

STRUCTURE-ACTIVITY STUDIES ON AGGREGATION
PHEROMONE COMPONENTS OF *Pityogenes*
chalcographus (COLEOPTERA: SCOLYTIDAE)
All Stereoisomers of Chalcogran and Methyl
2,4-Decadienoate

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Abstract—Syntheses of all four stereoisomers (2*S*,5*S*; 2*S*,5*R*; 2*R*,5*R*; and 2*R*,5*S*) of chalcogran, a major component of the aggregation pheromone of *Pityogenes chalcographus*, and of all four isomers (2*Z*,4*Z*; 2*Z*,4*E*; 2*E*,4*E*; and 2*E*,4*Z*) of methyl 2,4-decadienoate (MD), the second major pheromone component, are briefly described. Attraction responses of walking beetles of both sexes were tested to mixtures of the synergistic pheromone components or analogs. These bioassays showed that the *E,Z* isomer of MD is the most active when tested with chalcogran. When tested with (*E,Z*)-MD, (2*S*,5*R*)-chalcogran was the most active stereoisomer, while 2*R*,5*R* and 2*R*,5*S* isomers had intermediate activities, and the 2*S*,5*S* isomer was inactive. There was no evidence that the relatively less active stereoisomers of chalcogran inhibited or promoted attraction to (2*S*,5*R*)-chalcogran with (*E,Z*)-MD. Male beetles only produce the active *E,Z* isomer of MD (inactive alone) and their hindguts contain the most active (2*S*,5*R*)- and least active (2*S*,5*S*)-chalcogran. A mixture of all MD isomers with racemic chalcogran was not significantly different in attractivity compared to (*E,Z*)-MD with racemic chalcogran, indicating no synergistic or inhibitory effects of the inactive isomers of MD.

Key Words—Synergism, aggregation pheromone, *Pityogenes chalcographus*, Coleoptera, Scolytidae, chalcogran, methyl (2*E*,4*Z*)-2,4-decadienoate, enantiomers, isomers, stereoisomers, synthesis, bioassay, structure-activity.

INTRODUCTION

The six-spined spruce bark beetle, *Pityogenes chalcographus* (in Germany called *Kupferstecher*), is a serious pest in Europe of Norway spruce [*Picea abies* (L.) Karst.], especially the younger trees. Both sexes of these tiny beetles (2 mm long) aggregate on host trees in response to a pheromone (Vité, 1965). Francke et al. (1977) isolated from males a unique spiroketal, chalcogran, which when released in the forest at 15 mg/hr attracted conspecifics. They isolated chalcogran by treating 100,000 beetles of both sexes with a juvenile hormone analog to induce synthesis of chalcogran in males. They then used a differential diagnosis method (Vité and Renwick 1970) to compare the sexes for volatile chemical differences, which revealed *E* and *Z* isomers of chalcogran and 1-hexanol as being unique to the male. However, when 1-hexanol was added to synthetic racemic chalcogran (46% *E*:54% *Z*, Koppenhoefer et al., 1980), there was no apparent increase in attraction (Francke et al., 1977). Furthermore, due to the rather large amounts of chalcogran required to elicit attraction in the field (15 mg/hr), Francke et al. (1977) suggested that (1) "a second component may be necessary for maximum response" or (2) "it is also possible that the enantiomeric composition of the isomers is critical for maximum beetle response." Using gas chromatography (GC) on a chiral liquid phase, Koppenhoefer et al. (1980) were able to separate synthetic chalcogran containing all four stereoisomers into the individual components (two diastomeric pairs of enantiomers). Using the same technique, Schurig and Weber (1984) found that an extract of male beetles consists of a mixture of only two of the stereoisomers, namely the diastereomers 2*S*,5*R* 1 and 2*S*,5*S* 1 (Figure 1). However, the attraction activities of these, and the "unnatural" stereoisomers, have not been determined for male and female beetles.

Byers et al. (1988, 1989) further investigated the attractive pheromone system of *P. chalcographus* by undertaking an isolation of semiochemicals using chemical fractionation and behavioral bioassays. However, they used a new procedure, "subtractive combination" of gas chromatographic (GC) fractions and bioassay, which usually requires far fewer behavioral tests than the previously used and similar "additive combination" method (Silverstein et al., 1966, 1967, 1968; Pearce et al., 1975). Chalcogran and a "second" pheromone component were revealed by the new methods and found to be produced and released only when males feed on host-tree tissue. However, it was necessary to further isolate the second pheromone component from a complex mixture of insect and host volatiles by using two-dimensional GC (Deans, 1981) and bioassay (Byers et al., 1989). Methyl (2*E*,4*Z*)-2,4-decadienoate (*E,Z*-MD) was then isolated

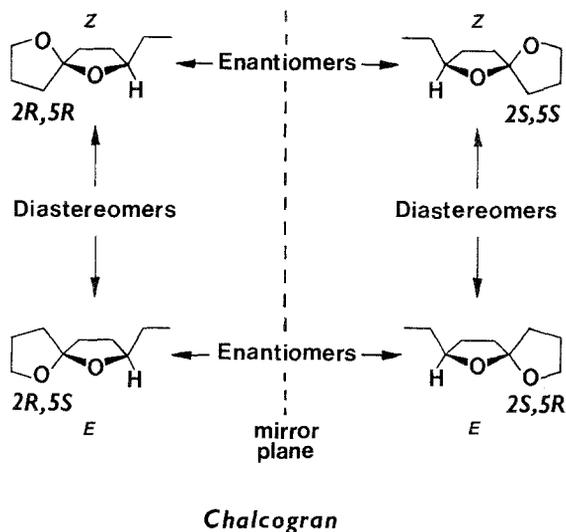


FIG. 1. Diastereomers and enantiomers of chalcogran. The two natural enantiomers found in male *Pityogenes chalcographus* are to the right of the mirror plane, with the behaviorally active one in the lower right.

and identified by GC-MS and found to be strongly synergistic with chalcogran (Byers et al., 1988, 1989). The males produce only the (*E,Z*)-MD isomer, but there are three other possible isomers that might have weaker or stronger synergistic effects on the attraction response when tested with chalcogran.

Here we briefly describe the syntheses of all four stereoisomers of chalcogran as well as the four isomers of methyl 2,4-decadienoate (MD) and their use in bioassay. The objectives were to determine which of the isomers/enantiomers of the pheromone components are bioactive by comparing (1) the attraction activities of each of the chalcogran stereoisomers when combined with the beetle-produced (*E,Z*)-MD and (2) the attraction activities of each of the MD isomers when combined with racemic chalcogran. We also wanted to determine if the mixture of chalcogran plus the four MD isomers has inhibitory or synergistic effects on attraction when compared to chalcogran plus the (*E,Z*)-MD isomer.

METHODS AND MATERIALS

Synthesis of Chalcogran Stereoisomers. Mixtures of the *E* and *Z* diastereomers of chalcogran were obtained enantiomerically pure using the synthetic pathway shown in Figure 2 (Högberg et al., 1987). The required γ -caprolactone enantiomers **2** (Figure 2) were conveniently obtained via resolution by chiral-

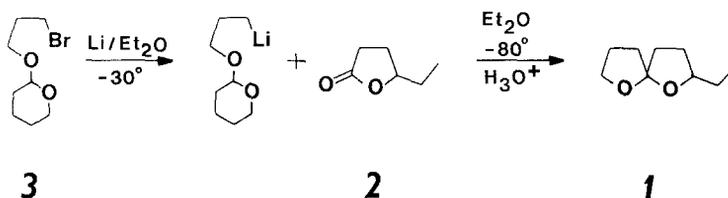


FIG. 2. Schematic pathway for synthesis of the chalcogran stereoisomers.

phase chromatography on microcrystalline triacetylated cellulose using the chromatographic system described by Isaksson and Roschester (1985). This gave (*R*)-(+)- and (*S*)-(–)- γ -caprolactone in 90% and 97% enantiomeric excess, respectively. These were subsequently reacted separately at -80°C with the alkyllithium obtained from the bromide **3** (Figure 2), each yielding a mixture of two diastereomers (*2R,5R/S*)-**1** and (*2S,5S/R*)-**1**, respectively. The diastereomers were separated by repeated silica gel chromatography using a gradient elution technique. All four isomers were obtained (Figure 1) in relatively high purity (Table 1) as shown by capillary GC on a chiral stationary phase (Schurig and Weber, 1984; Koppenhoefer et al., 1980).

Synthesis of Geometric Isomers of Methyl 2,4-Decadienoate. For details

TABLE 1. COMPOSITION OF CHALCOGRAN AND METHYL DECADIENOATE (MD) PHEROMONE COMPONENTS OR ANALOGS TESTED IN BIOASSAY

	Percent composition			
	<i>2S,5R</i>	<i>2R,5S</i>	<i>2S,5S</i>	<i>2R,5R</i>
(<i>E</i>)-Chalcogran				
<i>2S,5R</i>	96.2	1.7	2.1	<0.1
<i>2R,5S</i>	5.2	91.3	0.3	3.2
(<i>Z</i>)-Chalcogran				
<i>2S,5S</i>	1.0	<0.1	97.0	2.0
<i>2R,5R</i>	<0.1	0.9	3.8	95.3
Racemic chalcogran	23.0	23.0	27.0	27.0
	Percent composition			
MD	(<i>Z,Z</i>)	(<i>Z,E</i>)	(<i>E,E</i>)	(<i>E,Z</i>)
(<i>Z,Z</i>)	>99.9	<0.1	<0.1	<0.1
(<i>Z,E</i>)	<0.1	>99.9	<0.1	<0.1
(<i>E,E</i>)	<0.1	<0.1	99.0	<0.1
(<i>E,Z</i>)	<0.1	<0.1	<0.1	99.2

regarding the syntheses, see Baeckström et al. (1988). The *E,E* isomer was prepared (Figure 3) starting from methyl crotonate in a way similar to previously described procedures (Barneji and Pal, 1983; Crombie and Denman, 1984; Roush, 1980; Garigipati and Weinreb, 1983). NBS-bromination gave the product **4**, and an Arbuzov reaction with triethylphosphite yielded the phosphonate **5**. An Emmons-Horner-Wadsworth reaction with LDA and hexanal then gave (*E,E*)-MD. The isomeric purity was 93% and was improved to >99% using urea inclusion complexes (Leadbetter and Plimmer, 1979; Fieser, 1964). Since ethyl (*2E,4Z*)-2,4-decadienoate (**6**) is commercially available (Oril products Chimiques, Neuilly, France), the *E,Z* isomer was prepared simply by reesterification with sodium methoxide in methanol (Figure 3). The isomeric purity was increased from 85% to >99% with the urea inclusion procedure above. (*Z,E*)-MD was prepared from propiolic acid (Figure 3). HBr addition to the triple bond in the presence of cuprous bromide and subsequent esterification of the acid with sulfuric acid in methanol gave methyl (*Z*)-3-bromopropenoate (**7**) (Weir et al., 1980). This compound was coupled with (*E*)-1-heptenyl-1,3,2-benzodioxaborole (**8**) in the presence of Pd(PPh₃)₄ and sodium methoxide (Björkling et al., 1987). The cross-coupling reaction gave the *Z,E* isomer with 92% purity. Contaminating (*E,E*)-MD was removed by the use of urea inclusion

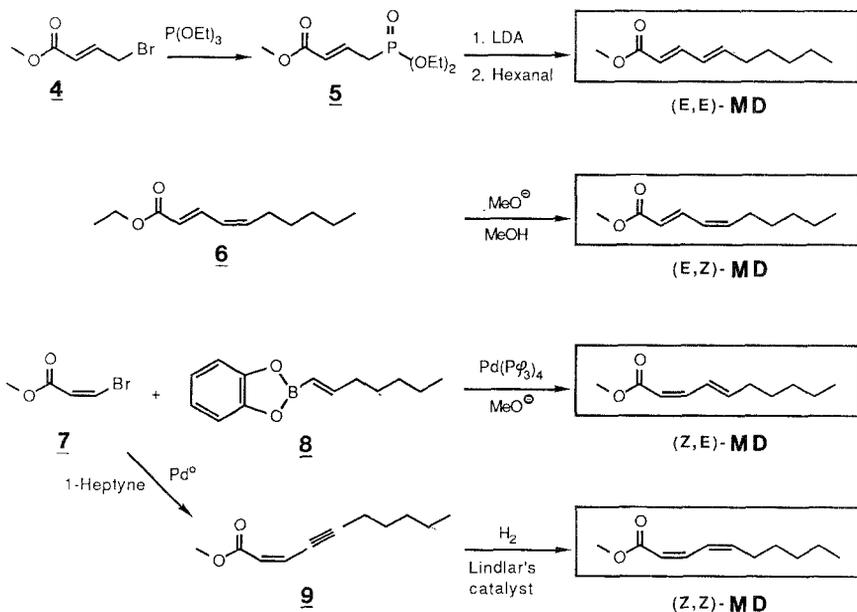


FIG. 3. Schematic pathway for synthesis of the geometrical isomers of methyl 2,4-decadienoate.

complexes. (*Z,Z*)-MD was prepared from **7** in two steps (Figure 3). A palladium-catalyzed cross-coupling with 1-heptyne according to Weir et al. (1980) yielded the enyne ester **9**. Reduction with hydrogen and Lindlar's catalyst (Rickards and Weiler, 1978) provided (*Z,Z*)-MD in 99% purity after preparative GC. The four MD isomers were analyzed and identified with GC-MS and [¹H]- and [¹³C]NMR, and the spectroscopic data were in agreement with those earlier reported (Stille and Groh, 1987).

Bioassay of Attraction Response of Pityogenes chalcographus to Chalcogran Stereoisomers and Methyl 2,4-Decadienoate Isomers. The open arena olfactometer for walking beetles was used for all bioassays (Browne et al, 1974; Byers and Wood, 1981; Byers et al., 1985). *P. chalcographus* (originally from Lardal, Norway) were obtained from a laboratory culture maintained on their natural host logs (28 × 10 cm diam.), Norway spruce, from Lund, Sweden. Beetles were separated according to sex and tested in groups of 10 in the olfactometer. They were released about 20 cm "downwind" from the odor source. Charcoal-filtered air was passed through a manifold to produce a laminar air flow across the arena of about 0.6 m/sec. Semiochemical mixtures were released in diethyl-ether solvent from a 5- μ l capillary (Drummond). Beetles that approached the odor source closer than 1 cm within the time of semiochemical release (about 2 min) were recorded as responding. Unresponsive beetles were tested a second time after refilling the capillary.

Purities of the chalcogran stereoisomers and MD isomers are shown in Table 1. Mixtures of each of the four chalcogran stereoisomers with the beetle-produced isomer of MD (Byers et al., 1988, 1989) were tested (Table 2) to see which stereoisomer was most active. Mixtures of racemic chalcogran (92.5% Shell Agrar, Table 1) with each of the four possible MD-isomers (Table 2) were also tested to determine their individual synergistic activities. Finally, tests were done to determine if the combination of all four MD-isomers were more active (synergism) or less active (inhibition) than the *E,Z* isomer when assayed with racemic chalcogran (Table 3).

RESULTS

The purities of the stereoisomers of chalcogran (Table 1) were sufficiently high to show differences in attraction activity (Table 2). In conjunction with (*E,Z*)-MD, (*2S,5S*)-chalcogran was the least active while the (*2S,5R*)-chalcogran was the most active for both sexes (Table 2). Again, in conjunction with (*E,Z*)-MD, racemic chalcogran [containing about 23% (*2S,5R*)-] was not significantly different from (*2S,5R*)-chalcogran in attraction activity, which indicates that the other stereoisomers of chalcogran are neither inhibitory nor synergistic (Table 2). The MD isomers were also of sufficiently high purity

TABLE 2. RESPONSES OF MALE AND FEMALE *Pityogenes chalcographus* IN LABORATORY BIOASSAY TO STEREOISOMERS OF CHALCOGRAN PRESENTED WITH (*E,Z*)-MD OR TO ISOMERS OF MD PRESENTED WITH RACEMIC CHALCOGRAN (JULY 27-28, 1986)^a

	Percent responding (95% BCL)	
	Females	Males
Chalcogran + (<i>E,Z</i>)-MD		
(<i>2S,5R</i>) + (<i>E,Z</i>)-MD	55.0 (42.5-66.9)a	55.0 (42.5-66.9)a
(<i>2R,5S</i>) + (<i>E,Z</i>)-MD	30.0 (19.9-42.5)b	23.3 (14.4-35.4)b
(<i>2S,5S</i>) + (<i>E,Z</i>)-MD	5.0 (1.7-13.7)c	1.7 (0.3-8.9)c
(<i>2R,5R</i>) + (<i>E,Z</i>)-MD	25.0 (15.8-37.2)b	23.3 (14.4-35.4)b
MD-isomers + chalcogran (<i>R</i>)		
(<i>Z,Z</i>)-MD + chalcogran (<i>R</i>)	25.0 (15.8-37.2)b	23.3 (14.4-35.4)b
(<i>Z,E</i>)-MD + chalcogran (<i>R</i>)	25.0 (15.8-37.2)b	25.0 (15.8-37.2)b
(<i>E,E</i>)-MD + chalcogran (<i>R</i>)	28.3 (18.5-40.8)b	25.0 (15.8-37.2)b
(<i>E,Z</i>)-MD + chalcogran (<i>R</i>)	60.0 (47.4-71.4)a	50.0 (37.7-62.3)a
Racemic chalcogran (<i>R</i>)	15.0 (8.1-26.1)b	11.7 (5.8-22.2)b
(<i>E,Z</i>)-MD	5.0 (1.7-13.7)c	5.0 (1.7-13.7)c

^aRacemic chalcogran (*R*) and each stereoisomer was released at 2.2×10^{-9} g/min and the MD isomers at 2.2×10^{-10} g/min. Parentheses enclose 95% binomial confidence limits for proportions (95% BCL) and 60 beetles of each sex were tested for each chemical blend. Percent responding followed by different letters were significantly different at $P < 0.01$, using a chi-square test.

TABLE 3. BIOASSAY FOR INHIBITORY OR SYNERGISTIC EFFECTS OF *E,E*, *Z,Z*, and *Z,E* ISOMERS OF METHYL DECADIENOATE (MD) ON RESPONSES OF MALE AND FEMALE *Pityogenes chalcographus* TO RACEMIC CHALCOGRAN PLUS (*E,Z*)-MD (OCTOBER 8, 1986)^a

	Percent responding (95% BCL)	
	Females	Males
Racemic chalcogran + (<i>E,Z</i>)-, (<i>E,E</i>)-, (<i>Z,E</i>)-, and (<i>Z,Z</i>)-MD	41.7 (30.1-54.3)	41.7 (30.1-54.3)
Racemic chalcogran + (<i>E,Z</i>)-MD	55.0 (42.5-66.9)	41.7 (30.1-54.3)

^aRacemic chalcogran was released at 2.2×10^{-9} g/min and each MD isomer at 2.2×10^{-10} g/min. Brackets enclose 95% binomial confidence limits for proportions (95% BCL) and 60 beetles of each sex were tested for each chemical blend. No significant differences were observed with a chi-square test, $P > 0.05$.

(>99%, Table 1) to yield differences in attraction activity (tested in combination with racemic chalcogran), with (*E,Z*)-MD being significantly more active than any other isomer or racemic chalcogran alone (Table 2). While (*Z,Z*)-, (*Z,E*)-, and (*E,E*)-MD tested with chalcogran were each somewhat more attractive than chalcogran alone, none of these combinations were statistically significantly different from chalcogran alone ($P > 0.05$, Table 2). The synergistically active (*E,Z*)-MD is not attractive when released alone (Table 2). When the mixture of all four isomers of MD plus chalcogran were compared to (*E,Z*)-MD plus chalcogran, there was no significant difference in attraction of either sex (Table 3), which indicates that the "inactive" MD isomers are neither synergistic nor inhibitory when combined with (*E,Z*)-MD.

DISCUSSION

Our bioassay tests revealed that the beetle-produced (*E,Z*)-MD was the only isomer of MD with significant synergistic activity with chalcogran. Apparently the configuration of both double bonds is important for the activity of the compound. The small activities of each of the other three MD isomers could be due to real effects or to the minute impurities of (*E,Z*)-MD. The expected release rates of any (*E,Z*)-MD impurity would have been on the order of 10^{-12} g/min or less, but this rate has produced a similar increase in response which is nearly or is statistically significant compared to response to chalcogran alone (Byers et al., 1989).

P. chalcographus males produce two of four possible stereoisomers of chalcogran (Shurig and Weber, 1984): (*2S,5R*)-1 and (*2S,5S*)-1, which we found were the most active and inactive, respectively, in our bioassay tests. There is some question as to the biosynthetic production of chalcogran because of rapid epimerization at carbon 5 to give a mixture of approximately 46% (*2S,5R*)-1 and 54% (*2S,5S*)-1 in solvents like benzene, ether, and chloroform (Smith et al., 1978; Francke et al., 1980). However, we found that our purified samples of chalcogran enantiomers were stable in hexane or pentane for over one year at -20°C by storage in alkaline-washed bottles. Thus, it is possible that the male beetle produces only one enantiomer that epimerizes in some part of the acidic gut to produce the 46:54 ratio as observed earlier (Francke et al., 1977; Shurig and Weber, 1984; Byers et al., 1989). Byers et al. (1989) found chalcogran predominantly in the abdomen (and more precisely in the hindgut; Birgersson and Byers unpublished) at 10.4 ± 2.4 ng (\pm SD) compared to only 2.6 ± 1.5 ng in the head/thorax. In contrast, (*E,Z*)-MD was found predominantly in the head/thorax of the male (11.2 ± 4.6 ng) compared to 2.3 ± 0.9 ng in the abdomen. This indicates different biosynthetic sites for the two pheromone components.

The structure-activities of the four chalcogran stereoisomers indicate that the configurations of all three major structural parts are important. Exchanging the ethyl and hydrogen groups at carbon 2 on the active (2*S*,5*R*)-chalcogran decreases response to an intermediate level for the (2*R*,5*R*)-**1** (Table 2). A 180° rotation of the center tetrahydrofuran ring on the active (2*S*,5*R*)-chalcogran also decreases response to an intermediate level for the (2*R*,5*S*)-**1**. Finally, 180° rotation of the end tetrahydrofuran ring at carbon 5 has the most severe effect, abolishing activity by forming (2*S*,5*S*)-chalcogran (Table 2).

Double-bond isomers of fatty acids of 12-, 14-, and 16-carbon chains are well known to have different behavioral effects or activities (Cardé and Baker, 1984). However, our report is the first case where such positional effects on long acetogenic carbon chains are important to bark beetles. It is well known that bark beetles use specific enantiomeric pheromone components (Wood et al., 1976; Borden et al., 1976; Wood, 1982), and *P. chalcographus* is yet another example. The *E* (2*S*,5*R*) and *Z* (2*S*,5*S*) chalcogran of *P. chalcographus* is analogous to *cis*- and *trans*-verbenol biosynthesis from α -pinene in *I. paracconfusus*, in which only one enantiomer of one compound is active, (–)-(*S*)-*cis*-verbenol (Silverstein et al., 1967; Renwick et al., 1976).

Our characterization of the synergistically bioactive stereoisomers of chalcogran (2*S*,5*R*)-**1**, and methyl 2,4-decadienoate, (*E*,*Z*)-MD, may be useful in control programs that use grids of pheromone-baited traps to catch the most vigorous (flying and responding) adults. This could reduce population levels below the tree-killing threshold so that tree resistance mechanisms can kill or repel the remaining potential colonizers. Pheromone trap-out may be a significant type of mortality which does not compete directly with other mortality factors such as adverse weather or desiccation/exhaustion of adults during the dispersal and host-seeking flight. The (*E*,*Z*)-MD and chalcogran components have recently been marketed under the name Chalcoprax, as a trap lure for *P. chalcographus* in Europe and northern Asia (Shell Agrar GmbH & Co. KG, D-6507 Ingelheim am Rhein).

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