ASSESSMENT OF SIX BENZYL-1,3-BENZODIOXOLE COMPOUNDS FOR ANTI-JUVENILE HORMONE ACTIVITY IN *CULEX PIPIENS*

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ABSTRACT. Fourth instar larvae of *Culex pipiens* were exposed to six benzyl-1,3-benzodioxole derivatives to assess the effectiveness of these compounds as anti-juvenile hormone agents. Mortality ranging from between 18 and 99% was observed in larvae and early pupae but the surviving adults showed no clearly defined anti-juvenile hormone effects. Adult effects included a reduction in number of eggs developed and the presence of degenerating eggs 4 days after the blood meal.

INTRODUCTION

Several benzylphenol and benzyl-1,3-benzo-dioxole derivatives synthesized by Leonard Jurd are effective insect sterilants (Van Mellaert et al. 1983). Some of these compounds also prevent adult emergence when dissolved in the rearing water of immature mosquitoes (Dame and Jurd 1983; Nelson et al. 1983a, 1983b, 1985) but have no deleterious effects on nontarget organisms such as bluegill sunfish, dytiscid larvae, clam shrimp, tadpole shrimp, and *Daphnia* spp. (Schaefer et al. 1984).

Van Mallaert et al. (1982) reported that certain benzylphenol and benzyl-1,3-benzodioxole analogues inhibit oogenesis in *Sarcophaga bullata* Parker by interfering with the uptake of vitellogenin by the ovarian follicles after the blood meal. They speculated that because juvenile hormone promotes patency (the formation of intercellular spaces between follicle cells allowing intake of vitellogenin) in several other species (Davey 1981) and yolk sequestration and vitellogenic growth in *Drosophila*, the inability of follicles to deposit yolk in *S. bullata* may be due to an anti-juvenile hormone effect produced by the compounds.

Van Mellaert et al. (1983) also reported that several of the Jurd compounds show a strong anti-juvenile hormone reaction when tested in the *Galleria* moth bioassay. Furthermore, they found that the most effective inhibitors of vitellogenesis in *S. bullata* elicit the strongest anti-juvenile hormone reaction in the *Galleria* bioassay.

In this study, we tested some of these compounds in an attempt to identify anti-juvenile hormones for use in mosquito control. As shown previously, both the initiation of biting behavior (Meola and Petralia 1980) and the development of previtellogenic stage follicles (Gwadz and Spielman 1973, Spielman 1974) are mediated by juvenile hormone in *Culex pipiens* Linn. There-

fore, compounds with anti-juvenile hormone activity would presumably be highly effective in preventing blood feeding and reproduction by blocking juvenile hormone release or its action in adult female mosquitoes.

MATERIALS AND METHODS

Five benzyl-1,3-benzodioxole derivatives selected by Jurd for their anti-juvenile hormone properties in the *Galleria* bioassay were tested. These compounds included: 5-methoxy-6-(1-[4-methoxyphenyl]-ethyl)-1,3-benzodioxole (J2710), 5-ethoxy-6-(4-methoxyphenyl)methyl-1,3-benzo-dioxole (J2581), 5-ethoxy-6-(1-[4-methoxyphenyl]ethyl)-1,3-benzodioxole (J2922), 5-[1-(4-methoxyphenyl) ethyl] - 6 - (2 - propenyloxy) - 1,3-benzodioxole (J3230), and 5-methoxy-6-[1-(4-methoxyphenyl)-1-methylethyl]-1,3-benzo-dioxole (J3770). The sixth compound, 6-[(4-methoxyphenyl) - 4 - morpholinylmethyl] - 1,3 - benzodioxole (J3386), was representative of a newer type of compound synthesized by Jurd.

Twenty-five fourth instar larvae of *Cx. pipiens* (USDA strain) were placed in a 190 ml styrofoam cup which contained 100 ml of water and the acetone-dissolved compound. One of the compounds was also tested in 0.473 liter glass canning jars and no significant difference was noted in percent mortality between mosquitoes reared in the two types of containers. During experiments, larvae were fed 60 mg of equal parts of Brewer's yeast, lactalbumin and finely ground laboratory animal chow. Eight replicates were made for each of several concentrations of the six compounds. Controls were reared in 0.1 ml or less of acetone per 100 ml of distilled water. All tests were conducted at 26°C, 75% RH, and 14:10 LD. Dead larvae and pupae were removed and counted daily. Pupae were rinsed 1 day after pupation and placed in fresh water because preliminary experiments showed that many adults (both experimental and control) died while emerging perhaps due to decomposing diet or to other detritus in the surface film.

Percentage mortalities for larval, pupal and adult stages were calculated. Adult mortality refers to death occurring during adult emergence. Percentage corrected mortality was de-

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termined by Abbott's formula (Abbott 1925), and the LC_{50}s for each compound were estimated by log-probit.

Newly emerged adults were maintained for 3 days with access to 10% sucrose solution and then examined by ovarian dissection for follicular development. Five typical follicles for each female were measured and the mean length was calculated. The percentage of females with mean follicular lengths ≥ 62 μm was determined from these measurements. In our colony, resting stage follicles usually range from 62 to 80 μm in length.

To assess the biting behavior, a small chick restrained in a stocking was left overnight with the mosquitoes and percentage biting was noted in the morning by counting the number of females that had fed. Bloodfed females were kept for 4 days in 22 x 22 x 30 cm cages with continuous access to a 10% sucrose solution and then examined for egg maturation.

For experiments involving injection of adult mosquitoes, the compounds were dissolved in olive oil and 0.5 μl of the mixture was injected into newly emerged females. Often it was necessary to dissolve the compounds first in a few drops of acetone. Compounds were injected with a hollow glass needle attached to a piece of rubber tubing fitted with a glass mouthpiece. The mosquito, anesthetized with nitrogen gas, was laid ventral side up on a glass slide and restrained with a plastic strap. Measured doses

of the compound were drawn into the needle and gently blown into the abdomen through one of the intersegmental membranes. Ovarian follicles of compound-injected females and olive oil-injected controls were measured 3 days after injection.

In other experiments, mosquitoes were injected either 1 hr or 4 days after emergence. Mosquitoes were blood fed on the night of the fifth day and follicular length was measured 4 days later. Mature eggs (length >500 μm) were counted and the number of eggs present in injected females versus olive oil-injected controls was determined.

Student's t-test was used to assess the significance of differences between two groups at the 99% confidence level.

RESULTS

Treatment of Fourth Instar Larvae. Table 1 shows the percentage mortality caused by each compound for each stage and the LC_{50}s occurring in *Cx. pipiens* treated as fourth instar larvae. Compound J2710 was the most active compound tested with an LC_{50} of 0.07 ppm followed by J2922, J3230, J2518, J3370 and J3386 in order of decreasing insect growth regulating (IGR) activity. Larval mortality was low for most compounds except at the highest concentrations tested. Most pupal mortality occurred during the early pupal stage before the cuticle

Table 1. Percent mortality and LC_{50}s of the test compounds on *Culex pipiens* treated as fourth instar larvae.

| Compound | Conc. (ppm) | Percent mortality | | | Corrected mortality (%) | LC_{50} (ppm) |
		Larval	Pupal	Adult		
J2710	0.00	10	14	7		
	0.03	15	19	4	13	
	0.07	17	51	2	55	0.07
	0.1	19	57	5	74	
J2922	0.00	4	17	3		
	0.07	6	20	7	12	
	0.1	35	35	4	66	0.09
	0.3	80	19	0	99	
J3230	0.00	27	7	1		
	0.1	13	21	6	8	
	0.2	3	71	2	64	0.17
	0.3	74	23	0	99	
J2518	0.00	4	17	3		
	0.3	4	18	7	7	
	0.5	9	30	2	22	0.58
	0.7	59	31	2	89	
J3370	0.00	12	16	6		
	0.3	13	18	11	12	
	0.7	10	28	10	21	0.88
	1.0	68	8	3	68	
J3386	0.00	20	6	0		
	1.0	6	12	8	0	
	5.0	22	10	0	8	7.75
	8.0	37	26	3	54	

had melanized. Adult mortality was low for all compounds tested.

Primary follicles were measured 3 days after emergence and the percentage reaching the resting stage of development was noted (Table 2). At the concentrations tested, none of the compounds prevented growth of the ovarian follicles to the resting stage. Biting behavior was tested at each concentration for each compound. However, because there was no difference in the percent biting when mosquitoes were reared in the higher vs the lower concentrations of the compounds, percent biting in Table 2 is given for only one concentration (usually 0.1 ppm).

Some adults emerging from larval treatments were fed blood and examined 4 days later for egg development. Eggs were scored as mature, undeveloped or degenerating (Table 3). Some mosquitoes contained only undeveloped (preresting stage) follicles. Other mosquitoes contained eggs, but the eggs were misshapen and degenerating. Frequently, degenerating eggs were mixed with previtellogenic follicles, indicating that the remaining eggs had been reabsorbed. Follicles that appeared abnormal in any of the above respects were scored as undeveloped or degenerating. One-third of the females treated with J2710 and J3370 showed abnormal follicle development; in contrast, 100% of the untreated

and 87% of the acetone controls had mature eggs.

Treatment of Adults. Test compounds were injected into newly emerged females to test their effect on development of resting stage follicles. Injected doses of 2.0 µg per female of compounds J2710, J3230, J2518 and J3386 did not prevent previtellogenic growth in 91 to 100% of the females injected. Compounds J2922 and J3370 suppressed follicular development in more than 50% of the females injected but the mosquitoes were moribund after treatment even though they remained alive 2 or 3 days. These results suggest that J2922 and J3370 were toxic at the concentrations used and that the reduced number of resting stage follicles was probably due to adverse metabolic effects rather than anti-juvenile hormone activity.

The compound J2710 injected into adult mosquitoes shortly after emergence did not affect the induction of biting behavior; i.e., both the olive oil-injected controls and the chemical-injected mosquitoes were similar in biting activity with 50% and 45% biting, respectively. Both groups of injected mosquitoes, however, showed a significant decrease in biting activity compared to the uninjected group where 79% blood fed. This effect may have related to stress associated with the injection rather than to the chemical itself.

Mosquitoes injected with J2710 either 1 hr or 4 days after emergence were examined 4 days after they were fed on blood. Sixty-six percent of the mosquitoes injected 1 hr after emergence had mature eggs. In the remaining 34%, undeveloped follicles were found in 7% and degenerating eggs in 27% of the mosquitoes. Significantly more (96%) of the olive oil-injected controls had mature eggs and in only 4% were the eggs degenerating. Degenerating eggs were noted in 56% of the mosquitoes injected 4 days after emergence. Several of these mosquitoes also contained secondary follicles approximately 125 µm long attached to degenerating eggs. In con-

Table 2. Development of resting stage follicles and biting behavior in adult *Culex pipiens* treated as fourth instar larvae with test compounds.

Compound	Conc. (ppm)	Adults with resting stage follicles (%)	Biting (%)
J2710	0.1	96	92
J2922	0.1	100	88
J3230	0.1	84	90
J2518	0.1	98	86
J3770	1.0	97	97
J3386	0.1	98	98
Acetone	100	97	96

Table 3. Condition of the ovaries 4 days after compound-reared *Culex pipiens* were fed blood.

Compound	Conc. (ppm)	Females (%) with follicles		
		Mature*	Undeveloped	Degenerating
J2710	0.1	65	5	30
J2922	0.1	92	8	—
J3230	0.1	89	3	8
J2581	0.3	96	—	4
J3370	1.0	63	14	23
J3386	8.0	95	—	5
Acetone	100	87	4	9
Untreated		100	—	—

* Differences between the experimental and untreated groups in the percentage of females with mature eggs considered significant (≥0.01) only in tests involving J2710 and J3370.

trast, mature eggs were found in 89% and degenerating eggs in only 11% of the olive-oil injected controls.

Although the ovaries often appeared normal in compound-injected mosquitoes, some ovaries appeared to contain fewer eggs than the ovaries of oil-injected controls. A count of the mature eggs in a sample of these mosquitoes revealed that the mean number of mature eggs/ovary in a J2710-injected group was 62 (n = 25) whereas the ovaries of olive oil controls contained 130 eggs (n = 9). This significant difference in number of eggs per ovary was probably due to degeneration of eggs in the compound-injected mosquitoes, since the mosquitoes in both groups appeared to ingest equivalent-sized blood meals.

DISCUSSION

All six compounds tested caused some mortality in *Cx. pipiens* larvae or pupae. Much of the mortality occurred in the early pupal stage before melanization of the cuticle. Mortality of early pupae was also reported by Dame and Jurd (1983), Nelson et al. (1983a, 1983b, 1985) and Schaefer et al. (1984) for other species of mosquitoes which were reared in these or related compounds. Jakob and Schoof (1972), working with another benzylphenol compound, MON-0585, determined that 0.1 ppm produced 95% mortality to *Culex quinquefasciatus* Say exposed as third instar larvae. We obtained slightly higher LC_{95} values with fourth instar larvae of *Cx. pipiens* reared in J2710 (0.19 ppm), J2922 (0.22 ppm) and J3230 (0.27 ppm). Dame and Jurd (1983), assessed 149 benzyl and cinnamyl substituted phenol and 2,4-benzodioxole derivatives against late third-early fourth instar larvae of *Anopheles quadrimaculatus* Say, and found that 19 of the most active compounds had $LC_{90}s$ of 0.02–0.099 ppm.

Schaefer and Wilder (1972) determined that MON-0585 was more effective when applied to early rather than late fourth instar larvae. We also observed a higher rate of mortality using early vs late fourth instar larvae when we reared *Cx. pipiens* in J2922. Early fourth instar larvae placed in 0.1 ppm of J2922 showed a significantly higher mortality (88%) than larvae placed with the compound 1 day later (61%). Most of the mortality in the first group occurred during the larval stage (64%), whereas in the second group most of the mortality (50%) occurred during the early pupal stage. We did not determine whether the early fourth instar stage was more sensitive to the compound as suggested by Schaefer et al. (1984) for MON-0585 or whether the higher larval mortality occurred because of longer exposure to J2922.

Nelson et al. (1983a, 1983b, 1985) showed that no significant larval mortality occurred in fourth instar larvae of *Cx. quinquefasciatus* and *Aedes aegypti* (Linn.) treated with J2532, J2645, J2706 and J2931. Likewise, Jakob and Schoof (1972) reported that MON-0585 produced no larval mortality in compound-treated *Ae. aegypti, Cx. quinquefasciatus, Culex tarsalis* Coquillett, *Aedes taeniorhynchus* (Wiedemann), *Anopheles albimanus* Wiedemann, and *Anopheles stephensi* Liston. In contrast, we found considerable larval mortality in *Cx. pipiens* at the highest concentration of most of the compounds tested.

Our results did not show an abbreviated larval period or premature metamorphosis which might be anticipated for anti-juvenile hormone agents. The larvae in the compound-treated containers took as long as the controls to complete their development. Staal (1986) also noted "... no evidence of AJH effects on larvae of any species" caused by benzylphenols and benzyl-1,3-benzodioxoles. Moreover, we found that the development of resting stage follicles and biting behavior did not appear to be affected by rearing fourth instar larvae in the test compounds.

Our rationale in injecting mosquitoes either immediately after emergence or 4 days later was to test the compounds in 2 different physiological milieu. The first group was injected before postemergent release of juvenile hormone had initiated biting behavior or development of pre-resting stage follicles. Thus, compounds were injected at emergence to determine whether they would block the synthesis or release of juvenile hormone or its action at receptor sites in the mosquito. The second group, injected 4 days after emergence, had already completed growth of previtellogenic follicles to the resting stage and no longer required active corpora allata for egg development. The compound J2710 was injected into these mosquitoes to assess its effect on vitellogenic growth of primary follicles after the blood meal.

Test compounds injected into newly emerged mosquitoes evidently did not suppress corpus allatum function; i.e., most of the injected mosquitoes initiated biting behavior and developed resting stage follicles. Meola and Readio (unpublished data) found that corpora allata of *Cx. pipiens* must remain *in situ* at least 2 days after emergence before significant numbers (>25%) of the females will develop biting behavior and the ability to mature eggs. Therefore, none of the test compounds appeared to suppress corpus allatum activity during this 2-day period.

Van Mellaert el al. (1982, 1983) postulated that benzyl-1,3-benzodioxoles such as J2710 and J2922, which showed strong anti-juvenile hormone effects in their *Galleria* assay, either compete for or inactivate juvenile hormone receptors or prevent binding of juvenile hormone to the

receptor sites. We detected no evidence for such effects when we injected these compounds into newly emerged mosquitoes. Thus, receptor activity in *Cx. pipiens* was apparently unaffected by the compounds tested in this study.

Injection of J2710 into 4-day-old females did not affect egg maturation. Nearly one-half of the females (44%) developed normal eggs after feeding on blood. The remaining 56% apparently developed eggs but the eggs degenerated either during or after maturation. Thus, in *Cx. pipiens*, J2710 did not affect patency or vitellogenin uptake which are juvenile hormone dependent events in most insects (Davey 1981, Van Mellaert et al. 1982). However, the fact that eggs often degenerated after maturation was clearly an abnormal effect caused by the test compound. In fact, this was the only significant effect observed from injecting the test compounds into adult mosquitoes.

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