Behavioral and Electrophysiological Activity of the Racemate and Enantiomers of a Monofluorinated Analog of European Corn Borer (Lepidoptera: Pyralidae) Sex Pheromone[1]

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ABSTRACT The racemate and individual enantiomers of 2-fluoro-Z-11-tetradecenyl acetate (2F-Z-11), analogs of a European corn borer moth, *Ostrinia nubilalis* (Hübner), female sex pheromone were compared with the natural pheromone, Z-11-tetradecenyl acetate, in field trapping experiments, flight tunnel studies, mating disruption assays and electrophysiological experiments. While the racemate and R-2F-Z-11 mimicked the natural female sex pheromone, they were not more biologically potent than the pheromone. The S-2F-Z-11 was largely ineffective in all assays and was, therefore, incompatible with the pheromone receptor system.

KEY WORDS *Ostrinia nubilalis,* 2-fluoro-Z-11-tetradecenyl acetate, R-2-fluoro-Z-11-tetradecenyl acetate, S-2-fluoro-Z-11-tetradecenyl acetate, insect sexual behavior, chirality of chemoreceptors

Chemical substitution of hydrogen with fluorine in biologically active natural products to modify or enhance their physiological properties is a well-established technique (Fried and Sabo 1954). In 1984, Camps et al. suggested that "replacement of hydrogen atoms by fluorine at definite sites of a given pheromone molecule could eventually disrupt the mating communication system by irreversible binding of these fluorinated analogs with specific pheromone receptors." Prestwich (1986) envisioned utility for fluorine-substituted pheromones. He speculated that fluorine substitution could make a pheromone analog more chemically stable than natural pheromone and make it a useful new tool against insects.

Widely ranging effects have been reported for fluorinated pheromones. In work with analogs of the aldehyde pheromones in *Heliothis virescens* (Fabr.), Prestwich, et al. (1986) reported that the acyl flourides were "hyperagonists that caused

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irreversible genitalia extension in that male moths. This work provided the first indication that fluorinated pheromone analogs could cause behaviorally disruptive effects in insects. More recently, Graham and Prestwich (1994) showed that fluorine-substituted analogs of the gypsy moth (*Lymantria dispar* L.) pheromone inhibited epoxide hydrolase, an enzyme found in antennal and leg homogenates of the insect, that is thought to be important in clearing pheromone from the insect.

Fluorinated pheromone analogs may be behaviorally inert (Linn et al. 1992), mimic natural pheromone (Bengtsson et al. 1990, Klun et al. 1994, Lucas et al. 1993, Masnyk et al. 1989, Parrilla and Guerrero 1993), have diminished electophysiological activities (Prestwich et al. 1986, Wu et al. 1993), or inhibit upwind flight of moths to natural pheromone in field traps (Nikonov et al. 1994). In one case, an ω-monofluorinated pheromone analog was toxic to moths (McLean et al. 1989).

Despite many studies, we are aware of no fluorinated pheromone analogs currently employed for insect monitoring or mating disruption. The impetus for our continuing research into fluorinated pheromone analogs of the European corn borer, *Ostrinia nubilalis* (Hübner), comes from the notion that useful new behavioral chemicals may yet be discovered in this class of compounds that will have increased utility as pest management tools. We also think that studies with fluorinated pheromone analogs can provide insight into the physical-chemical interaction of pheromone with the pheromone receptor system (Prestwich 1993, Klun et al. 1994).

We report the comparative biological evaluation of a monofluorinated pheromone analog, 2-fluoro-Z-11-tetradecenyl acetate (2F-Z-11), of the European corn borer sex pheromone, Z-11-tetradecenyl acetate (Z-11). The 2-fluoro substituted compound was selected for study because similarly substituted aliphatic acids are known to inhibit β-oxidation in insects (Rosell et al. 1992, Plettner et al. 1996). Because antennal pheromone catabolism in the European corn borer is thought to involve β-oxidation (Klun et al. 1992), we speculated that the racemate (2F-Z-11) or its individual 2-fluoro substituted enantiomers might have biological properties that would make them more effective than natural pheromone as lures or as behavioral disruptants. To our knowledge, this is the only instance in which the biological activity of an asymmetric monofluorinated insect sex pheromone analog has been investigated.

Materials and Methods

Chemicals. Z-11 (98:2, Z:E) came from our laboratory stock of the compound. The synthesis of 2F-Z-11 (95:5, Z:E) has been described by Oliver et al. (1994), and that of the individual enantiomers, S-2F-Z-11 and R-2F-Z-11 [95:5 (**S:R**) and 3:97 (**S:R**), respectively] by Khrimian et al. (1996). Gas chromatographic analyses of polar and non-polar capillary columns showed that all compounds used in the bioassays were 95 to 98% chemically pure. The chemical structures of the compounds are shown in Fig.1.

Insects. European corn borer moths were from a laboratory colony of the **Z** pheromonal type (Klun and Huettel 1988) that was established from insects collected near Beltsville, MD, USA. The culture had been infused three times with additional field-collected moths since it was established. All insects used in laboratory assays were isolated individually in jelly cups as pupae. Adult

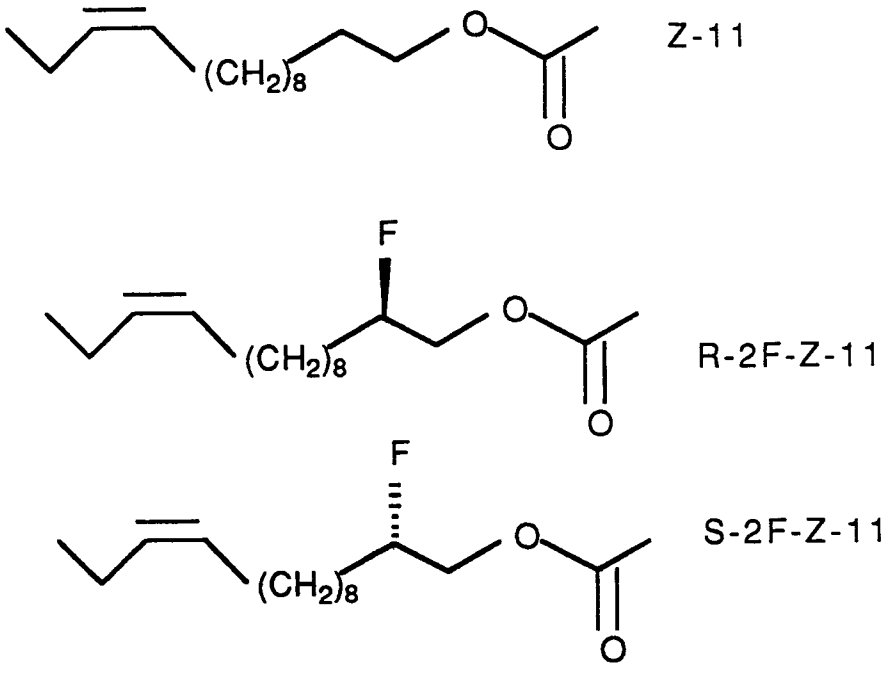


Fig. 1. Structures of European female sex pheromone (Z-11), and the R and S enantiomers of a monofluorinated pheromone analog that were used in the studies.

males were caged, provided with water, and held in reversed diurnal cycle incubators at 16h (25°C) light: 8 h (20°C) dark and 80% RH. Moths were used in assays when they were 3 to 4 days old.

Flight tunnel bioassays. Conditions in the flight tunnel (Raina et al. 1989) were: 18-20°, 40-60% RH, 2.5 lux red light, and wind speed of 50 cm/sec. Males were tested between the second through the fifth hour of scotophase. In the test, 2F-Z-11 (1.2 µmol), R-2F-Z-11, S-2F-Z-11 (0.6 µmol each) and Z-11 (0.6 µmol) in 10 µL heptane were each applied to red rubber serum bottle stoppers (#1780J07, Thomas Scientific, Swedesboro, NJ 08085). The test involved random exposure of 63 individual European corn borer males to each of the four treatments in the flight tunnel over nine test days using a randomized block design. One set of four stoppers was used throughout the experiment and they were stored (0°C) in sealed vials between test days. A treated stopper was positioned 3 m upwind of an individual moth in the tunnel, and male responses to the stimulus were classified into four categories: None = no upwind flight

to the source of compound; Plume = upwind flight in the odor plume but not arriving within 3 cm of source; 3 cm = upwind flight within 3 cm of the source; Land = flew to source, landed, and displayed precopulatory behavior on the septum. A logistic regression model for ordinal categorical data was fitted to the responses of the moths using the SAS procedure, LOGISTIC (SAS Institute 1989). Comparisons among the compounds were made using Wald χ^2 tests.

Field-Trapping tests. Trapping experiments were conducted using *Heliothis* traps (Great Lakes IPM, Vestaburg, MI 48891). The traps were positioned approximately 1 m from the ground and 50 m apart at the perimeter of corn fields near Beltsville, MD. The test was conducted during the 1995 second brood flight of the European corn borer. Traps were baited with rubber stoppers treated with same µmol loads of compound that were used in the flight tunnel bioassays and with stoppers having a one-tenth dose. The field test was arranged as a randomized complete block design with nine treatments and three blocks. The treatments consisted of four compounds (2F-Z-11, R-2F-Z-11, S-2F-Z-11, and Z-11) at two doses, 01x and 1x each, plus an untreated control. Male trap captures were recorded daily over 35 days. On each day, the treatments were rerandomized within blocks. Freshly-treated stoppers were placed in the traps weekly. The used stoppers were recovered and analyzed for residual compound by using the internal standard gas chromatographic analytical method. The total number of trapped moths over the 35 dates was used as the response variable in analysis of variance. The square-root of (x + 1) transformation was used to stabilize the variance. Comparisons among treatments were made using the least significant difference test on the transformed scale.

Electrophysiology. Electoantennogram (EAG) techniques were a modification of a previous technique used by Schneider (1957) and are described in detail by Dickens et al. (1993). Ag-AgCl wires were inserted into two capillary electrodes filled with *Drosophila* Ringer or 0.1 M KCl. Antennae excised at the base with one or two terminal segments removed were mounted between the two glass electrodes. The signal was amplified 100X by a Grass P-16 microelectrode D.C. amplifier and viewed on a Tektronix 5111A storage oscilloscope. EAGs were recorded on a Houston Instruments strip-chart recorder.

Serial dilutions of experimental odorants from 1 to 10^{-4} µg/µL solvent were prepared in heptane. Stimuli were delivered as 1 µL aliquots on Whatman no. 1 filter paper (7 mm × 20 mm) inserted into glass cartridges (80 mm long × 6 mm ID). These odor cartridges were oriented toward the preparation from 1 cm. Odor molecules evaporating from the filter paper were delivered over the antennal preparation in a 1 sec pulse of dry, charcoal filtered air (1.3 L/min) from the cartridge. Interstimulus times of 2 to 3 min allowed for recovery of the EAG. The atmosphere around the preparation was continuously exhausted.

Two experimental EAG series were done with the European corn borer. In the first experiment with antennae of male moths, Z-11, 2F-Z-11, R-2F-Z-11 and S-2F-Z-11 were used in dose-response studies. In the second experiment, female moth antennae were exposed to odorous pulses of one µg stimulus loads of the same compounds. Five replicates were recorded for the first experiment with males and three replicates were recorded for the second experiment with females. For both experiments, 1-hexanol (10 µg stimulus load) served as a standard. Stimulation with the standard occurred after every two experimental

odorants. Compounds, chosen randomly, were tested from the lowest to the highest dose. Mean responses to experimental stimuli were calculated as a percentage response to the two nearest responses to the standard. Since a small response was often elicited to a solvent control (1 μl heptane), mean responses to stimulation with the solvent at the beginning and end of each dose-response series was subtracted from responses to all intervening experimental stimuli. Mean EAG responses were transformed ($\log_{10}$ Percentage EAG+10) to stabilize variances. Comparisons among treatments were made by using the least significant difference test on the transformed scale.

Quantitation of odor molecules delivered in EAG. Because of potential differences in the volatility of Z-11 and 2F-Z-11, we used tritiated analogs (Klun et al. 1992) of the compounds to determine relative release rates from the filter paper. Filter paper strips identical to those used in the EAG tests were treated with 1, 10, 26.9, 43.6, 60, and 100 ng doses of tritiated Z-11 (41 Ci/mmol) and 2F-Z-11 (45 Ci/m mol) and placed in stimulus delivery cartridges identical to those used in the EAG studies. The radiolabeled compounds used in this study were prepared as described by Klun et al. (1992). Two Rainin automatic pipette tips were cut to form 3.6 cm long tapered tubes with a 6.5 mm ID opening on one end and 3.5 mm on the other. The 3.5 mm end of each tube was plugged with 10 mg glass wool and each tube was charged with approximately 50 mg crushed dry ice through the 6.5 mm end. The 3.5 mm end of one tube was inserted into the 6.5 mm end of the other. The 3.5 mm end of the combined the combined trap units was then inserted into the 6 mm ID delivery cartridge. Once the traps were in place, a pulse generator was activated to make a 1 sec air pulse through the tritium-loaded glass cartridge and into the traps. The respective traps were immediately disconnected and dropped into liquid scintillation vials containing Ecoscint scintillant. The purpose of the second in-line trap was to permit verification that breakthrough of radioactivity through the first trap was minimal. Liquid scintillation counts were made for 10 min for each sample, and the amount of compound collected in a pulse was calculated from the total disintegrations per min observed in both traps. The air pulse-collection test was replicated four times on two dates for each of the six doses of two compounds. A mixed linear model was fitted to the data, where date within compound was considered a random effect. The data were analyzed on the $\log_{10}$ scale.

Mating disruption assays. The mating disruption assay was patterned after that used by Klun and Robinson (1970). For this study, mating disruption is defined as the suppression of spermatophore transfer to the female under conditions were the sexes are 35.2 cm or less removed from one another. This is the furthest distance possible between male and female moths held in 20.3 cm square screened cages that were used in the study. We placed three to seven 20.3 cm square screened cages each containing six European corn borer virgin male-female pairs (2 to 3 days old) into one or more 623-liter Powers incubators containing a 64-cm^2 glass wool (0.7 g) dispenser pad treated with a 1 ml acetone solution of test compound. The incubators were programmed: 16 h (25°C) light, 8 h (20°C) dark and 80% RH. The assay began at the onset of a scotophase. Another incubator of the same design and conditions was fitted with an equal number of caged European corn borer moths but without compound, to

serve as control. At the end of the scotophase, the dispensers were recovered, extracted with acetone and analyzed for residual compound by capillary gas chromatography using the internal standard method of quantitation. Females were dissected to determine incidence of mating. The presence of a spermatophore in a female (Drecktrah and Brindley 1967) was proof that a female had mated. Tests were replicated a minimum of four times over days using 9.3, 4.6, 2.3, 0.93, and 0.46 µmol doses of Z-11, 2F-Z-11, R-2F-Z-11 and S-2F-Z-11 and mean percentage mating disruption (%D) was calculated:

$$\left[1 - \frac{\%\ \text{females mated in treatment}}{\%\ \text{females mated in control}} \right] \times 100 = \%D$$

Results and Discussion

Flight tunnel bioassays show that the 1.2 µmol 2F-Z-11 and 0.6 µmol R-2F-Z-11 doses elicited equal responses and that they were each equivalent to the natural pheromone (Table 1). Of all treatments, the chi-square test showed that S-2F-Z-11 was the least effective in eliciting responses from the males.

Field trapping tests showed that traps baited with S-2F-Z-11 at 0.1X dose were not different from a blank trap (Table 2). Traps baited with 2F-Z-11 and R-2F-Z-11 captured males with equal effectiveness at both doses but always less effectively than Z-11. These results differed from those of the flight tunnel where the activities of Z-11, 2F-Z-11 and R-2F-Z-11 were equivalent to one another. Contradictions between flight tunnel and field-test results are not uncommon for this insect (Schwarz et al. 1989). All treatments that caused male captures significantly greater than a blank trap were more effective at the 0.1X dose than at the 1X dose. Thus, although captures with 2F-Z-11 and R-2F-Z-11 at both doses were significantly different from those of a blank trap, the numbers at 1X were not statistically different from those of the S-2F-Z-11. The diminished difference between these treatments at the high dose is attributed overall decline of captures and to the amount of R-2F-Z-11 in S-2F-Z-11 [95:5 (S:R)]. At the high dose, the males were most likely responding to the 0.03 µmol R in the 1X dose of S-2F-Z-11. Residue analyses of stoppers that had been in traps for one week showed that evaporation rates of the fluorinated analogs and Z-11 were not significantly different.

Electrophysiological studies of antennal responses of males and females to the pheromone and its fluorinated analogs provided a basis for understanding of the activity of the optical isomers and the mechanism of action of the compounds in the mating disruption studies. In our first experiment, dose response curves constructed from EAGs of male moths to serial stimulus loads of the pheromone and its analogs were similar in shape for each odorant (Fig. 2A). After reaching threshold (about 1 ng), EAGs were significantly greater for pheromone, R-2F-Z-11, and Racemic 2F-Z-11, than for S-2F-Z-11 except for the highest stimulus load tested. EAGs elicited by the pheromone were equal to or greater than those elicited by the fluorinated compounds at all stimulus loads. Thus, greater activities of R-2F-Z-11 and the racemate relative to the S-enantiomer observed in the behavioral studies can be explained by the greater sensitivity of the pheromone receptor system to the R-enantiomer. The EAG data

Table 1. Male European cornborer (n = 63) responses in a flight tunnel to 1.2 μ mol racemate (2F-Z-11) and 0.6 μ mol each of R-2F-Z-11, S-2F-Z-11, Z-11 on rubber septa (None = no upwind flight in plume, Plume = up wind flight in plume but not getting within 3 cm of source, 3 cm = upwind in plume to 3 cm of source, land = land on source).

	Response percentages estimated with the logistic model.			
Compound	None	Plume, 3 cm or land	3 cm or land	land
2F-Z-11	25	75	71	66
R-2F-11	23	73	73	68
S-2F-Z-11	56	44	38	33
Z-11	19	81	77	73

Null Hypothesis	Wald χ^2	*P* value
2F-Z-11 = R-2F-Z-11	0.1	0.8
2F-Z-11 = S-2F-Z-11	11.1	0.0009*
2F-Z-11 = Z-11	0.6	0.5
R-2F-11 = S-2F-Z-11	12.7	0.0004*
R-2F-Z=11 = Z-11	0.2	0.6
S-2F-Z-11 = Z-11	15.8	0.0007*

*The null hypothesis is rejected as determined by Chi-square test.

complemented the behavioral test results in the sense that the S-enantiomer elicited the weakest electophysiological response.

The electrophysiological results suggest that the 2F-Z-11 may stimulate the same peripheral neurons within trichoid sensilla of male moths that respond to Z-11 (Roelofs et al. 1987). Alternatively, our results might be explained if 2F-Z-11 differentially stimulated other receptor neurons which are known to respond to *E*-11-tetradecenyl acetate (*E*-11) or Z-9-tetradecenyl acetate (Hansson et al. 1987, Kafka et al. 1989, Hallberg et al. 1994). However preliminary recordings from neurons within trichoid sensilla of European corn borer males show that both Z-11 and 2F-Z-11 stimulate a neuron with a large amplitude spike while E-11 stimulates a neuron with a small amplitude spike (Dickens, unpubl. data).

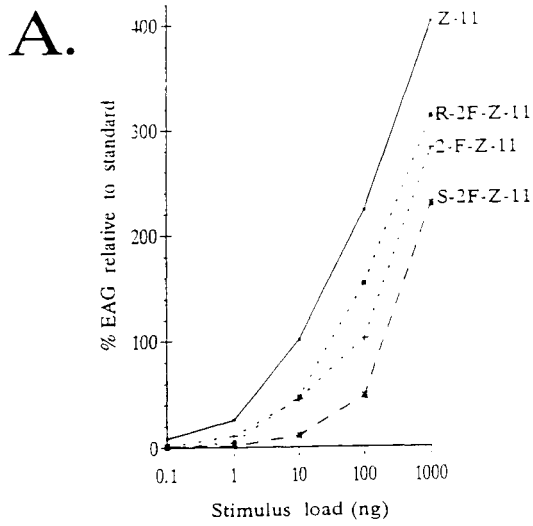
Table 2. European corn borer male trap captures in the field in response to natural pheromone (Z-11) racemate (2F) and R- and S- enantiomers of the phoermone analog at 0.1X and 1X doses.

Dose	Dose µ mol Compound	Total Male captures/trap
0.1X	0.12 2F-Z-11	52 b
	0.06 R-2F-Z-11	37 b
	" S-2F-Z-11	9 d
	" Z=11	112 a
1X	1.20 2F-Z-11	22 bc
	0.60 R-2F-Z-11	18 c
	" S-2F-Z-11	12 c
	" Z-11	53 b
Blank		7 d

Values followed by the same letter are not significantly different at the 5% level according to the least significant difference test ($LSD_{0.05}$ = 1.4) on the transformed data. The de-transformed values are listed in the table.

We understand that what was being compared in the EAG study was stimulus loading and not concentration of molecules reaching the antennal preparation and, ultimately, receptive olfactory dendrites. However, experiments using tritiated pheromone and racemic 2F-Z-11 showed the pheromone to be more volatile than the analog (Fig. 2B). The data show that the amount of 2F-Z-11 delivered per 1 sec pulse from the odorant cartridge was significantly less than the amount of Z-11 delivered at all doses. The comparatively lower rate of 2F-Z-11 delivery is probably the result of a slowed rate of evaporation caused by an interaction of the fluorine with the highly polar hydroxyl groups of the cellulose which makes up the filter paper. (Residue analyses of rubber stoppers used in trapping tests and glasswool used in mating disruption tests showed no significant evaporation differences). Accordingly, we can postulate that antennal receptors may be even more sensitive to the fluorinated analogs than is apparent from our dose-response curves.

In our second EAG experiment, neither the pheromone nor its fluorinated analogs at the one µg elicited EAG responses from female moths. This indicates that females of this species are quite insensitive to their own sex pheromone. Thus, it is likely that the effects of the pheromone and the fluorinated analogs observed in mating disruption tests were mediated by antennal



Compound/Load (ng)	*0.1*	*1*	*10*	*100*	*1000*
Z-11	8.6*a*	26.3*a*	99.6*a*	224.4*a*	397.3*a*
R-2F-Z-11	0.2*a*	4.7*bc*	47.5*b*	155.9*ab*	313.5*a*
2F-Z-11	1.2*a*	11.3*ab*	44.9*b*	102.2*b*	285.1*a*
S-2F-Z-11	1.5*a*	1.4*c*	11.3*c*	48.8*c*	229.8*a*

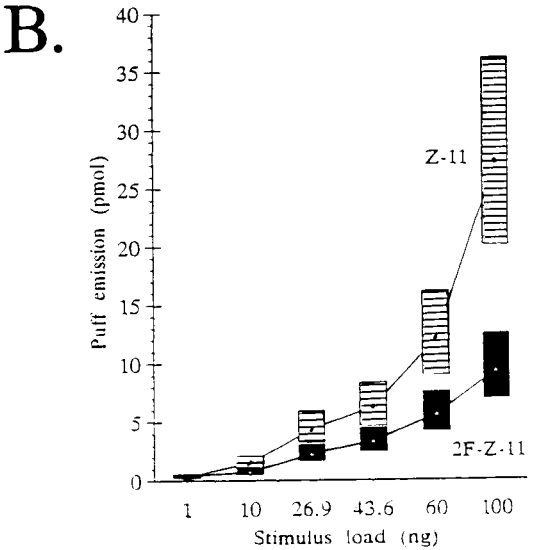


Fig. 2. (A.) EAG responses of European corn borer males to analogs and pheromone. Tabular means followed by the same letter are not significantly different at $P = 0.05$. (B.) Median amount of pheromone and analog released in air pulses from different loads of compound on filter paper (95% CI indicated).

Table 3. Mean (SE) percentage suppression of European corn borer spermatophore transfer in mating disruption assays.

	Compound			
μ mol. dose	Z-11	2F-Z-11	R-2F-Z-11	S-2F-Z-11
9.3	60 (9) a	67 (8) a	80 (11) a	14 (11) b
4.6	80 (13) a	65 (11) ab	91 (10) a	35 (7) c
2.3	77 (12) a	57 (12) a	79 (12) a	38 (12) c
0.93	15 (11) b	49 (11) a	35 (12) a	21 (12) b
0.46	32 (13) a	33 (11) a	39 (7) a	11 (11) b

Any two means, A and B, that are separated by more than $2(SE_A^2 + SE_B^2)^{1/2}$ (about 24 for most means) are significantly different at the 0.05 confidence level. Means followed by the same letter in a row are not significantly different from one another.

receptors on the male.

Results of the mating disruption tests show that Z-11, 2F-Z-11 and R-2F-Z-11 caused significant and similar levels of disruption at 9.3, 4.6, and 2.3 μmol doses (Table 3). 2F-Z-11 and R-2F-Z-11 continued to express disruptive activity at 0.93 μmol dose while Z-11 expressed no significant disruptive activity at that dose. Thus, the R-enantiomer and racemate expressed a slight mating-disruption advantage over the natural pheromone. Observation of a slight disruptive advantage in the laboratory is encouraging but this does not ensure a parallel benefit in a field setting. Only tests in the outdoors could validate such a comparison.

Residue analyses of the dispenser pads used in the mating disruption test showed that rates of evaporation of Z-11 and fluorinated analogs from the pads were not significantly different (data not reported). Therefore, the atmospheric concentrations of compounds in the incubators were comparable for the pheromone and for the analogs.

The data of Table 3 also show that S-2F-11 was essentially ineffective as a mating disruptant. A small effect was detected at 2.3 μmol, however, this effect probably was a spurious event because no significant disruptive effects were observed at any other dose.

The S-enantiomer lacked any significant biological activity by itself in any of our bioassays, and its presence in the racemate did not interfere with the activity of the R-enantiomer. This indicates that some steric or electronic peculiarity of the pheromone receptor system prevents fruitful interaction of it with the S-enantiomer. The differing biological activity of the R-2F-Z-11 and S-2F-Z-11 enantiomers confirms the earlier work of Chapman et al. (1978) that the Z type European corn borer pheromone receptor system is chiral, although the natural pheromone is not.

Moreover, test results show that only 2F-Z-11 and R-enantiomer of the fluorinated analog mimicked the natural pheromone in the behavioral and

electrophysiological assays and only these forms proved to be mating disruptants. The mechanism by which mating was disrupted probably involved loss of male sensitivity to female sex pheromone by central nervous system habituation or sensory adaptation (Cardé and Minks 1995). The diminished male sensory acuity ostensibly reduced expression of male precopulatory behavior toward the female and by that disrupted mating.

2F-Z-11 and R-2F-Z-11 showed a modest advantage over the pheromone in the mating-disruption assay, and it is plausible that this might be related to resistance of the fluorinated analog to β-oxidation or some other aspect of postchemoreceptive processing of the fluorinated compound in the insect. Evaluation of this possibility will require appropriate metabolic investigations.

Although our analogs did not possess extraordinary properties that would lead us to believe that they could be significantly more useful than natural pheromone as attractants or mating disruptants, it is plausible that fluorine substitution at other sites in the pheromone could yield analogs with more potent activity. Only continued pursuit of the problem with additional structure-activity relationship studies can reveal the validity of the notion that a useful class of behavioral-modifying chemical can be discovered through selective fluorination of pheromone.

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