Reduction of *Carpophilus freemani* Dobson (Coleoptera: Nitidulidae) Aggregation Pheromone Response by Synthetic Analogues

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Analogues of (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-nonatriene, the major component of the aggregation pheromone of *Carpophilus freemani* Dobson (Coleoptera: Nitidulidae), were synthesized and the potency of these compounds in suppressing the response of *C. freemani* to its pheromone in a wind tunnel bioassay was determined. The most potent compounds reduced behavioral response to pheromone 83−96% when the inhibitors were present in 10-fold excess. These compounds are (1*Z*,3*E*,5*E*)-1-methoxy-3-ethyl-5-methyl-1,3,5-heptatriene, (1*E*,3*E*,5*E*)-1-cyclopropyl-3-ethyl-5-methyl-1,3,5-heptatriene, and (1*Z*,3*E*,5*E*)-1-cyclopropyl-3-ethyl-5-methyl-1,3,5-heptatriene. In the presence of fermenting bread dough (a pheromone synergist), the most potent inhibitory compound, (1*Z*,3*E*,5*E*)-1-cyclopropyl-3-ethyl-5-methyl-1,3,5-heptatriene, was less effective in reducing mean landings (69% vs 99%) than when dough was absent. This inhibitory compound causes a reduction of response to pheromone but does not cause a reduction of response to fermenting food-type volatiles such as fermenting bread dough. Analogues of pheromones that strongly reduce response to pheromones by insects might be useful as biochemical probes to study the pharmacophoric (three-dimensional structure) requirements for pheromone perception.

Keywords: *Triene; hydrocarbon; Freeman sap beetle; pheromone analogues; structure−activity relationships*

INTRODUCTION

Males of the Freeman sap beetle, *Carpophilus freemani* Dobson (Coleoptera: Nitidulidae), produce an aggregation pheromone that attracts beetles of both sexes, and the major component of the pheromone was identified as (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-nonatriene (**1a**) (Bartelt et al., 1990a). The major component, by itself, is sufficient to elicit attraction. The natural pheromone blend is composed of triene hydrocarbons **1a** and **2**−**5** and tetraene hydrocarbons **6**−**8** (Figure 1). Compound **3** does not attract *C. freemani*. The attraction response of *C. freemani* to **1a** is synergized by **7** (Bartelt et al., 1990a). Response of *Carpophilus* species to their aggregation pheromones is also synergized by volatiles from fermenting food materials (Bartelt, 1997).

Compounds that could suppress the response of the target insect to its own pheromone would be useful as biochemical probes to help elucidate the pharmacophoric (three-dimensional structure) requirements for pheromone perception (Tumlinson and Teal, 1987). That knowledge might lead to species-specific insect pest control strategies that would be less harmful to the environment. If the reduction of behavioral response to pheromone was strong enough to substantially reduce aggregation and mating under natural field conditions then a new species-specific control agent could be developed.

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We chose to study *C. freemani* because the major component of its aggregation pheromone is the simplest structure of all the major components of the known nitidulid aggregation pheromone blends (Bartelt, 1997). Therefore, target compounds would be easier to prepare by chemical synthesis. Knowledge has been acquired regarding the rearing of this insect, a wind-tunnel bioassay, and the synthesis of its pheromone components (Bartelt et al., 1990a,c). Therefore, experiments could be conducted without having to acquire this preliminary knowledge.

It has been previously shown that the response of *C. freemani* to **1a** in a wind tunnel bioassay was inhibited 26−47% ($P < 0.01$) by a 10-fold excess of (2*E*,4*E*,6*Z*)-5-ethyl-3-methyl-2,4,6-nonatriene (**1b**), a double-bond configurational isomer (Petroski and Weisleder, 1997). This artifact of synthesis is not produced by *C. freemani*.

The response of *C. freemani* to **1a** in a wind tunnel bioassay was also inhibited by a 10-fold excess of **5** (Bartelt et al., 1995), which is the major component of the aggregation pheromone for *C. mutilatus* (Bartelt et al., 1993). A semiochemical mechanism for *C. freemani* to avoid competition with *C. mutilatus* under natural field conditions is suggested (Bartelt et al., 1995). A site colonized by both species would be less attractive to *C. freemani* than one with only *C. freemani*.

We felt that we could improve on the level of reduction of behavioral response to pheromone that has already been observed in the case of *C. freemani* by compounds **1b** and **5**. Analogues were prepared to explore structure−activity relationships.

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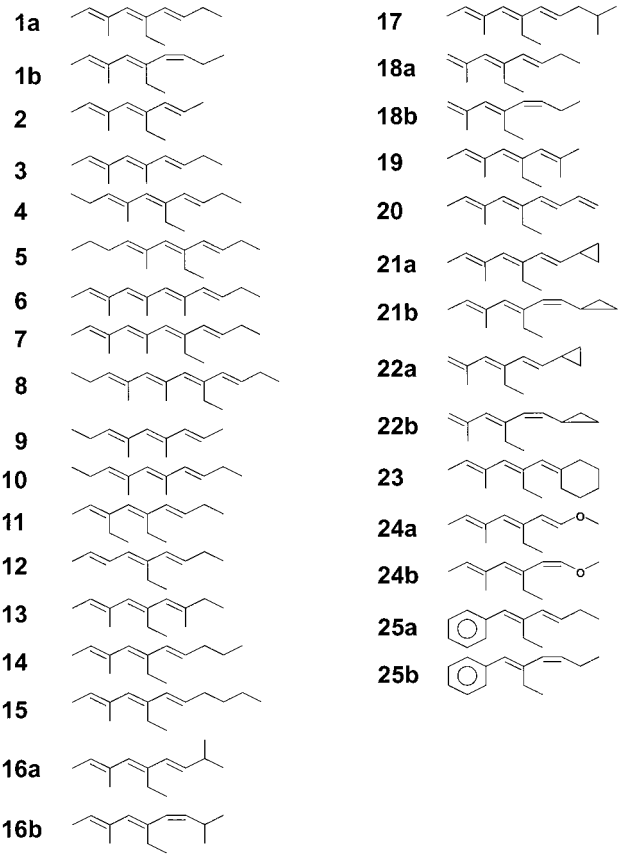


Figure 1. Structures of compounds used in the *Carpophilus freemani* study of structure−activity relationships.

We also wanted to study the effect of **7** and synergists such as propyl acetate (Bartelt et al., 1990a) and fermenting food volatiles on reduction of beetle response to pheromone. We felt that fermenting bread dough (Alm et al., 1985) could serve as a "field-like" synergist because a number of volatile compounds are present and it has been used as the "standard" in field research studies (Bartelt, 1997).

MATERIALS AND METHODS

Beetle Culture. The *C. freemani* culture was the same as for earlier pheromone identification (Bartelt et al., 1990a). This culture was originally collected in an area of oak woods and corn fields near Bath, IL, in 1989 and has been maintained using the artificial diet described previously (Dowd, 1987), except that the pinto beans were replaced by an equal weight of brewer's yeast.

Synthesis, Purification, and Structural Confirmation of Pheromone Analogues. Compounds **1**−**11** were available from previous work (Bartelt et al., 1990a). The identity and purity of each compound was checked by GC/MS and liquid chromatography was used as needed to improve purity.

New compounds, **12**−**25**, were prepared by the general synthetic procedures described by Bartelt et al. (1990c). For example (Figure 2), compounds **21a** and **21b** were prepared by a sequence of reactions in which tiglic aldehyde was condensed with triethyl-2-phosphonobutyrate in a Wittig−Horner reaction; after reduction of the ester product to an alcohol with lithium aluminum hydride and subsequent oxidation of the alcohol to an aldehyde with manganese dioxide, a Wittig condensation with cyclopropylmethyltriphenylphosphonium bromide afforded a mixture of the target compounds (**21a,b**), which were separated by liquid chromatography.

A silica HPLC column coated with $AgNO_3$ (Heath and Sonnet, 1980) was used to separate and purify the *E*- and *Z*-isomers of pheromone analogues. The solvent was 0.5% 1-hexene in hexane using a flow rate of 0.6 mL/min. The HPLC equipment was described previously (Bartelt et al., 1990b). Removal of remaining artifacts of synthesis and impurities from 1-hexene was achieved with size exclusion chromatography using a PL GEL 5μ 100A column (Polymer Laboratories) with hexane as the solvent at a flow rate of 0.6 mL/min.

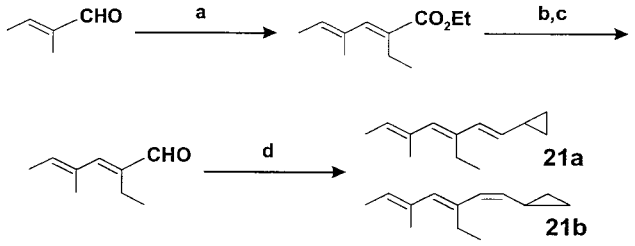


Figure 2. Synthetic method for pheromone analogues exemplified by compounds **21a** and **21b**. Abbreviations: a = Wittig−Horner condensation with triethyl-2-phosphonobutyrate, b = reduction of ester with lithium aluminum hydride, c = partial oxidation of alcohol with manganese dioxide, d = Wittig condensation with (cyclopropylmethyl)triphenylphosphonium bromide.

HPLC fractions were analyzed by gas chromatography (GC) using a Hewlett-Packard (HP) 5890 Series II instrument equipped with flame ionization detector, splitless injector, and an HP 7673 autosampler and interfaced to an HP 3396 integrator. The oven temperature was programmed from 50 to 250 °C at 10 °C/min; the injector temperature was 220 °C, and the detector temperature was 270 °C. A DB-1 capillary column (15 m × 0.25 mm, 1.0-μm film thickness, J&W Scientific, Folsom, CA) was used with 1-μL sample injections. In the occasional case of slightly more dilute samples, 2-μL injections were used. An internal standard (*n*-nonadecane) was used for isomer quantitation.

Electron impact mass spectra (70 eV) were obtained with an HP 5970 MSD instrument, with a DB-1 capillary GC column as described above. Proton NMR spectra (C_6D_6) were obtained on a Bruker (Bellerica, MA) ARX 400-MHz spectrometer; *J* is in hertz. Compound names, MS data, and ^{1}H NMR spectral data (C_6D_6) follow:

(2*E*,4*E*,6*E*)-5-Ethyl-2,4,6-nonatriene (12). MS *m*/*z* (% base) 150 (M^+, 22), 135 (6), 121 (43), 107 (9), 105 (11), 93 (100), 91 (33), 79 (30), 77 (22); ^{1}H NMR δ 1.00 (3 H, t, J = 7.5), 1.12 (3 H, t, J = 7.5), 1.69 (3 H, br d, J = 6.8), 2.09 (2 H, m, J = 7.5), 2.39 (2 H, q, J = 7.5), 5.66 (1 H, m, J = 6.8), 5.73 (1 H, m, J = 15.5), 6.07 (2 H, dd), 6.48 (1 H, m, J = 11.1).

(2*E*,4*E*,6*E*)-3,7-Dimethyl-5-ethyl-2,4,6-nonatriene (13). MS *m*/*z* (% base) 178 (M^+, 12), 149 (72), 121 (100), 119 (16), 107 (56), 93 (37), 91 (16), 79 (24), 77 (20); ^{1}H NMR δ 1.05 (3 H, t, J = 7.5), 1.10 (3 H, t, J = 7.5), 1.65 (3 H, br d, J = 6.9), 1.78 (3 H, br s), 1.84 (3 H, br s), 2.07 (2 H, m, J = 7.5), 2.39 (2 H, q, J = 7.5), 5.58 (1 H, m), 5.81 (br s), 5.84 (1 H, br s).

(2*E*,4*E*,6*E*)-5-Ethyl-3-methyl-2,4,6-decatriene (14). MS *m*/*z* (% base) 178 (M^+, 11), 149 (10), 135 (33), 121 (12), 119 (9), 107 (100), 105 (21), 93 (24), 91 (33), 79 (17), 77 (18); ^{1}H NMR δ 0.93 (3 H, t, J = 7.5), 1.19 (3 H, t, J = 7.5), 1.42 (2 H, m, J = 7.5), 1.63 (3 H, br d, J = 6.9), 1.76 (3 H, br s), 2.09 (2 H, m, J = 6.9), 2.52 (2 H, m, J = 7.5), 5.59 (1 H, m, J = 6.9), 5.72 (1 H, dt, J = 15.6, 6.9), 5.97 (1 H, br s), 6.11 (1 H, br d, J = 15.6).

(2*E*,4*E*,6*E*)-5-Ethyl-3-methyl-2,4,6-undecatriene (15). MS *m*/*z* (% base) 192 (M^+, 6), 163 (4), 149 (3), 135 (34), 121 (11), 119 (6), 107 (100), 105 (15), 93 (21), 91 (29), 79 (17), 77 (17); ^{1}H NMR δ 0.93 (3 H, t, J = 7.0), 1.19 (3 H, t, J = 7.5), 1.27 (4 H, br s), 1.63 (3 H, br d, J = 6.8), 1.77 (3 H, br s), 2.13 (2 H, m, J = 7.0), 2.54 (2 H, q, J = 7.5), 5.60 (1 H, m, J = 7.0), 5.73 (1 H, dt, J = 15.5, 7.0), 5.97 (1 H, s), 6.12 (1 H, br d, J = 15.5).

(2*E*,4*E*,6*E*)-3,8-Dimethyl-5-ethyl-2,4,6-nonatriene (16a). MS *m*/*z* (% base) 178 (M^+, 11), 163 (3), 149 (6), 135 (49), 121 (9), 119 (9), 107 (100), 105 (15), 93 (14), 91 (23), 79 (11), 77 (11); ^{1}H NMR δ 1.05 (6 H, d, J = 6.7), 1.18 (3 H, t, J = 7.5), 1.63 (3 H, m, J = 6.9), 1.76 (3 H, br s), 2.34 (1 H, m, J = 7.0), 2.52 (2 H, q, J = 7.5), 5.59 (1 H, m, J = 6.9), 5.71 (1 H, dd, J = 15.8, 7.0), 5.98 (1 H, br s), 6.09 (1 H, d, J = 15.8).

(2*E*,4*E*,6*Z*)-3,8-Dimethyl-5-ethyl-2,4,6-nonatriene (16b). MS *m*/*z* (% base) 178 (M^+, 9), 163 (5), 149 (8), 135 (51), 121 (12), 119 (9), 107 (100), 105 (17), 93 (18), 91 (26), 79 (13), 77 (12); ^{1}H NMR δ 1.03 (6 H, d, J = 7.0), 1.08 (3 H, t, J = 7.5), 1.62 (3 H, d, J = 6.8), 1.74 (3 H, br s), 2.35 (2 H, q, J = 7.5), 3.03 (1 H, m, J = 10.1), 5.33 (1 H, dd, J = 11.5, 10.1), 5.55 (1 H, m, J = 6.8), 5.84 (1 H, d, J = 11.5), 6.04 (1 H, br s), 6.09 (1 H, d, J = 15.8).

(2*E*,4*E*,6*E*)-3,9-Dimethyl-5-ethyl-2,4,6-decatriene (17). MS *m*/*z* (% base) 192 (M^+, 4), 163 (2), 149 (5), 135 (26), 121 (12), 119 (7), 107 (100), 105 (17), 93 (23), 91 (30), 79 (18), 77 (19); ^{1}H NMR δ 0.94 (6 H, d, J = 6.7), 1.19 (3 H, t, J = 7.5), 1.63 (3 H, br d, J = 6.9), 1.76 (3 H, br s), 2.03 (2 H, m, J = 7.0), 2.54 (2 H, q, J = 7.5), 5.59 (1 H, m, J = 13.8), 5.73 (1 H, dt, J = 15.7), 5.97 (1 H, br s), 6.10 (1 H, br d, J = 15.7, 7.0).

(3*E*,5*E*)-4-Ethyl-2-methyl-1,3,5-octatriene (18a). MS *m*/*z* (% base) 150 (M^+, 7), 135 (2), 121 (45), 107 (6), 105 (22), 93 (100), 91 (35), 79 (21), 77 (29); ^{1}H NMR δ 0.99 (3 H, t, J = 7.5), 1.13 (3 H, t, J = 7.5), 1.85 (3 H, br s), 2.07 (2 H, m, J = 7.5), 2.51 (2 H, q, J = 7.5), 5.04 (2 H, br s), 5.74 (1 H, dt, J = 15.7), 5.89 (1 H, br s), 6.03 (1 H, br d, J = 15.7)

(3*E*,5*Z*)-4-Ethyl-2-methyl-1,3,5-octatriene (18b). MS *m*/*z* (% base) 150 (M^+, 8), 135 (5), 121 (62), 107 (12), 105 (16), 93 (100), 91 (42), 79 (25), 77 (31); ^{1}H NMR δ 0.96 (3 H, t, J = 7.5), 1.04 (3 H, t, J = 7.5), 1.83 (3 H, br s), 2.28 (2 H, m, J = 7.5, 1.7), 2.35 (2 H, q, J = 7.5), 5.02 (2 H, br s), 5.48 (1 H, dt, J = 11.6, 7.3), 5.87 (1 H, m, J = 11.6, 1.7), 5.95 (1 H, br s).

(4*E*,6*E*)-2,6-Dimethyl-4-ethyl-2,4,6-octatriene (19). MS *m*/*z* (% base) 164 (M^+, 26), 149 (56), 135 (43), 121 (100), 119 (30), 107 (49), 105 (44), 93 (31), 91 (44), 79 (25), 77 (30); ^{1}H NMR δ 1.09 (3 H, t, J = 7.5), 1.64 (3 H, br d, J = 6.8), 1.76 (6 H, br s), 1.82 (3 H, br s), 2.37 (2 H, q, J = 7.5), 5.57 (1 H, m, J = 6.8), 5.78 (1 H, br s), 5.91 (1 H, br s).

(3*E*,5*E*,7*E*)-5-Ethyl-7-methyl-1,3,5,7-nonatetraene (20). MS *m*/*z* (% base) 162 (M^+, 43), 147 (34), 133 (63), 119 (60), 105 (100), 93 (20), 91 (86), 79 (31), 77 (43); ^{1}H NMR δ 1.05 (3 H, t, J = 7.5), 1.59 (3 H, br d, J = 6.8), 1.69 (3 H, br s), 2.34 (2 H, q, J = 7.5), 5.10 (1 H, br d, J = 10.0), 5.26 (1 H, br d, J = 16.9), 5.53 (1 H, m, J = 6.8), 5.90 (1 H, d, J = 11.3), 6.10 (1 H, br s), 6.15 (1 H, t, J = 11.3), 7.08 (1 H, m, J = 16.9).

(1*E*,3*E*,5*E*)-1-Cyclopropyl-3-ethyl-5-methyl-1,3,5-heptatriene (21a). MS *m*/*z* (% base) 176 (M^+, 15), 161 (23), 147 (40), 133 (34), 121 (7), 119 (88), 107 (37), 95 (100), 93 (24), 91 (100), 79 (44), 77 (57); ^{1}H NMR δ 0.36 (2 H, m), 0.61 (2 H, m), 1.15 (3 H, t, J = 7.5), 1.30 (1 H, m), 1.63 (3 H, br d, J = 6.9), 1.77 (3 H, br s), 2.49 (2 H, q, J = 7.5), 5.26 (1 H, dd, J = 8.6, 15.6), 5.59 (1 H, m, J = 6.9), 5.95 (1 H, br s), 6.21 (1 H, d, J = 15.6).

(1*Z*,3*E*,5*E*)-1-Cyclopropyl-3-ethyl-5-methyl-1,3,5-heptatriene (21b). MS *m*/*z* (% base) 176 (M^+, 21), 161 (24), 147 (45), 133 (34), 121 (7), 119 (89), 107 (40), 95 (97), 93 (24), 91 (100), 79 (39), 77 (53); ^{1}H NMR δ 0.30 (2 H, m), 0.61 (2 H, m), 1.17 (3 H, t, J = 7.5), 1.63 (3 H, br d, J = 6.9), 1.77 (3 H, br s), 1.95 (1 H, m), 2.45 (2 H, q, J = 7.5), 4.81 (1 H, dd, J = 11.5, 10.0), 5.60 (1 H, m, J = 6.9), 5.90 (1 H, d, J = 11.5), 6.29 (1 H, br s).

(1*E*,3*E*)-1-Cyclopropyl-3-ethyl-5-methyl-1,3,5-hexatriene (22a). MS *m*/*z* (% base) 162 (M^+, 17), 147 (20), 133 (49), 119 (34), 117 (14), 105 (100), 93 (37), 91 (77), 79 (26), 77 (39); ^{1}H NMR δ 0.34 (2 H, m, J = 4.7, 2.0), 0.60 (2 H, m, J = 4.1, 2.0), 1.11 (3 H, t, J = 7.5), 1.33 (1 H, m), 1.86 (3 H, br s), 2.48 (2 H, q, J = 7.5), 5.05 (1 H, br s), 5.25 (1 H, dd, J = 8.7, 15.6), 5.89 (1 H, br s), 6.16 (1 H, d, J = 15.6).

(1*Z*,3*E*)-1-Cyclopropyl-3-ethyl-5-methyl-1,3,5-hexatriene (22b). MS *m*/*z* (% base) 162 (M^+, 15), 147 (25), 133 (53), 119 (50), 117 (19), 105 (100), 93 (41), 91 (96), 79 (32), 77 (52); ^{1}H NMR δ 0.28 (2 H, m), 0.58 (2 H, m), 1.13 (3 H, t, J = 7.5), 1.35 (1 H, m), 1.86 (3 H, br s), 2.12 (2 H, m, J = 7.5), 2.45 (2 H, q, J = 7.5), 4.79 (1 H, dd, J = 9.9, 11.5), 5.04 (1 H, m), 5.05 (1 H, m), 5.84 (1 H, d, J = 11.5), 6.24 (1 H, br s).

(2*E*,4*E*)-5-(Cyclohexylidenemethyl)-3-methyl-2,4-heptadiene (23). MS *m*/*z* (% base) 204 (M^+, 32), 175 (40), 147 (64), 133 (78), 122 (40), 119 (85), 107 (100), 105 (58), 93 (26), 91 (18), 79 (42), 77 (36); ^{1}H NMR δ 0.93 (3 H, t, J = 7.0), 1.12 (3 H, t, J = 7.5), 1.52 (6 H, m), 1.64 (3 H, br d, J = 6.8), 1.77 (3 H, br s), 2.18 (2 H, m), 2.38 (2 H, q, J = 7.5), 2.44 (2 H, m), 5.58 (1 H, m, J = 6.8), 5.77 (1 H, br s).

(1*E*,3*E*,5*E*)-1-Methoxy-3-ethyl-5-methyl-1,3,5-heptatriene (24a). MS *m*/*z* (% base) 166 (M^+, 21), 151 (46), 137 (100), 135 (29), 123 (55), 119 (84), 107 (90), 105 (66), 93 (30), 91 (85), 79 (48), 77 (57); ^{1}H NMR δ 1.13 (3 H, t, J = 7.5), 1.65 (3 H, br d, J = 6.8), 1.77 (3 H, br s), 2.41 (2 H, q, J = 7.5), 3.23 (3 H, br s), 5.56 (1 H, m, J = 6.8), 5.57 (1 H, d, J = 12.9), 5.86 (1 H, br s), 6.73 (1 H, d, J = 12.9).

(1*Z*,3*E*,5*E*)-1-Methoxy-3-ethyl-5-methyl-1,3,5-heptatriene (24b). MS *m*/*z* (% base) 166 (M^+, 22), 151 (34), 137 (100), 135 (22), 123 (48), 119 (73), 107 (79), 105 (60), 93 (28), 91 (78), 79 (42), 77 (52); ^{1}H NMR δ 1.32 (3 H, t, J = 7.4), 1.64 (3 H, br d, J = 6.8), 1.79 (3 H, br s), 2.76 (2 H, q, J = 7.4), 3.10 (3 H, br s), 4.86 (1 H, d, J = 7.3), 5.57 (1 H, d, J = 7.3), 5.63 (1 H, d, J = 6.8), 6.33 (1 H, br s).

(1*E*,3*E*)-2-Ethyl-1-phenyl-1,3-hexadiene (25a). MS *m*/*z* (% base) 186 (M^+, 8), 157 (55), 141 (10), 129 (100), 115 (34), 91 (34), 77 (10); ^{1}H NMR δ 1.01 (3 H, t, J = 7.5), 1.17 (3 H, t, J = 7.5), 2.08 (2 H, m, J = 7.5, 6.6), 2.50 (2 H, q, J = 7.5), 5.79 (1 H, dt, J = 6.6, 9.1), 6.15 (1 H, br d, J = 15.7), 6.47 (1 H, s), 7.10 (1 H, m, J = 7.4), 7.23 (2 H, m, J = 7.4), 7.34 (2 H, br d, J = 7.4).

(1*E*,3*Z*)-2-Ethyl-1-phenyl-1,3-hexadiene (25b). MS *m*/*z* (% base) 186 (M^+, 7), 157 (45), 141 (9), 129 (100), 115 (19), 91 (24), 77 (10); ^{1}H NMR δ 0.75 (3 H, t, J = 7.5), 1.07 (3 H, t, J = 7.5), 1.90 (2 H, m, J = 7.5, 1.7), 2.20 (2 H, q, J = 7.5), 5.36 (1 H, dt, J = 11.6, 7.5), 6.00 (1 H, br d, J = 11.6), 6.35 (1 H, br s), 7.07 (1 H, m, J = 7.4), 7.20 (2 H, m), 7.49 (2 H, br d, J = 7.4).

Bioassay. The wind tunnel bioassays were conducted as described by Bartelt et al. (1990a). The wind tunnel contained ca. 500−1000 beetles, and ca. 100 were in flight at any time during bioassays. Two different treatment preparations to be compared were applied to pieces of filter paper, and those were hung side by side in the upwind end of the wind tunnel. Each bait was tested at each side to minimize bias resulting from position effects. Behavioral responses by the beetles to an active preparation included an upwind, casting flight, followed by alighting on the filter paper. Each test lasted 3 min, and the numbers of landings were recorded. Propyl acetate (20 μL, 1% solution in mineral oil) was added as a synergist in some bioassay treatments; previous studies have shown that mean landings are enhanced by the presence of food-related compounds such as small esters (Bartelt et al., 1990a,b). Controls consisted of filter paper alone (studies conducted in the absence of synergist) or filter paper containing only propyl acetate (studies conducted in the presence of synergist). Tests were replicated as indicated in the tables. Mean landings varied somewhat from day to day; for this reason, comparisons between test compounds were made relative to the response to a standard (the pheromone **1a** alone or with synergist as indicated in tables).

To investigate relationships between the chemical structure of the pheromone analogues and biological activity, all compounds were first tested for attractancy (four replications each) and then for their ability to reduce beetle response to pheromone (four replications each) in the wind tunnel behavioral bioassay. Compounds with at least slight activity in the preliminary screen were retested for attractancy or inhibitory properties by using more replications, as given in the tables.

Results of retests for attractive or inhibitory properties are presented either as an index of attraction or as the percent reduction of mean landings. Index of attraction is defined as (test compound mean landings − control)/(compound **1a** mean landings − control) × 100. Percent reduction of mean landings is defined as (compound **1a** mean landings − mean landings of the mixture of **1a** and the test compound)/(compound **1a** mean landings) × 100.

We studied the effect of the presence of pheromone component **7** on the reduction of mean landings caused by analogues of **1a**. Responses of beetles to a blend of **1a** plus **7** alone (both at 1.3 ng), **1a** plus **7** (both at 1.3 ng) plus **21b** at a dose of 13 ng, and **1a** plus **7** (both at 1.3 ng) plus **21b** at a dose of 39 ng were compared in a balanced incomplete block design experi-

ment (10 replications per treatment). In a separate experiment to verify the earlier report of synergism between **1a** and **7** (Bartelt et al., 1990), we compared the beetle response to blank filter paper alone, **1a** alone (1.3 ng), **7** alone (1.3 ng), and a combination of **1a** plus **7** (both at 1.3 ng) at one spot on the same filter paper in a balanced incomplete block design experiment (10 replications per treatment).

Effect of Dough on Reduction of Response to Pheromone. Fermenting whole wheat bread dough (Alm et al., 1985) was prepared from 15 g of whole wheat flour, 70 mg of active dry yeast, 10 g of cane sugar, and 25 mL of water. After stirring, 1-mL aliquots of the resulting suspension were dispensed into 5.5-mL vials. The vials were covered with cheesecloth and the cloth was held in place with a stiff copper wire; the remaining portion of wire was bent into a hook for the purpose of hanging the attractant-containing vials directly in back of filter paper containing treatments. Prior to use in wind tunnel bioassay experiments, vials containing the bread dough suspension, cheesecloth cover, and wire hanger were placed in an incubator held at 27 °C for 18 h to ferment.

The effect of fermenting bread dough on the reduction of mean landings caused by the most potent compound (**21b**) was measured. All possible pairs of treatments, with and without fermenting bread dough, including blank filter paper, pheromone **1a** (1.3 ng), and pheromone **1a** (1.3 ng) plus inhibitory compound **21b** (13 ng) were compared in a balanced incomplete block design experiment using 10 replications of each of the six different treatments. The data were analyzed by contrast comparisons within analysis of variance. In a separate experiment, we studied the effect of **21b** on the response of beetles to fermenting bread dough alone (eight replications per treatment).

Statistical Analysis and Experimental Design. Wind tunnel data were transformed to the $\log(X + 1)$ scale before analysis to stabilize variance. Analysis of variance, within the Statistix program 4.1 (Analytical Software, Tallahasse, FL), was used to compare two treatments. When experiments involved comparisons among three or more treatments, these were tested in pairs in all possible combinations by using a balanced incomplete block design and data were analyzed by the method of Yates (1940).

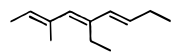
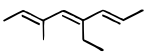
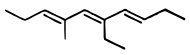
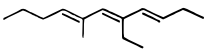
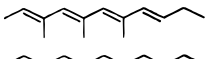
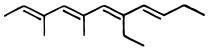
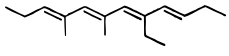
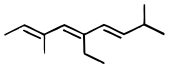
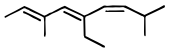
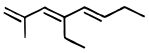
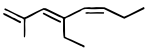
Computational Chemistry. Molecular modeling studies were conducted using Sybyl version 6.4 software (Tripos Associates, St . Louis, MO) and Spartan version 5 software (Wave function Inc., Irvine, CA). The 3D structures of compounds **1a**, **16a**, and **21a** were constructed by using structure **1a** as a template. The bonds in structure **1a** were assumed to be rotatable with a reasonable energy barrier in terms of all states being populated at room temperature, based on previous work with similar compounds (Petroski and Vaz, 1995). The template structure was subjected to conformational searching about the rotatable bonds by using the Tripos 5.2 molecular mechanics force field (Clark et al., 1989).

The minima encountered in the conformational search of the template compound were optimized with the AM1 Hamiltonian and the minimum energy conformation was used as the template for the construction of compounds **16a** and **21a.** Structures **16a** and **21a** were optimized with the AM1 Hamiltonian. Conformations described here are vapor phase.

RESULTS

Of all the newly prepared pheromone analogues, only the 6*E*-isomer of the isopropyl analogue, compound **16a**, was found to be somewhat active as an attractant (Table 1). Its 6*Z*-isomer, compound **16b**, is inactive. Compounds **18a** and **18b**, which lack the left-hand terminal methyl group, are active as attractants but only at a level of less than 3% of **1a**. The oxygen-containing bioisostere of **1a**, (1*E*,3*E*,5*E*)-1-methoxy-3-ethyl-5-methyl-1,3,5-heptatriene (**24a**) (shown in Figure 1), is inactive as an attractant. Compounds **1a**, **2**, and **4**−**8** are

Table 1. Attractiveness of Test Compounds to *C. freemani* in a Wind Tunnel Bioassay[a]

compd	structure	index of attraction[b]
1a		100***
2		27***
4		16***
5		0.6**
6		2.3**
7		6.0***
8		1.7***
16a		25***
16b		0 ns
18a		2.9**
18b		1.6*
3, 9−15, 17, 19−25	see Figure 1	0 ns

[a] See Materials and Methods for bioassay conditions. [b] Index of attraction is defined as (test compound mean landings − control)/(compound **1a** mean landings − control) × 100. Significance: ns means not significant difference from control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Attractants other than **1a**, such as **2** or **16a**, were significantly less attractive than **1a** ($P < 0.001$). Eight replications per treatment, balanced incomplete block design.

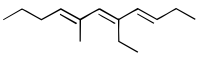
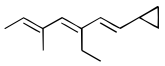
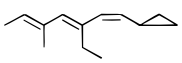
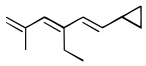
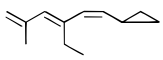
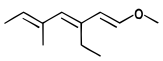
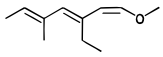
constituents of the natural pheromone blend and are known attractants of this insect species (Bartelt et al., 1990a).

Of all the compounds tested (Figure 1), only **5**, **24b**, **21a**, and **21b** reduced the response of *C. freemani* to **1a** in the wind tunnel bioassay (Table 2). The most potent compounds tested were **21a, 21b**, and **24b**. The oxygen-containing bioisostere of **1b**, compound **24b**, effectively reduces behavioral response to pheromone in the absence of the synergist (94%, $P < 0.001$). Compound **1b** was also inhibitory but at a level of only 26% ($P < 0.001$) (Petroski and Weisleder, 1997). The reduction in mean landings caused by **24b** and the lower dose of **21a** was not as substantial in the presence of the propyl acetate synergist. Neither of the cyclopropyl-group-containing analogues lacking the left-hand terminal methyl group, **22a** nor **22b** shown in Figure 1, caused a reduction in mean landings ($P > 0.9$, four replications each).

The effect of **21b** on the response to a pheromone blend composed of **1a** + **7** was determined at two doses of **21b**: 10-fold excess, 30-fold excess (Table 3). A greater reduction of response to pheromone was observed at the higher dose of **21b**. As part of this study, an experiment was conducted to recheck an earlier report of synergism between **1a** and **7** (Bartelt et al., 1990a).

The results of a study conducted to measure the effect of fermenting bread dough on the attractancy of **1a** and reduction in mean landings caused by **21b** are shown in Table 4. Mean landings for the pheromone **1a** increased from 32 to 65 in the presence of dough. Compound **21b** was 30% less effective (change from 99% to 69%, $P < 0.001$) in causing a reduction in mean landings in the presence of dough.

Table 2. Reduction of *C. freemani* Response to Aggregation Pheromone (1a) by Test Compounds

				without synergist[a]			with synergist		
compd	structure	dose, ng	no. of replications	mean[b] landings **1a**	mean landings **1a** + test compound	reduction,[c] %	mean landings **1a** + test compound	mean landings **1a** + test compound	reduction, %
5		13	8	16	3.2	80***	19	3.2	83***
21a		1.3	16	42	19	55***	69	57	17 ns
		13	16	41	6.9	83***	54	8.6	84***
21b		1.3	16	35	19	44***	99	56	43***
		13	16	36	1.3	96***	70	4.4	94***
22a		13	4	21.4	21.3	0.5 ns		not tested	
22b		13	4	20.1	19.9	1 ns		not tested	
24a		13	8	14	12	14 ns		not tested	
24b		13	8	14	0.9	94***	21	9.8	53***

[a] The synergist was propyl acetate (20 μL, 1% solution in mineral oil). [b] Mean landings are the number of beetles flying upwind to the filter paper baits and alighting during the 3-min tests. [c] Percent reduction is defined as 100[mean landings **1a** − mean landings (**1a** + test compound)]/(mean loadings **1a**). ns means not significant; *** $P < 0.001$.

Table 3. Response Relationships between 1a, 7, and 21b

A. Effect of **21b** on Response to a Pheromone Blend Composed of **1a** and **7**

dose of **21b**, ng	mean landings in response to blend[a,b]	reduction,[c] %
0	41	
13	11	74***
39	3.7	91***

B. Effect of **21b** on Response to Pheromone **1a**

mean landings		
1a	**1a** + **21b** (13 ng)	reduction,[d] %
36	1.3	96***

C. Effect of Natural Pheromone Blend Component **7** on Response to **1a**

mean landings		
1a	**7**	**1a** + **7**
54	0.5	74[e]

[a] Mean landings are the numbers of beetles flying upwind to the filter paper baits and alighting during the 3-min tests. [b] Both **1a** and **7** at 1.3 ng each. [c] Percent reduction is defined as [mean landings (**1a** + **7**) − mean landings (**1a** + **7**) + **21b**]/[mean landings (**1a** + **7**)] × 100. Significance: *** $P < 0.001$. [d] Percent reduction is defined as [mean landings (**1a**) − mean landings (**1a** + **21b**)/[mean landings (**1a**)] × 100. Significance: *** $P < 0.001$. [e] The difference between the response to the combination treatment (**1a** + **7**) was significantly different than the sum of the response for **7** alone plus the response for **1a** alone (74 vs 54.5, 10 replications per treatment, $P < 0.05$). Experiments A, B, and C were conducted separately.

Table 4. Response Relationships between Fermenting Dough, 1a, and 21b

A. Effect of Fermenting Bread Dough on Reduction of Response to Pheromone

mean landings[a]				
dough	blank	**1a**	**1a** + **21b**	reduction,[b] %
absent	0	32	0.2	99
present	9	65	20	69

B. Effect of **21b** on Response to Fermenting Bread Dough

mean landings[c]		
dough	dough + **21b**	reduction,[d] %
8.3	7.1	14 ns[e]

[a] Mean landings are the number of beetles flying upwind to the filter paper baits and alighting during the 3-min tests. Compounds **1a** and **21b** were used at doses of 1.3 ng and 13 ng, respectively. The presence or absence of dough, pheromone **1a**, and (**1a** + **21b**) were all significantly ($P < 0.001$) different as described by contrast comparisons in analysis of variance. [b] Percent reduction is defined as [mean landings **1a** − mean landings (**1a** + **21b**)]/(mean landings **1a**) × 100. The difference in percent reduction when dough was absent (99%) vs when dough was present (69%) was significant at the $P < 0.001$ level. [c] Mean landings are the numbers of beetles flying upwind to the filter paper baits and alighting during the 3-min tests. [d] Percent reduction is defined as [mean landings dough − mean landings (dough + **21b**)]/(mean landings dough) × 100. [e] Eight replications per treatment, $P = 0.70$. Experiments A and B were conducted separately.

DISCUSSION

Analogues were prepared to study structure−activity relationships, by exploring three-dimensional space at the putative pheromone recognition site. Exploration of molecular space was accomplished by extension of the carbon chain, addition or subtraction of methyl groups at various positions in the molecule, and substitution of more bulky substituents such as cyclopropyl for ethyl (Figure 1). Compounds **12**−**25** are new synthetic pheromone analogues. Compounds **1**−**11** were available from previous work (Bartelt et al., 1990a).

Compounds **12**, **13**, **16a**,**b**, **17**, **18a**,**b**, and **19** feature addition or subtraction of methyl groups at various positions in the molecule (Figure 1). For example, compounds **18a** lacks the terminal methyl group comprising carbon one of **1a**. Compounds **14** and **15** were prepared to examine the effect of lengthening the saturated hydrocarbon chain distal to carbon 1.

Compounds **21a**,**b**, **22a**,**b**, **23**, and **25a**,**b** were prepared to study the effect of placing other forms of steric bulk at various molecular sites. For example, compounds **21a**,**b** and **22a**,**b** contain a cyclopropane ring located in place of the terminal ethyl group distal from carbon 1.

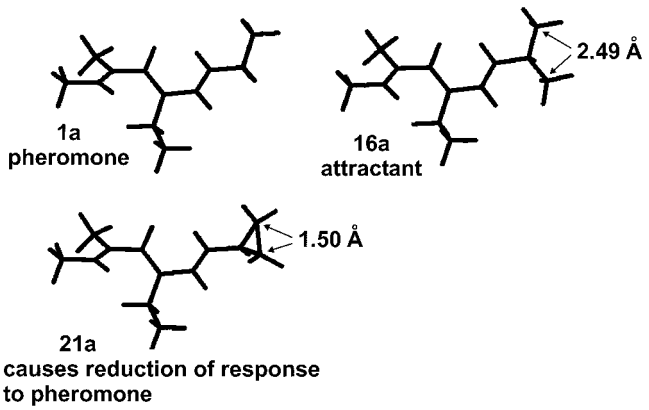


Figure 3. Structures of **1a** (upper left) and 6*E*-isomers of pheromone analogues **16a** (upper right) and **21a** (lower left) in a low-energy conformation arrived at by semiempirical quantum mechanics calculations (see **Computational Chemistry** under Materials and Methods).

The effect of other structural modifications was also examined. Compound **20** contains an extra double bond to form a conjugated tetraene. Compounds **24a** and **24b** were prepared to study the effect of an oxygen atom located in place of the methylene portion of the terminal ethyl group of **1a** and **1b**, respectively.

The presence of the terminal methyl group comprising carbon one of **1a** is important for pheromonal activity. Compounds **18a** and **18b**, which lack the terminal methyl group, are only slightly active as attractants (index of attraction <3%).

The 6*Z*-isomer of the isopropyl analogue, compound **16b,** is inactive as an attractant, just as **1b** is inactive (Petroski and Weisleder, 1997). Therefore, the *E*-geometry about the 6-double bond is also important for pheromonal activity.

The lack of attractiveness of the oxygen-containing bioisostere of **1a**, (1*E*,3*E*,5*E*)-1-methoxy-3-ethyl-5-methyl-1,3,5-heptatriene (**24a**) (Figure 1), suggests a requirement for a hydrophobic group at this position in the molecule. The presence of an oxygen atom introduces the possibility of hydrogen bonding at the putative pheromone recognition site, which might lower attractiveness or even cause a reduction of response to pheromone. The presence of an oxygen atom in **24b** as opposed to **1b** (Petroski and Weisleder, 1997) enhances reduction of response to pheromone.

Molecular modeling studies were conducted to examine similarities and differences between attractive and inhibitory *E*-isomer compounds in a low-energy conformation. Figure 3 shows the structures of 6*E*-isomer energy-minimized compounds (AM1 geometry optimization): upper left shows **1a**, upper right shows the attractive 6*E*-isobutyl analogue **16a**, and the lower left shows the inhibitory 6*E*-cyclopropyl analogue **21a**. The interatomic distance between the methyl carbons of the *gem*-dimethyl group in **16a** is 2.49 Å as determined by computational chemistry calculations within Spartan. In the case of inhibitory **21a**, the cyclopropane ring places two methylene groups only 1.50 Å apart.

We do not know why the isopropyl analogue, **16b**, is not inhibitory, whereas the cyclopropyl analogue, **21b**, is the most potent inhibitor tested, but a partial explanation may involve interatomic distances. The interatomic distance between the methyl carbons of the *gem*-dimethyl group in **16b** is 2.49 Å, whereas in **21b**, the cyclopropane ring places the methylene carbons only 1.50 Å apart. Both analogues **16b** and **21b** have the *Z*-double-bond configuration at the 6-position.

The reason for the reduction in mean landings caused by the cyclopropyl-group containing analogues **21a** and **21b** is unknown; however, Guo et al. (1990) reported that the presence of a cyclopropyl group at the 14-position of the housefly, *Musca domestica* (L.), sex pheromone, (*Z*)-9-tricosene, inhibited catabolism of the pheromone analogue to its 9−10 epoxide and thus clearance of the compound from the natural pheromone recognition site was impaired. Cyclopropyl-group-containing analogues **22a** and **22b** lack the terminal methyl group that comprises carbon one of **1a** or **1b** and are inactive as inhibitors of the *C. freemani* behavioral response; this shows a requirement for a methyl group at this position in the molecule for the analogues to be properly recognized and suggests that **21a** and **21b** are affecting pheromone reception specifically.

In the presence of **7**, a synergistic component of the natural *C. freemani* pheromone blend, **21b** was less effective in reducing mean landings than when **7** was absent, but the effect of **7** can be somewhat overcome by using a higher dose of **21b**. We were unable to test the effect of **21b** on **7** alone because of an insufficient bioassay count for **7** (index of attraction of only 6, Table 1).

Compound **21b**, by itself, might not be successful as a new agrochemical to control *C. freemani*. The compound is 30% less effective in reducing mean landings in the presence of fermenting food volatiles as represented by fermenting bread dough. The compound is also less effective in reducing mean landings in the presence of **7**. The fact that **21b** reduced the response of *C. freemani* to **1a** but not the response to only fermenting bread dough suggests that **21b** specifically inhibits the response of *C. freemani* to its aggregation pheromone **1a** instead of acting as a nonspecific repellent. A single synergist, propyl acetate, lowered the effectiveness of compound **21a** at an equivalent dose of the pheromone and a test compound (both at 1.3 ng). The same synergist also lowered the effectiveness of compound **24b** at a 10-fold excess.

The pheromone analogues encountered in this study may reduce the pheromonal response in a variety of ways: the analogue may compete with the natural pheromone for the pheromone binding protein, the pheromone analogue may arrive at the pheromone receptor site and not be cleared from this site rapidly enough to allow navigation in a "trail" of pheromone molecules (possibly by inhibition of catabolism), or the analogue might arrive at a separate recognition site that elicits a response from a distinctly different region of the insect brain. Evidence for pheromone component-specific recognition sites exists in Lepidoptera (Todd et al., 1995, 1997). We have no evidence to support any of the above possible mechanisms of reduction of response to pheromone.

The possibility that the pheromone and some of the compounds that reduce response to pheromone, encountered in this study, actually bind to the same recognition site is supported by a study of the oriental fruit moth, *Grapholita molesta* (Busck), conducted by Hoskovec et al. (1996). The replacement of the acetate function of (*Z*)-dodecenyl acetate, the major pheromone component, with a less hydrophobic chloroformate function (chlorine of chloroformate vs methyl group of acetate) resulted in a pheromone perception inhibition of 60% when the

pheromone analogue was present at a 10-fold excess. Electrophysiological evidence showed that the pheromone analogue was competing with the natural pheromone for the same recognition sites on the male antennal sensilla.

Compounds **5** (*C. mutilatus* pheromone), **21a** or **21b** (analogues contain a cyclopropyl group), and **24b** (analogue contains an oxygen atom) may reduce response to pheromone by different mechanisms. These compounds may be useful as biochemical probes in future studies of pheromone perception in *C. freemani*.

CONCLUSIONS

The results presented in this paper show the complexity of the response of *C. freemani* to its aggregation pheromone. Efforts to reduce pheromonal response by the use of pheromone analogues are confounded by host plant volatile synergists, as artificially represented by propyl acetate or fermenting bread dough, as well as other components of the aggregation pheromone blend.

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