Journal of Chemical Ecology, Vol. 16, No. 3, 1990

ISOLATION OF PHEROMONE SYNERGISTS OF BARK BEETLE, *Pityogenes chalcographus*, FROM COMPLEX INSECT-PLANT ODORS BY FRACTIONATION AND SUBTRACTIVE-COMBINATION BIOASSAY

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(Received January 27, 1989; accepted May 8, 1989)

Abstract—Capillary gas chromatography with columns of different polarity and two-dimensional fractionation of effluents were used with novel subtractive-combination bioassays to rigorously isolate host- and insect-produced pheromone synergists of the bark beetle *Pityogenes chalcographus* (Coleoptera: Scolytidae). Methyl (*E,Z*)-2,4-decadienoate (*E,Z*-MD) and the previously identified chalcogran were found to be synergistically attractive to both sexes. *E,Z*-MD was produced sex-specifically in males, and only when they had fed on host-plant tissue. A Norway spruce monoterpene fraction (including α-pinene, β-pinene, and camphene) increased the attractive response to the pheromone components. Dose-response curves for *E,Z*-MD and chalcogran in the laboratory bioassay indicated the two components are highly synergistic. The isolation methods are important for further progress in identifying certain semiochemical synergists found in trace amounts in complex chemical mixtures, such as when insects must feed in host plants in order to produce pheromone.

Key Words—Pheromone, bark beetle, Coleoptera, Scolytidae, *Pityogenes chalcographus*, methyl (*E,Z*)-2,4-decadienoate, chalcogran, *Picea abies*, synergist, subtractive-combination bioassay, two-dimensional fractionation.

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INTRODUCTION

In Europe, *Pityogenes chalcographus* (Kupferstecher) is a serious pest of Norway spruce, *Picea abies* (L.) Karst., and is especially damaging to young trees. These tiny bark beetles (2 mm long) aggregate on certain host trees in response to a male-produced pheromone (Francke et al., 1977). Francke et al. (1977) isolated a unique spiroketal, chalcogran (2-ethyl-1,6-dioxaspiro[4.4]nonane), from *P. chalcographus*, which, when released in the forest at 15 mg/hr, attracted conspecifics. They used a differential diagnosis method (Vité and Renwick, 1970) whereby the gas chromatographic elution patterns and mass spectra of chemicals are compared between the sexes for unique differences. About 100,000 beetles of both sexes were treated with juvenile hormone analog (ethyl-3,7,11-trimethyl-2-dodecenoate), but only the male was induced to produce chalcogran. However, since the natural release rates of chalcogran were not known and combinatorial bioassays of fractions in order to detect synergists were not performed, Francke et al. (1977) were not certain whether "a second component may be necessary for maximum response." Chalcogran also was not tested alone, but only with spruce bark (which is unattractive alone), so it was not known whether host volatiles play a role in the orientation response. Thus, we wanted to determine whether host-plant and/or other pheromone components participate with chalcogran in eliciting the attraction response of the beetle.

The problem with isolation of semiochemicals is that solvent extracts of insects or their volatile effluents may contain hundreds of compounds along with the few bioactive components of interest (Millar et al., 1985; Burger et al., 1985; Jackson et al., 1984; Birgersson et al., 1984; Byers et al., 1985) (Figure 1 below). In addition, many insects release trace amounts of pheromones and other semiochemicals only when feeding on host plants, which makes these isolations more difficult because of the complexity of the odors. Thus, in many isolation studies workers have chromatographically fractionated extracts of complex chemical mixtures into successively smaller portions until an active fraction in bioassay contains just a few compounds (Doss and Shanks, 1984; Jackson et al., 1984; Silk et al., 1985; Heath et al., 1986; Burger et al., 1985; Chuman et al., 1985; B.H. Smith et al., 1985; Löfstedt and Van Der Pers, 1985; Teal et al., 1985). These chemicals are usually identified by spectrometric methods, and then the corresponding synthetic compounds are bioassayed to confirm their behavioral role. However, these methods are inadequate for isolating synergistic components, where several in different fractions may be required together for biological activity, the usual case for the multicomponent insect pheromones (Silverstein and Young, 1976; Silverstein 1981).

With this synergistic problem in mind, the additive-combination bioassay of fractions was developed in the 1960s for the isolation of pheromone syner-

gists. Unfortunately, relatively few studies have used this methodology (Silverstein et al., 1966, 1967, 1968; Pearce et al., 1975; Millar et al., 1985) because it is laborious to test all combinations of many fractions for any number of possible synergists. L.E. Browne, while at the University of California, Berkeley, proposed a subtractive method for isolation of synergists (personal communication c. 1976). We have developed this novel idea into a subtractive-combination bioassay of gas chromatographic (GC) fractions, which is as rigorous as the additive method but requires far fewer tests. Our objective was to use this methodology to isolate from a complex, but natural, mixture of plant and insect volatiles all the pheromone synergists that are attractive to the six-spined spruce bark beetle *Pityogenes chalcographus*. If one or more additional pheromone components were discovered, we wanted to elucidate their synergistic properties with chalcogran.

METHODS AND MATERIALS

P. chalcographus beetles were reared in a laboratory culture originating from Lardal, Norway, on freshly cut Norway spruce logs at 27°C. At emergence, adults were collected and maintained at 4°C for several days until used in experiments (Anderbrant et al., 1990).

To determine whether host and/or other pheromone components may participate with chalcogran in eliciting the attraction response, we collected odors from air passed over appropriate host logs or beetle-infested host logs inside glass containers (Byers et al., 1985). Odor collections from logs infested with 40 males were made on 11 occasions as well as twice from uninfested host logs (from December 1982 to May 1985) to obtain enough material for bioassays and identification. Volatiles were collected from headspace air by passage through Porapak Q traps (300 mg, 80–100 mesh) at rates of 100–250 ml/min for two to five days, beginning one day after releasing males onto the logs. The absorbent was subsequently extracted with 2 ml diethyl ether to remove the trapped volatiles.

Each extract from a collection period was concentrated by allowing the diethyl ether to evaporate at room temperature from a tapered glass vial (W. Francke, University of Hamburg, personal communication) before further analysis or bioassay. The attractiveness of extracts and of various ratios of pheromone components were determined in a laboratory bioassay that tested the proportion of walking females in an olfactometer for their upwind responses to odor sources (Byers et al., 1985; Lanne et al., 1987). Beetles that did not respond the first time were given a second chance to reach the source. The light intensity in the bioassay arena was 180 lumens/m^2.

The subtractive-combination method of bioassay compares activity of the

whole blend of recombined fractions to the activities of all such blends, each with a different fraction missing or subtracted. If synergists occur, then one or more blends with a fraction removed will be found to have significantly less bioassay activity than does the whole blend. Thus, each of the removed fractions from these less active blends must contain at least one synergist. Further fractionation and subtractive-combination bioassays are then performed only with these "active" fractions (the fractions that were removed causing the loss in activity).

The concentrated extracts were analyzed on a Hewlett-Packard gas chromatograph (GC) model 5830 using a polar column (No. 1, Table 1) in order to formulate a sequence plan for fractionation by preparative GC. Fractionation was performed on a Carlo Erba model 4160 GC outfitted with a revolving microfraction collector (Wassgren and Bergström, 1984). The sequence GC fractionation of the whole extract is shown in Figure 1. A fused silica capillary

TABLE 1. GAS CHROMATOGRAPHIC COLUMNS[a] USED IN ANALYTICAL AND PREPARATIVE GC AND IN COUPLED GAS CHROMATOGRAPHY–MASS SPECTROMETRY

Analytical GC: Instrument: Hewlett-Packard, model 5830.
Polar GC column 1: Fused silica; length = 46 m, ID = 0.35 mm; OV-351, df = 0.59 μm.
Temperature program: 50°C for 5 min, 5°C/min to 200°C, then isothermal for 20 min.
Mobile phase: N_2 at 20 cm/sec.

Preparative GC: Instrument: Carlo Erba, model 4160.
Polar GC column 2: Fused silica; length = 24 m, ID = 0.35 mm; OV-351, df = 0.5 μm.
Temperature program: 50°C for 2 min, 5°C/min to 220°C, then isothermal for 20 min.
Mobile phase: N_2 at 30 cm/sec.
Nonpolar GC column 3: Fused silica; length = 26 m, ID = 0.32 mm; SE-33 cross-linked, df = 1.0 μm.
Temperature program: 80°C for 2 min, 5°C/min to 220°C, then isothermal for 20 min.
Mobile phase: N_2 at 30 cm/sec.

Analytical GC-MS: Instrument: Finnigan, model 4021.
Polar GC column 4: Fused silica; length = 23 m, ID = 0.20 mm; OV-351, df = 0.5 μm.
Temperature program: 50°C for 4 min, 8°C/min to 200°C, then isothermal for 10 min.
Mobile phase: He at 20 cm/sec.
Nonpolar GC column 5: Fused silica; length = 12.5 m, ID = 0.20 mm; SE-54 cross-linked.
Temperature program: 80°C for 3 min, 8°C/min to 220°C, then isothermal for 10 min.
Mobile phase: He at 20 cm/sec.

Quantitative GC-MS: Instrument: Finnigan, model 4021.
Polar GC column 6: Fused silica; length = 25 m, ID = 0.15 mm; Superox FA, df = 0.3 μm.
Temperature program: 50°C for 4 min, 8°C/min to 200°C, then isothermal for 10 min.
Mobile phase: He at 20 cm/sec.

[a]GC column 5, Hewlett-Packard, other GC columns prepared at Department of Chemical Ecology, Göteborg University.

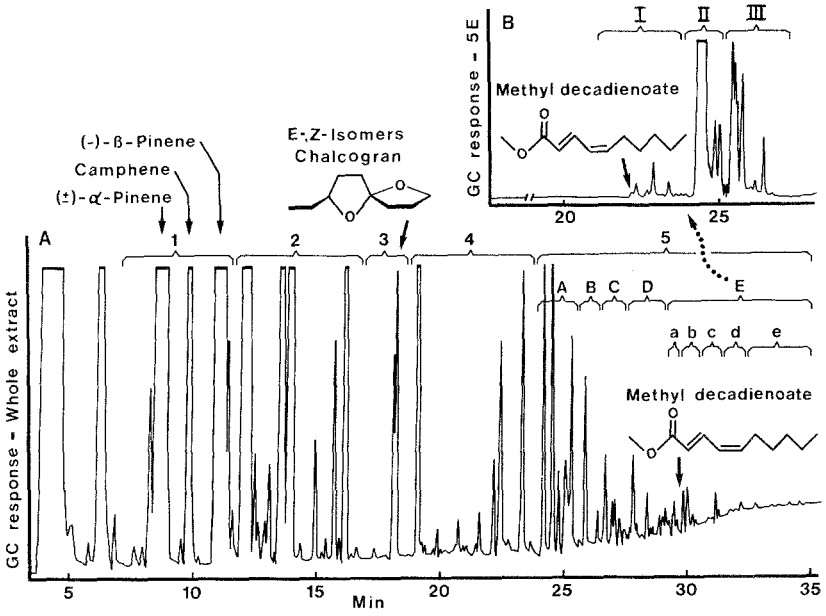

FIG. 1. Gas chromatograms (FID) of volatiles from a 40-male-infested Norway spruce log (25 × 8 cm diam.). Numbers and letters above peaks indicate the effluent collection periods for the respective fractions. Methyl (*E*,*Z*)-2,4-decadienoate cannot be discriminated visually in chromatogram A on a polar GC phase (column 1, Table 1) but was located by EICP-GC-MS and can be seen in chromatogram B as a small side peak (nonpolar GC phase, column 3).

column (No. 2, Table 1) coated with a polar stationary phase was used to obtain fractions 1–5, 5A–5E, and $5E_a$–$5E_e$ (Figure 1A). A second nonpolar GC column (No. 3, Table 1) was used to chromatograph fraction 5E, which contained the synergistic activity with chalcogran (Figure 1B). The identities of the indicated semiochemicals (Figure 1) were confirmed by matching their retention time and mass spectra with authentic standards using GC-mass spectrometry (GC-MS, columns 4–5, Table 1). Further details concerning the GC fractionation (Figure 1) and behavioral bioassays (Tables 2 and 3) used during the isolation of the synergists will be given with the results.

RESULTS AND DISCUSSION

The attractive activity of an extract of odor collection from a log infested with 40 males was compared to a similar extract of an uninfested log and to synthetic chalcogran in the bioassay. Volatiles from the infested and uninfested

logs were released in the olfactometer at the natural collection rates of 40 males/log/min and 1 log/min, respectively. The synthetic chalcogran (from W. Francke, University of Hamburg, West Germany), consisting of a racemic mixture of 46% (*E*)-(2*S*,5*R*- and 2*R*,5*S*-) and 54% (*Z*)-chalcogran (2*S*,5*S*- and 2*R*,5*R*-) stereoisomers, was released at 2 ng/min. The uninfested log extract or synthetic chalcogran alone were not appreciably attractive (both $< 10\%$ female response, $N = 30$–60 each) compared to the extract of volatiles from the male-infested log (73.3% response, $N = 30$, significantly different $P < 0.01$, χ^2). Natural chalcogran amounts were quantified in the extract and estimated to be released from the infested log in amounts up to 10 ng/min in the bioassay or by feeding males at up to 360 ng/beetle/day. A 0.1 rate of release (1 ng/min) from the infested logs of natural chalcogran (two stereoisomers, of which 0.46 ng was the active 2*S*,5*R*-isomer; Byers et al., 1989) still elicited a high female response (56.7%, $N = 30$) compared to the low response ($< 10\%$) to 2 ng synthetic chalcogran/min (four stereoisomers, of which 0.46 ng was the active 2*S*,5*R*-isomer. These results indicated that one or more synergists, in addition to chalcogran, elicit the attraction of the beetle. It was also possible that the 2*R*,5*S*- and 2*R*,5*R*-isomers of chalcogran were blocking response since the beetle does not produce these isomers (Schurig and Weber, 1984), and it has been shown that unnatural enantiomers can inhibit response in some species (Borden et al., 1976). However, in subsequent experiments (Byers et al., 1989), we have not found evidence of either synergistic or inhibitory interactions among the stereoisomers of chalcogran.

Using the subtractive-combination method of fractionation and bioassay (Figure 1A), we confirmed that natural chalcogran is an essential pheromone component of *P. chalcographus*. The combination of all fractions (1–5) released at 8 male/0.2 log/min attracted 65% ($N = 40$ females) but when fraction 3, containing chalcogran, was subtracted from the blend released at the same rate, the response dropped to 0% (Table 2). However, fraction 3 alone was not appreciably attractive (13.3%, $N = 30$). This is in agreement with the relatively low response to synthetic chalcogran reported above. Furthermore, the 65% response to the whole blend was reduced to 12.5% (Table 2) when fraction 5 was subtracted, indicating that this fraction contained one or more additional synergists. Only one other major fraction (No. 1) contributed to the attraction of the whole blend, as indicated by a slight decrease in response when subtracted (37.5% compared to 65% response, Table 2). This fraction contained the host-tree monoterpenes, α-pinene, camphene, and β-pinene (Figure 1A). These monoterpenes were shown later to play a role in host recognition by the beetle (Byers et al., 1988).

Thus, the subtractive bioassay required $1 + 5 = 6$ tests to isolate the synergistic activity in the major fractions (1–5). This is compared to the additive-combination method which would have required $1 + C_1^5 + C_2^5 + C_3^5 =$

TABLE 2. ATTRACTION OF FEMALE *Pityogenes chalcographus* IN THE LABORATORY BIOASSAY TO MIXTURES OF GAS CHROMATOGRAPHIC FRACTIONS (POLAR GC COLUMN 2, TABLE 1) OF AIRBORNE VOLATILE EXTRACTS FROM MALES BORING IN NORWAY SPRUCE LOGS

Stimulus[a]	Females (%) responding[b] (N = 30–40)	95% CI[c]
Fractions tested March 15, 1983 (cf. Figure 1A)		
1 + 2 + 3 + 4 + 5 = total blend	65.0a	49.5–77.9
Total blend − 1	37.5b	24.2–53.0
Total blend − 2	60.0a	44.6–73.7
Total blend − 3	0.0d	0.0–12.8
Total blend − 4	63.3a	45.5–78.1
Total blend − 5	12.5c	5.5–26.1
3 (natural chalcogran)	13.3c	5.3–29.7
Fractions tested April 23, 1983 (cf. Figure 1A)		
1 + CH[d] + 5A + 5B + 5C + 5D + 5E = blend		
Blend − 5A	63.3a	45.5–78.1
Blend − 5B	60.0a	42.3–75.4
Blend − 5C	50.0a	33.2–66.8
Blend − 5D	46.7a	30.2–63.9
Blend − 5E	10.0b	3.5–25.6
CH[d] + 5E	46.7a	30.2–63.9
Fractions tested May 10, 1983 (cf. Figure 1A)		
CH[d] + $5E_a$ + $5E_b$ + $5E_c$ + $5E_d$ + $5E_e$ = blend	50.0a	33.2–66.9
Blend − $5E_a$	50.0a	33.2–66.9
Blend − $5E_b$	46.7a	30.2–63.9
Blend − $5E_c$	46.7a	30.2–63.9
Blend − $5E_d$	50.0a	33.2–66.9
Blend − $5E_e$	40.0a	24.6–57.7
Fractions tested May 22, 1983 (cf. Figure 1A)		
CH[d] + $5E_a$	56.7a	39.2–72.6
CH[d] + $5E_b$	70.0a	52.1–83.3
CH[d] + $5E_c$	3.3b	0.6–16.7
CH[d] + $5E_d$	6.7b	1.8–21.3
CH[d] + $5E_e$	10.0b	3.5–25.6

[a] Release rate was 8 males/0.2 bolt/min.
[b] Values followed by the same letter within a test period were not significantly different ($\alpha = 0.05$, χ^2).
[c] Confidence interval for proportions.
[d] Synthetic chalcogran (46% E:54% Z) released at 2 ng/min.

26 tests to obtain the same information, assuming only three synergistically active fractions (1, 3, and 5 as shown here). However, if there had been 10 initial fractions and three synergists, then the subtractive method would have required 11 tests while the additive method would need 171 tests for the same level of isolation efficiency. However, even more tests than 171 would be needed if there were more synergists, in order to be assured of locating all the fractions with synergists.

In our initial fractionation, fraction 5 was clearly of importance in synergizing the activity of fraction 3 containing chalcogran. Thus, fraction 5 was subfractionated to further isolate the synergistic activity (when combined with chalcogran). As shown in Table 2, fraction 5 released at a rate equivalent to 8 male/0.2 log/min was little affected by the subtraction of fractions 5A–5D from the tested blends (47–63%), but the blend without 5E was of low attractiveness (10%). However, when fraction 5E was subfractionated, subtractive-combination bioassays could not determine which of the five fractions ($5E_a$–$5E_e$) contained essential components since all such blends with one of these fractions subtracted had a similar activity (40–50%, each $N = 30$). This implied that either there were at least two synergists (in addition to chalcogran) in different subfractions that were substitutive or, more likely, that one synergist had been split during fractionation between two consecutive fractions. The second hypothesis was supported when each of the five fractions ($5E_a$–$5E_e$) was tested alone with synthetic chalcogran. In these bioassays, both fractions $5E_a$, attracting 57% of the females, and $5E_b$, attracting 70% of the females, had significant activity compared to the other three fractions, $5E_c$–$5E_e$, each attracting $< 10\%$ of the females (Table 2). It was still possible that mutually substitutive synergists were present in these two fractions, although we know of no cases like this in species with three (or more?) synergistic pheromone components (Byers, 1989).

The GC elution of compounds of fractions $5E_a$ and $5E_b$ showed that several host sesquiterpenes, including γ-cadinene, δ-cadinene and α-curcumene, and myrtenol were present (GC-MS No. 6, Table 1). These compounds could have accounted for the loss of bioassay activity when the fractions were removed from the blend. Thus, volatiles were collected from uninfested host logs, and the extracts were fractionated, first on a polar column 2 to obtain 5E, and then on nonpolar column 3 (Table 1) to obtain two fractions. Each of these fractions were assayed at rates of 1.6 log/min with 4 ng/min of synthetic chalcogran. One host fraction, including δ-cadinene and myrtenol, elicited 13% response ($N = 60$), while the other fraction, with α-curcumene, elicited 6.7% response ($N = 60$), and the two fractions combined with chalcogran still only elicited 13.3% ($N = 30$) compared to a 50% response to the male infested log (fraction 5E with chalcogran) released at 8 male/0.2 log/min (significantly different, $P < 0.01$, χ^2). Since we could not discriminate differences in the GC elution

patterns of volatiles between fraction 5E from the host alone and fraction 5E from male-infested host logs, but the beetles could detect differences between responses to these fractions, we concluded that the beetle-produced synergist(s) needed to be further separated from the host constituents.

In order to separate the constituents of fraction 5E into a different GC elution pattern that might isolate the beetle-produced synergist(s), we utilized two-dimensional fractionation. We chromatographed fraction 5E (activity in $5E_{a+b}$) on nonpolar column 3 (Table 1, Figure 1B) and collected the effluent into three fractions (I–III). Our fractionation method is similar to the concept of two-dimensional GC described by Deans (1981), where the specific portion is reinitialized on the other column, i.e., all compounds begin at the same starting time. The synergistic activity of fraction 5E was found only in fraction 5E:I of three fractions (Figure 1B, Table 3). This fraction eluted prior to most of the sesquiterpene hydrocarbons. However, there were still several unidentified compounds in this fraction. By using a differential diagnosis technique, we compared every compound in fraction 5E:I of the nonpolar column to compounds eluting in the region between fractions $5E_a$ and $5E_b$ on the polar column using GC-MS extracted ion current profiles (EICP)(Chen, 1979; Garland and Powell, 1981). Only one compound (bp = 81; m/z = 111, 97% bp; M^+ = 182) was found both in the area of bioassay activity on the nonpolar (fraction 5E:I) and polar columns (split between fractions $5E_a$ and $5E_b$).

Once the active compound was located, more material was obtained from

TABLE 3. ATTRACTION OF FEMALE *Pityogenes chalcographus* IN LABORATORY BIOASSAY TO MIXTURES OF GAS CHROMATOGRAPHIC FRACTIONS (NONPOLAR GC COLUMN 3, TABLE 1) OF AIRBORNE VOLATILE EXTRACTS FROM MALES BORING IN NORWAY SPRUCE LOGS

Stimulus[a]	Females (%) responding[b] (N = 30)	95% CI[c]
Fractions tested April 3, 1984 (cf. Figure 1B)		
CH[d] + 5E:I + 5E:II + 5E:III = blend	60.0a	42.3–75.4
Blend − 5E:I	16.7b	0.7–33.6
Blend − 5E:II	53.3a	36.1–69.8
Blend − 5E:III	53.3a	36.1–69.8
CH[d] + 5E:I	40.0a	24.6–57.7
CH[d] + 5E:II	13.3b	0.5–29.7
CH[d] + 5E:III	13.3b	0.5–29.7

[a] Release rate was 8 males/0.2 bolt/min.
[b] Values followed by the same letter were not significantly different (α = 0.05, χ^2).
[c] Confidence interval for proportions.
[d] Synthetic chalcogran (46% *E*:54% *Z*) released at 2 ng/min.

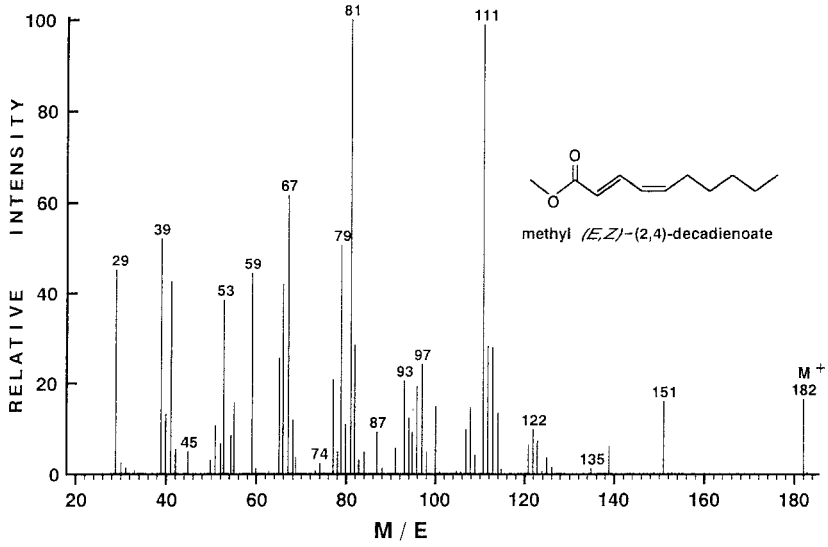

FIG. 2. Mass spectrum of methyl (*E,Z*)-2,4-decadienoate obtained from hindguts of 80 male *Pityogenes chalcographus* that had fed in a Norway spruce log for 42 hr (March 20, 1985). The molecular weight is indicated by M^+.

volatile collections and purified by preparative GC (columns 2 and 3, Table 1) to determine its mass spectra (Figure 2) and hydrogenation products. These spectra indicated the compound was methyl 2,4-decadienoate. Authentic standards of the four possible isomers [from reesterified ethyl (*E,Z*)-2,4-decadienoate, Oril produits chemiques, Neuilly-sur-Seine, France, or synthesized by R. Unelius, Department of Organic Chemistry, Royal University of Technology, Stockholm] were compared by GC-MS using retention times, coinjection, and MS data to prove that the isolated compound was methyl (*E,Z*)-2,4-decadienoate (*E,Z*-MD). *E,Z*-MD could not be found in extracts of host volatiles or in gut extracts of females feeding in the host for 48 hr. However, small amounts (about 10 ng/male) were located in feeding males but not in unfed males (Birgersson et al., 1990).

An unusual aspect of the two pheromone components of *P. chalcographus* is that they are acetogenic and not terpenic, as are most other bark beetle pheromones (Vanderwel and Oehlschlager, 1987; Byers, 1989). Esters such as *E,Z*-MD have, up to now, not been discovered in Scolytidae. *E,Z*-MD has not been identified before in insects and is a representative of a new class of semiochemical esters.

The synergistic properties of *E,Z*-MD and chalcogran mixtures were evaluated in the laboratory bioassay by varying each component over five orders of

magnitude in concentration while keeping the other component constant (Figure 3). *P. chalcographus* is exceptionally sensitive to both components, which is in agreement with the relatively low nanogram amounts released by males feeding in host logs. Recently we tested synthetic chalcogran and *E,Z*-MD in the forest (Byers et al., 1988), both together and alone, as well as together with the major host monoterpenes that were indicated to have activity in our initial fractionation (Figure 1A, fraction 1). We found that when chalcogran was released at 1 mg/day, it attracted few beetles (total of 39); when chalcogran was released with 18 μg *E,Z*-MD/day the catch increased by about 35-fold. The effect of the host monoterpenes, ($\pm$)-α-pinene, camphene, and ($-$)-β-pinene, when released in conjunction with the two pheromone components, was to increase the landing rate as well as induce both sexes to enter 2-mm-diameter holes in the artificial host (Byers et al., 1988).

Schurig and Weber (1984) found that two species of *Pityogenes* (*chalcographus* and *quadridens*) both contain the same stereoisomers of chalcogran. Apparently the beetles produce either (2*S*,5*R*)- or (2*S*,5*S*)-chalcogran or both

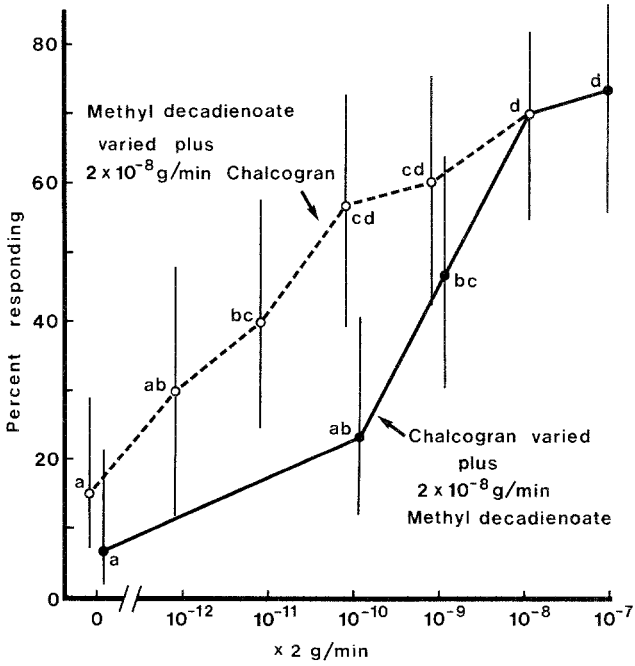

FIG. 3. Response of *Pityogenes chalcographus* to varying concentrations and ratios of its pheromone components, methyl (*E,Z*)-2,4-decadienoate and (46% *E* + 54% *Z*) chalcogran in the laboratory bioassay. The bars represent 95% confidence limits for proportions. Points with the same letter were not significantly different ($\alpha = 0.05$, χ^2).

but, in the acidic gut, they epimerize to the 46:54 ratio of 2*S*,5*R*:2*S*,5*S* isomers. *P. chalcographus* only contains these isomers of which the 2*S*,5*R*-isomer is the most active synergist with *E*,*Z*-MD, while 2*S*,5*S* is the least active (Byers et al., 1989). The other two possible isomers, 2*R*,5*S* and 2*R*,5*R* are not present in the beetle and are of intermediate activity. Pheromone discrimination between the *Pityogenes* species may reside in the *E*,*Z*-MD component or its isomers (*E*,*E*-, *Z*,*E*-, and *Z*,*Z*-). Recently, Baader (1989) investigated six species of *Pityogenes* (*chalcographus*, *quadridens*, *bidentatus*, *conjunctus*, *calcaratus*, and *carinulatus*) for volatiles released from attacked host material. Only the first two species produced chalcogran while only *P. chalcographus* released *E*,*Z*-MD.

Francke et al. (1977), in addition to (*E*)- and (*Z*)-chalcogran, found another male-specific compound, 1-hexanol, which had no apparent synergistic effect with chalcogran, although they stated that "it is also possible that the enantiomeric composition of the isomers (of chalcogran) is critical for maximum beetle response." In our samples, 1-hexanol was found in fraction 2 (Figure 1A), and this fraction did not exhibit synergistic activity since its subtraction from the whole extract did not significantly affect the attraction response.

Isolations of trace (nanogram) levels of synergistic pheromones have been accomplished in several species of moth (Roelofs and Cardé, 1977; Löfstedt et al., 1982, 1985), but these studies have often investigated relatively less complex odors from calling females alone. The number of compounds is often less than 20, but still there are numerous cases where additional pheromone components are discovered in well-researched species (Löfstedt and Van Der Pers, 1985; Teal et al., 1985; Silverstein et al., 1966, 1968; Roelofs and Cardé, 1977; Löfstedt et al., 1982, 1985; Booij and Voerman, 1985). More widespread adoption of our subtractive-combination method would facilitate the outcome of unequivocal studies with a minimum of work.

While multicomponent insect pheromones have become the paradigm (Silverstein and Young, 1976; Silverstein 1981), it seems that one-compound ideas are still ingrained in our "test each fraction" thinking about isolation of insect host stimulants (Kirk, 1985; Dicke et al., 1985; Stubbs et al., 1985; McKibben et al., 1985) and ovipositional stimulants (Maeshima et al., 1985; Kim et al., 1985; Hanula et al., 1985). In some of these cases there may be synergistic components, and without use of the subtractive-combination method there is the possibility of overlooking important components. Synergism between plant toxicants is known in plant–insect interactions (Berenbaum and Neal, 1985). Thus, the subtractive-combination method would also be a more efficient and systematic way of elucidating toxicant synergists. This also applies to isolating synergistic chemicals found in any biological or pharmacological system (cf. Norman, 1985).

A.B. Smith et al. (1985) have recently described a new approach for analysis of chemical signals using statistical methods of pattern recognition to detect differences between chromatograms. This is a variation of the differential diagnosis method (Vité and Renwick, 1970). However, these approaches presume that trace components can be discriminated by the particular chromatographic column. Also, these methods do not employ behavioral assays to determine which components are active either alone or, more importantly, in synergistic combinations. Thus, the subtractive-combination bioassay of chromatographic fractions is a more rigorous method that can be used to isolate all synergists, no matter when they elute and even if they are not visible with a particular chromatographic column used initially.

Acknowledgments—We thank E. Wijk for laboratory assistance, E. Jirle for rearing and sex determination of beetles, and J. Jönsson of construction for the exhaust system of the olfactometer. A.-B. Wassgren prepared the GC columns and assisted in the laboratory. We thank Gerda Lange, Department of Chemistry, University of Würzburg, Würzburg, West Germany, for helpful mass spectral assistance. The study was supported primarily by the research project "Odour Signals for Control of Pest Insects," funded by the Swedish Research Councils (NFR, FRN, SJFR, STU).

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