatiles des mâles et des femelles de *P. pubescens* et les antennes des deux sexes ont répondu au (*E*)-(+)-pityol lors des essais électrophysiologiques. Dans un olfactomètre à deux choix, les mâles ont montré une préférence significative pour (*E*)-(+)- pityol et (*E*)-(±)-pityol aux doses de 1, 10, et 100 ng par rapport au contrôle, alors que les femelles seulement ont montré une préférence pour (*E*)-pityol à la dose de 1 ng.

Introduction

Most species of twig beetles, *Pityophthorus* Eichhoff (Coleoptera: Curculionidae: Scolytinae), are considered polygamous (Bright 1981; Wood 1982), but there is evidence of monogamy in a few species (Pfeffer 1976; Bright 1981; Dallara *et al.* 2000). In polygamous species, a male selects the host, starts constructing an egg gallery with a nuptial chamber, and attracts females, which extend the egg gallery (Bright 1981; Kirkendall 1983).

Little is known about pheromone-based aggregation in the genus *Pityophthorus*. Vité (1965); however, Chararas (1966, 1975) observed that males of *P. confertus* Swaine, *P. annectans* LeConte, and *P. pityographus* (Ratzeburg) attract conspecific females. Francke *et al.* (1987) identified (2*R*,5*S*)-2-(1-hydroxy-1-methylethyl)-5-methyltetrahydrofuran (*E*-(+)-pityol) and *cis*-1-(2-hydroxyethyl)-1-methyl-2-(1-methylethenyl) cyclobutane ((±)-grandisol) from males of *P. pityographus*, and showed that both compounds were active in the field. (*E*)-(+)-Pityol was also found in males of *P. carmeli* Swaine and females of *P. nitidulus* Mannerheim and *P. setosus* (Blackman) (Dallara *et al.* 2000), and has been reported as the female-produced sex pheromone of the cone beetles *Conophthorus resinosae* Hopkins, *C. coniperda* (Schwarz), and *C. ponderosae* Hopkins (Coleoptera: Curculionidae: Scolytinae) (Birgersson *et al.* 1995; Pierce *et al.* 1995;

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Miller *et al.* 2000). In addition to pityol, the spiroacetal (5*S*,7*S*)-(–)-7-methyl-1,6-dioxaspiro[4.5]decane (conophthorin) has been identified as a semiochemical in species of *Pityophthorus*. Conopthorin is a component of the aggregation pheromone emitted by males of *P. carmeli* (Dallara *et al.* 2000) and is a male-produced repellent in some other scolytines (Kohnle *et al.* 1992; Birgersson *et al.* 1995; Pierce *et al.* 1995; de Groot and DeBarr 2000). (*E*)-(–)-Conophthorin, by itself, is not attractive to *Pityophthorus* species, and significantly reduces catches of *P. setosus* (predominantly males) to (*E*)-pityol (Dallara *et al*. 2000), suggesting that it acts as a synomone to reduce intraspecific competition between *P. setosus*, *P. nitidulus*, and *P. carmeli*, three species that cohabit in *Pinus radiata* D. Don (Pinaceae) stands in central coastal California (Dallara *et al.* 2000).

Pityophthorus pubescens (Marsham) is the only *Pityophthorus* species known from *P. radiata* stands in the Basque Country (northern Spain) (López *et al*. 2007). It is associated with *Fusarium circinatum* (Niremberg and O'Donnell) (Hypocreales: Nectriaceae), the pathogen causing pitch canker disease (Romón *et al.* 2007). *Pityophthorus setosus* and *P. carmeli* have been associated with pitch canker-infected Monterey pines in California (Storer *et al.* 2004; Sakamoto *et al.* 2007). Our objectives were to identify the aggregation pheromone of *P. pubescens* and evaluate its biological activity in electroantennographic (EAG) and behavioral tests in the laboratory.

Materials and methods

Beetles

Specimens of *P. pubescens* were collected from infested branches of *P. radiata* from a stand located at Gorosika (43°15′N, 02°42′W), Basque Country. Infested branches were maintained in an incubator at 25 °C and 65% RH under a 10L:14D photoperiod, and beetles were collected by dissecting the branches with a microscalpel under a binocular microscope.

Chemicals

Racemic (*E*)-pityol (93.4% chemical purity) was purchased from Contech Inc. (Delta British Columbia, Canada) and (*E*)-(+)-pityol (99%) (Mori and Puapoomchareon 1987) was provided by Prof. W. Francke (Institute of Organic Chemistry, University of Hamburg, Hamburg, Germany).

Collection of volatiles

Volatiles of *P. pubescens* were adsorbed on a Porapak Q column (50/80 mesh, Supelco, Bellefonte, Pennsylvania) using 200 beetles of each sex caged in a glass flask and exposed to a stream of charcoal-filtered air at a flow rate of 1 L/min for 40 h. Three independent sets of this system containing males, females, or no beetles (control) were operated simultaneously and two volatile collections and blanks (control) were made for each sex. Each column was extracted with 300 µL of dichloromethane and the extract was stored at –40 °C until used.

Additional volatile collections were obtained using a polydimethylsiloxane fiber (100 µm) for solid-phase microextraction (SPME) (Supelco) (Belardi and Pawliszyn 1989; Matich *et al.* 1996). Two hundred individuals of each sex were placed in separate 40 mL vials (29 mm × 81 mm) with a SPME fiber for 36 h under laboratory conditions (mean temperature 23 °C, 65% RH, 14L:10D). The fiber had been conditioned prior to use by inserting it into the injection port of a gas chromatograph (GC) for 15 min. Two replicates were done for each sex, using 200 different beetles each time.

Volatiles from both collection methods were analyzed on a Thermo Finnigan Trace 2000 GC system coupled to a Trace MS quadrupole mass spectrometer (ThermoFisher Scientific, Madrid, Spain) using helium (1 mL/min) as the carrier gas. The samples were introduced in splitless mode at 250 °C. The column used for analysis was a 30 m × 0.25 mm i.d. × 0.25 µm HP-5MS fused silica capillary (Agilent Technologies, Madrid, Spain). The following chromatographic conditions were used: injection at 60 °C for 5 min, increasing by 5 °C/min to 280 °C, and then maintained at this temperature for 10 min. Mass spectra were obtained

under electron impact ionization mode at 70 eV in the 40–400 m/z range.

EAG assays

The EAG instrument was obtained from Syntech (Kirchzarten, Germany). EAG recordings were performed using Ag-AgCl glass microcapillaries filled with Ringer solution. Each beetle was fixed upside down on a piece of double-sided sticky tape, and the head was excised using a microscalpel. The recording electrode was connected to the tip of an antenna and the reference electrode was inserted into the occipital foramen using MP15 micromanipulators (Syntech). Humidified pure air (1000 mL/min) was continuously directed over the antenna. The signals were amplified (100 ×) and filtered (DC to 1 kHz) with a IDAC-2 interface (Syntech), digitized on a PC, and analyzed with the EAG Pro program. The EAG system was set up in a Faraday cage (70 cm × 65 cm × 60 cm) to preclude external electric signals. A log dilution series of (*E*)-(+)-pityol in hexane was prepared at doses of 0.1, 1, 10, and 100 ng/μL. Odor stimuli consisted of applying 10 μL of a given concentration to a filter-paper strip (2.5 cm i.d.) that was then placed inside a Pasteur pipette. After evaporation of the solvent, puffs of 400 ms duration through the Pasteur pipette placed 2 cm from the antennal setup were pulsed with a stimulus controller, CS-01 (Syntech). The recovery time for the antenna between two consecutive stimuli was established at 1–1.5 min. Eight individuals of each sex were used with each dose of (*E*)-(+)-pityol and only one antenna was used per beetle. Three puffs of each dose of (*E*)-(+)-pityol were applied and the mean amplitude of depolarization was subtracted from that in response to puffs of the hexane control, before and after each stimulus. Stimuli were delivered in order of increasing dose.

Behavioral response

The behavioral responses of male and female *P. pubescens* to three different doses of (*E*)-(+)-pityol and (*E*)-(±)-pityol were evaluated using a Y-tube olfactometer designed for small beetles (5 mm i.d., main arm 5 cm long, short arms 4 cm long, 90° angle between short arms). Each short arm of the olfactometer was connected to a glass chamber containing the odor source. One of the arms contained 10 μL of hexane on a circle of filter paper (2.5 cm diameter) as a control, while the other contained a piece of filter paper of similar size treated with the test chemical. Different doses of the semiochemical were obtained from 10 μL of decadic dilutions in hexane containing 0.1, 1, and 10 ng/μL. Filter papers were replaced in each arm for every second beetle. Incoming air was filtered through activated charcoal and the airflow was maintained at 820 mL/min.

All tests were conducted at 23 ± 1 °C, 50 ± 9% RH, and beetles were acclimatized to conditions for 15 min before the assays. Each beetle was observed for a maximum of 5 min and was used only once. A response was considered positive when the beetle walked at least 3 cm into one of the arms. The arms were reversed after five beetles were tested, to avoid directional bias. After 10 individuals were tested, the olfactometer was cleaned first with soap and water and then with absolute ethanol, and left to dry until the solvent had completely evaporated. In total, 35–40 different beetles were used for each sex and dose.

Statistical analysis

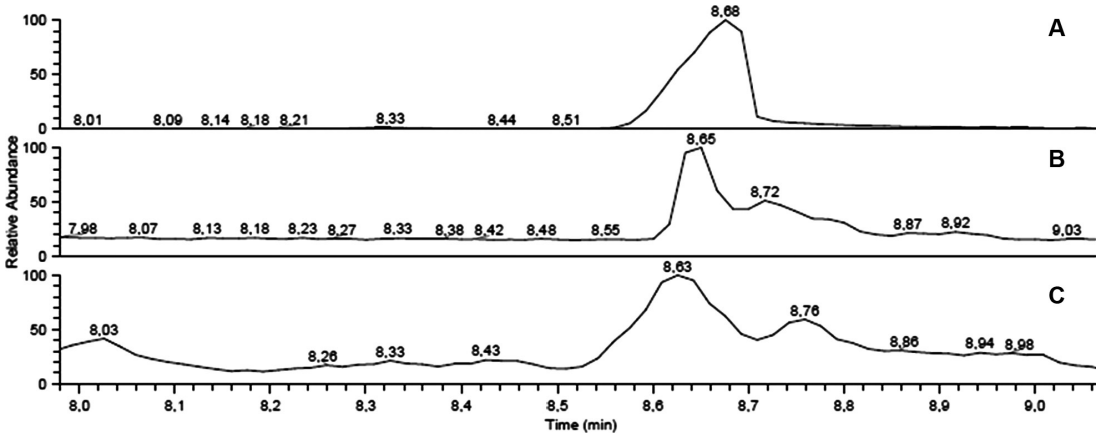
Data on the EAG response to (*E*)-(+)-pityol concentrations and sex were subjected to two-way ANOVA, followed by Tukey's post-hoc tests at a significance level of $\alpha = 0.05$. In olfactometer trials, the null hypothesis that *P. pubescens* showed no preference for either olfactometer arm (a response equal to 50:50) was analyzed by a χ^2 test.

Results

Volatile collections

GC-mass spectrometry analyses of volatile collections trapped on Porapak Q or by SPME revealed the presence of (*E*)-pityol in both sexes of *P. pubescens* by comparing its retention time and mass spectra with those of

Fig. 1. Amplified region of the gas chromatogram of a standard sample of (*E*)-(+)-pityol (A) and a dichloromethane extract of headspace volatiles of male (B) and female (C) *Pityophthorus pubescens* trapped on Porapak Q. The numeral above each peak represents the retention time (minutes). See Materials and methods for gas chromatographic conditions.



an authentic standard (Francke *et al*. 1987) (Figs. 1, 2). All spectra exhibited a base peak at m/z 59, suggesting a tertiary alcohol, and prominent peaks at 85, 102, and 129 m/z. The intense fragment ion of m/z 85 in the mass spectrum reflects the presence of a tetrahydropyran or methyl-substituted tetrahydrofuran ring as a partial structure. The amount of (*E*)-(+)-pityol emitted was not compared between the sexes. As expected, no traces of (*E*)-pityol were found when the controls of both sexes were analyzed.

EAG response of *P. pubescens* to (*E*)-(+)-pityol

(*E*)-(+)-Pityol elicited electrophysiological responses from the antennae of both sexes (0.1–0.2 mV, on average) (Fig. 3). The mean amplitude of depolarization was not significantly affected by sex ($F_{1,56} = 1.050$, $P = 0.310$) or dose ($F_{3,56} = 0.700$, $P = 0.476$), but the interaction between sex and dose was nearly significant ($F_{3,56} = 2.356$, $P = 0.083$). Therefore, we compared responses among all eight sex-dose combinations using Tukey's post-hoc test. From this analysis it was clear that male antennae responded most strongly to the lowest dose of (*E*)-(+)-pityol (1 ng), whereas female antennae responded most strongly to the highest dose (1000 ng) (Fig. 3).

Behavioral response

Male *P. pubescens* significantly preferred the olfactometer arm containing either racemic (*E*)-pityol or (*E*)-(+)-pityol at all three doses tested (Fig. 4A). The lowest doses (1 ng for the racemic material and 1–10 ng for the chiral material) proved to be the most attractive. However, females were attracted only to the lowest dose (1 ng) of racemic (*E*)-pityol and (*E*)-(+)-pityol (Fig. 4B). Moreover, they significantly avoided racemic (*E*)-pityol at doses of 10 and 100 ng.

Discussion

We isolated and identified (*E*)-pityol in volatiles of male and female *P. pubescens,* and demonstrated an electrophysiological response to (*E*)-(+)-pityol in the antennae of both sexes, as well as a dose-dependant behavioral response of both sexes to racemic (*E*)-pityol and (*E*)-(+)-pityol in olfactometer bioassays. These results suggest that (*E*)-(+)-pityol may be a key compound in the chemical ecology of *P. pubescens*. Prior to our study, (*E*)-(+)-pityol had been detected in several *Pityophthorus* species but always in one sex only, *e.g.*, males of *P. carmeli* and *P. pityographus* and females of *P. setosus* and *P. nitidulus*. In field tests, males of *P. setosus*, a monogamous species,

Fig. 2. Mass spectra of a standard sample of (*E*)-(+)-pityol (A), male *Pityophthorus pubescens* (B), and female *P. pubescens* (C). Note the presence of fragment ions of m/z 59, 85, 102, and 129 in all three spectra.

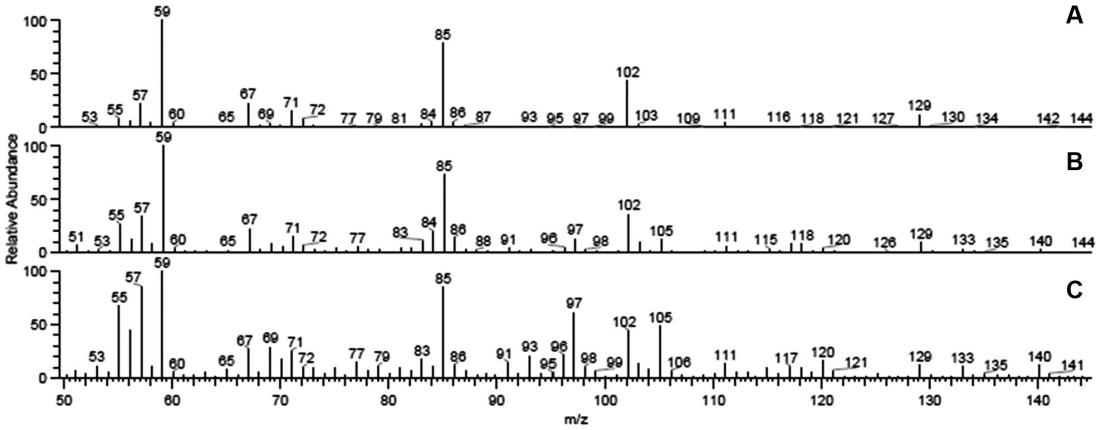
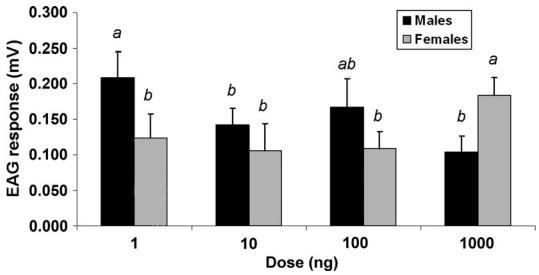


Fig. 3. Absolute EAG responses (mean ± SE) of *Pityophthorus pubescens* males (dark shading) and females (light shading) to serial dilutions containing 1, 10, 100, and 1000 ng of (*E*)-(+)-pityol. A different letter above the bar indicates a significant difference (two-way ANOVA followed by Tukey's multiple range test ($P \leq 0.05$), $n = 8$).

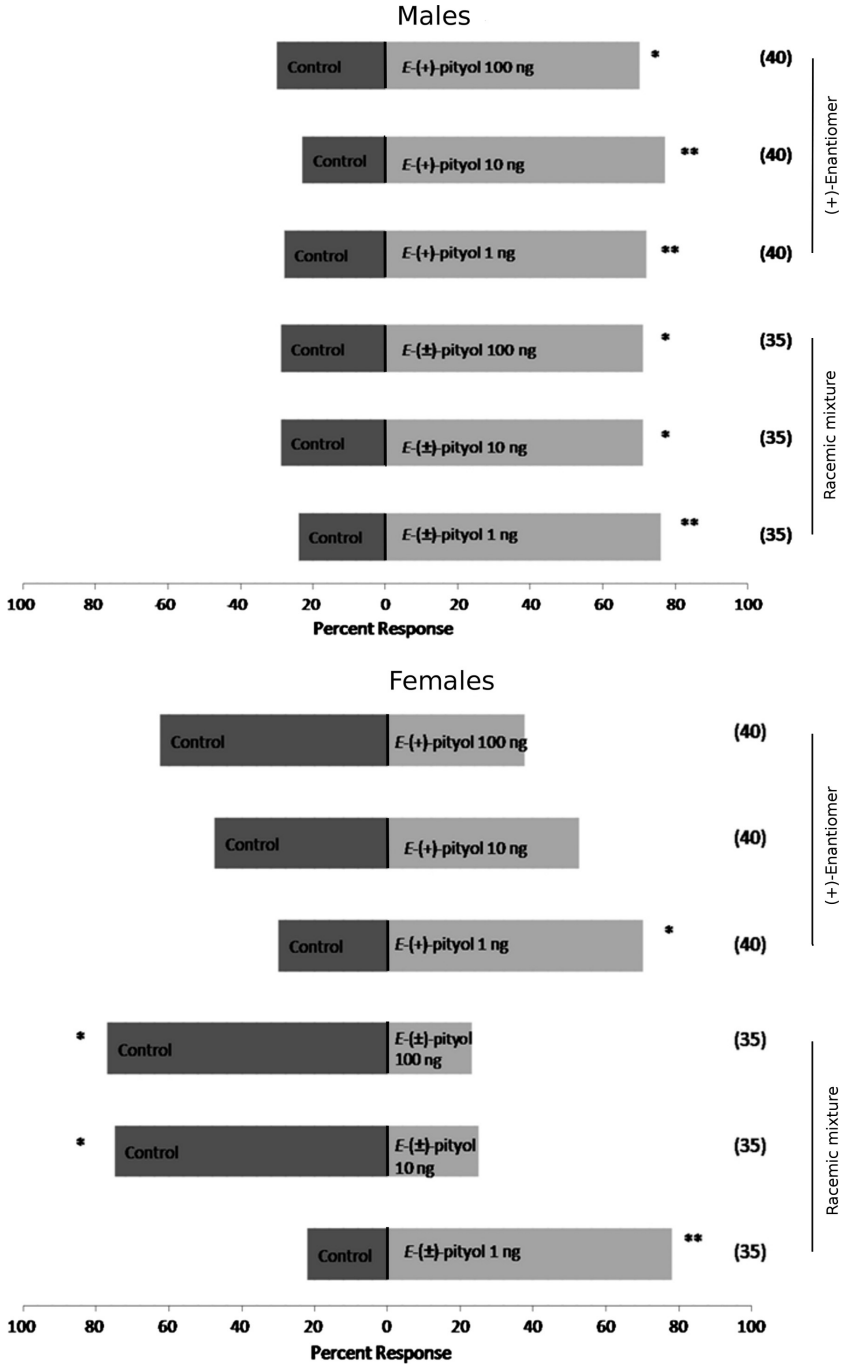


responded strongly to (*E*)-(+)-pityol alone (Dallara *et al*. 2000), whereas *P. pityographus*, a polygamous species, was attracted by the combination of grandisol and (*E*)-(+)-pityol (Francke *et al*. 1987). Another polygamous species, *P. carmeli*, was attracted only by the combination of (*E*)-(+)-pityol and (*E*)-(−)-conophthorin (Dallara *et al*. 2000). Similarly, in species of *Conophthorus* Hopkins (a genus considered to be phylogenetically closely related to *Pityophthorus*) (Cognato *et al*. 2005), (*E*)-(+)-pityol is known to be the major compound of its sex pheromone and has been found only in females (Birgersson *et al.* 1995; Pierce *et al.* 1995; Miller *et al.* 2000).

During dissection of naturally infested branches to collect beetles, we observed that all galleries contained a single mating pair of *P. pubescens*, and had a longitudinal pattern without a nuptial chamber, which is consistent with monogamy. However, it would be necessary to carry out a more extensive study of the gallery patterns from more naturally collected branches to allow us to draw accurate conclusions.

Although we found (*E*)-pityol in both sexes, the enantiomeric composition of the natural material has not been elucidated. Chirality plays an important role in determining pheromone specificity. In 60% of species and sex/aggregation systems studied to date, only a single enantiomer is bioactive, and its opposite enantiomer does not inhibit the response to the active stereoisomer in racemic blends (Mori 2007), but in some species the antipode can significantly reduce the attractive response to the active enantiomer (Birch *et al.* 1980; Leal 1996; Lacey *et al.* 2004). Owing to the lack of the (−)-enantiomer in our behavioral assays, its biological activity cannot be inferred. However, the racemic mixture was attractive, as was the pure (+)-enantiomer, to male *P. pubescens* when tested in the olfactometer, suggesting that (*E*)-(−)-pityol could be behaviorally inactive for males. This is consistent with the lack of response of other *Pityophthorus* species to (*E*)-(−)-pityol (Francke *et al.* 1987; Dallara *et al.* 2000;

Fig. 4. Responses of *Pityophthorus pubescens* males and females to different doses of (*E*)-(+)-pityol and racemic (*E*)-pityol in Y-tube olfactometer trials (*, $P < 0.05$; **, $P < 0.01$). Numbers in parentheses denote the number of beetles that responded.



W. Francke, personal communication). In contrast, females were only attracted to the lowest dose in both cases, showing a significant preference for the blank arm when the racemic mixture was tested (and it is evident that increasing the (*E*)-(+)-pityol dose leads

to an apparent and progressive decrease of the positive response, although this is not statistically significant). In light of our results we cannot assert that this behavior is caused by the presence of (*E*)-(−)-pityol; further studies would be needed to determine the biological influence of each enantiomer on *P. pubescens*.

We report the first electroantennographic assays performed on a species of *Pityophthorus* and show that the antennae of both sexes of *P. pubescens* respond to (*E*)-(+)-pityol. Interestingly, whereas female antennae showed the greatest response to the highest dose, the reverse was true for male antennae, *i.e.*, they showed the greatest response to the lowest dose.

In summary, for the first time we detected the presence of (*E*)-pityol as a possible aggregation pheromone in male and female volatiles of *P. pubescens*, and demonstrated the biological activity of the (+)-enantiomer and the racemate in electrophysiological and behavioral studies. Studies should be carried out to confirm the attraction of both sexes of *P. pubescens* in the field for these chemicals.

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