often presents problems such as nonuniform uptake of the test compound by the flies.

The most effective reproduction inhibitors injected into female flies were the two pyridine analogues **11** and **12** (71 and 75% inhibition of hatch, respectively, at 10 μg/fly). Less effective but still possessing appreciable sterilizing activity were the thiazole analogue **5**, the pyrimidine **18**, and the benzoxazole **22**. None of the compounds were tested against males since diflubenzuron had no effect on males by the injection method.

In house fly feeding tests, only **11** and **25** inhibited reproduction: i.e., both completely inhibited pupal formation at dietary concentrations of 0.5 and 1%, respectively. Treated males were unaffected by either compound at similar dietary concentrations. Despite the sterilizing effectiveness of the aforementioned compounds in flies, none was as effective as diflubenzuron which completely inhibited hatch when administered by injection (10 μg/fly) or orally (0.1%).

None of the compounds, including diflubenzuron, induced sterility in fall armyworms by our test procedure. Perhaps sublethal concentrations of compounds in the larval medium were too low to induce sterility in the surviving adults.

In summary, we have identified some new heterocyclic analogues of diflubenzuron that markedly inhibit the growth of immature stages of the fall armyworm and house fly. Several of the compounds also inhibit reproduction of the house fly. Of those compounds tested, the 2-pyridyl derivatives were the most effective. From our results we speculate that further synthesis of analogues bearing the pyridine or thiazole systems could lead to compounds of even greater activity.

Supplementary Material Available: A listing of analytical data and recrystallization solvents for the heterocyclic analogues (2 pages). Ordering information is given on any current masthead page.

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Acute Toxicity and Developmental Effects of Analogues of Ethyl α-(4-Chlorophenoxy)-α-methylpropionate on Two Insects, *Oncopeltus fasciatus* and *Tenebrio molitor*

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Forty analogues of the hypocholesterolemic agent clofibrate were synthesized and bioassayed on the yellow mealworm, *Tenebrio molitor*, and the large milkweed bug, *Oncopeltus fasciatus*. The compounds demonstrated only weak morphogenic activity in *T. molitor* but moderate acute toxicity in *O. fasciatus*. The acute symptoms of clofibrate analogues in *O. fasciatus* were similar to those induced by the antijuvenile hormone precocene. Clofibrate analogues resulted in delayed or blocked development of *O. fasciatus* nymphs, yet failed to produce clear antijuvenile hormone activity.

Insect juvenile hormone (JH) mimics (juvenoids) hold promise as insect control agents which disrupt insect development or reproduction by inundating the insect's system with exogenous hormone or providing hormone activity at stages during development when the JH should be absent. The recent reports on anti-JH's (Bowers, 1976; Bowers et al., 1976) have stimulated interest that pest control agents may be developed which inhibit JH production. Such compounds may be found by attempting to inhibit key reactions in the JH biosynthetic pathway in vitro (Mumby et al., 1976), random screening with an appropriate in vivo bioassay (Bowers, 1976), or directed screening with an in vivo bioassay in an attempt to inhibit reactions in the JH biosynthetic pathway based on analogy with other organisms.

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Chronologically, the insect endocrine system appears to have evolved late enough that there are large differences in the basic endocrine biology between insects and other organisms. For instance, terpenes have not been shown to have a regulatory function in any organism other than insects, and homoterpenes (as JH I or II) have not been reported from any other plant or animal (Schooley et al., 1973). A survey of compounds which might inhibit the synthesis of the JH terpenoid backbone thus seemed warranted.

Of the large number of antilipemic agents in man, the hypocholesterolemic agent clofibrate [ethyl α-(4-chlorophenoxy)-α-methylpropionate, ECPIB, Chlorpenisate, ICI-28257, CPIB, Atromid-S, Regelan] appeared promising. Many potential modes of action have been suggested for clofibrate, including displacement of thyroxine and fatty acids from albumin, inhibition or stimulation of fatty acid or triglyceride metabolism, changes in lipid distribution, disruption of mitochondrial function, and a host of other actions (Bencze et al., 1969; Bach, 1970; Levinson and Levinson, 1973; Cederbaum et al., 1976). It is widely accepted that clofibrate, at least in vitro, inhibits steroid biosynthesis prior to squalene formation (Azarnoff et al., 1965; Witiak et al., 1969; Suzuki, 1976). Although insects do not synthesize steroids, it was hoped that clofibrate or its analogues might act to block terpene biosynthesis leading to JH analogous to the blockage of squalene synthesis in mammals. Newman et al. (1973) and Witiak et al. (1976) report that small changes in clofibrate-like molecules resulted in major changes in their relative hypocholesterolemic and hypotriglyceridemic activities, so 40 clofibrate analogues were synthesized and assayed for acute and delayed effects on two insect species.

MATERIALS AND METHODS

Chemicals. Analogues of the hypocholesterolemic drug α-(4-chlorophenoxy)-α-methylpropionate (clofibrate) were synthesized by refluxing the appropriate phenol (20 mmol) or thiophenol with ethyl 2-bromoisobutyrate, ethyl 2-bromobutyrate, ethyl 2-bromopropionate, or ethyl 2-bromoacetate (20 mmol) in dry dimethoxyethane with potassium carbonate (60 mmol) (48 h, N_2) (Bach, 1970; Witiak et al., 1968). Yields averaged 80% after workup. The *p*-nitrophenyl compound (Table I, **24**) was found to give better yields when sodium bicarbonate rather than potassium carbonate was used as the base (50% vs. 7%, respectively).

Analogues with α-ethyl or α-propyl groups were prepared by refluxing the appropriate ketone in chloroform with *p*-chlorophenol and sodium hydroxide (Julia et al., 1956; Melandri et al., 1963). Following workup by solvent partitioning, chromatography, and recrystallization, the acid was converted to its ethyl ester by reaction with diazoethane. Esters were interconverted by refluxing the ethyl ester with the appropriate alcohol and potassium cyanide (Mori et al., 1975) for 24 h in the case of primary alcohols and 72 h in the case of secondary alcohols.

All compounds showed a single spot on thin-layer chromatography and an appropriate infrared spectrum. Before bioassay, all compounds were judged to be >97% pure by the integration of their ^1H NMR spectrum. Most of the compounds reported in this study have been prepared earlier for evaluation as herbicides or pharmaceuticals (see review by Bencze et al., 1969).

An isomer mixture of JH I was provided by A. J. Manson (Ayerst Research Laboratories), while precocene II was a gift of William Bowers (New York State Agricultural Experiment Station). Phenylmethylsulfonyl fluoride was purchased from Calbiochem, and DDT was synthesized by an established pathway.

Bioassays. Morphogenetic activity was determined by assaying the compounds on *Tenebrio molitor* as described by Hammock et al., 1974. Last instar larvae of *T. molitor* were obtained from California Worm Warehouse (Riverside, Calif.). The juvenoid, 1-(4′-ethylphenoxy)-3,7-dimethyl-6,7-epoxy-*trans*-2-octene, has an EC_{50} of 5 ng/pupa under the conditions of this assay.

The bioassay for acute toxicity and developmental effects used was essentially that of Bowers (1976) with the large milkweed bug, *Oncopeltus fasciatus* (Dallas), reared in cages similar to those described by Gordon (1974) (18:6 L:D, 27 °C). The compound to be tested was added in acetone (4 mL) to Whatman No. 1 filter paper (9 cm) in a petri dish (100 × 15 mm). After the acetone evaporated, a cotton-plugged water tube (9 × 75 mm) and sunflower seeds were added along with 15–20 second instar *O. fasciatus* which can survive to adulthood without further care in this chamber. Mortality was recorded at 48 h and the LD_{50} calculated from an eye fitted probit (Finney, 1952) after corrections were made for control mortality (<5%). The 48 h LD_{50}'s are expressed as nmol/cm^2 of filter paper (Table I). The *O. fasciatus* were reared to adulthood and observations made on mortality, speed of development, JH-like effects (Jacobson et al., 1972), or precocious development.

Fatty Acid Analysis. Third instar *O. fasciatus* (~5 mg/insect) were held for 48 h on filter paper treated with 29 or 83 nmol/cm^2 of clofibrate or 22 or 63 nmol/cm^2 precocene. The insects were weighed and total lipid extracted with chloroform–methanol (1:2 v/v). After saponification with KOH in ethanol, the lipid was esterified with boron trifluoride–methanol and analyzed on a 15% diethylene glycol succinate column with appropriate standards (Bligh and Dyer, 1959; Morrison and Smith, 1964). The lipid content and fatty acid composition of the four treated populations were compared with control insects and extracts of the sunflower seed diet (Table II).

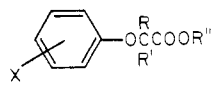
RESULTS

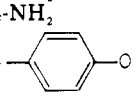
None of the compounds in Table I resulted in the formation of >50% pupal adult intermediates when applied to 0–24-h-old *T. molitor* pupa at 200 μg/pupa, thus all ED_{50}'s are >200 μg/pupa using the bioassay described by Hammock et al. (1974). Only at very high doses did clofibrate (**1**), and some of its analogues which were very active on *O. fasciatus*, cause the morphogenetic effects often observed with juvenoids (Jacobson et al., 1972).

The relative 48-h toxicity of the clofibrate analogues to *O. fasciatus* are shown in Table I. Esters of unbranched alcohols (**1, 2, 3, 5**) are more active than branched chain esters (**4, 6, 7**). All primary alcohol esters except isobutanol have similar activity with the activity increasing from butyl to ethyl and the methyl ester (**1, 13, 15**) showing again a lower activity. The free acid of clofibrate (not in Table I) is much less active at 48 h (>2000 nmol/cm^2), but the mortality increases with longer exposure times (LD_{50} ~120 nmol/cm^2 at 10 days). Assuming that the compounds act as their free acids in insects as in mammalian systems (Bach, 1970), the biological activity correlates roughly with the predicted ease of ester hydrolysis, except for the methyl ester which may experience a reduced penetration rate due to its polarity and the free acid which undoubtedly penetrates very slowly. Esters of branched-chain alcohols should be more stable to esterases and, as expected, demonstrate lower biological activity. The thioethers (**39, 40**) are less active than their corresponding ethers (**2, 17**).

It was hoped that increased length of an α-carbon branch might increase the resemblance of the compound

Table I. Toxicity of Substituted Phenoxyacetic Acid Esters to the Milkweed Bug



No.	X	R	R′	R″	48 h LD_{50}, nmol/cm²
			Ethers		
1	4-Cl	CH_3	CH_3	CH_3	48
2	4-Cl	CH_3	CH_3	C_2H_5	40
3	4-Cl	CH_3	CH_3	n-C_3H_7	47
4	4-Cl	CH_3	CH_3	i-C_3H_7	78
5	4-Cl	CH_3	CH_3	n-C_4H_9	56
6	4-Cl	CH_3	CH_3	s-C_4H_9	130
7	4-Cl	CH_3	CH_3	i-C_4H_9	120
8	4-Cl	H	H	C_2H_5	110
9	4-Cl	H	CH_3	C_2H_5	88
10	4-Cl	H	C_2H_5	C_2H_5	62
11	4-Cl	H	i-C_3H_7	C_2H_5	140
12	4-Cl	CH_3	C_2H_5	C_2H_5	130
13	4-Cl	CH_3	C_2H_5	CH_3	160
14	4-Cl	CH_3	n-C_3H_7	C_2H_5	120
15	4-Cl	CH_3	n-C_3H_7	CH_3	160
16	H	CH_3	CH_3	C_2H_5	240
17	4-F	CH_3	CH_3	C_2H_5	160
18	4-Br	CH_3	CH_3	C_2H_5	90
19	4-I	CH_3	CH_3	C_2H_5	>1000
20	4-CH_3	CH_3	CH_3	C_2H_5	460
21	4-i-C_3H_7	CH_3	CH_3	C_2H_5	420
22	4-CH_3CO	CH_3	CH_3	C_2H_5	720
23	4-CH_3O	CH_3	CH_3	C_2H_5	430
24	4-NO_2	CH_3	CH_3	C_2H_5	390
25	4-NH_2	CH_3	CH_3	C_2H_5	>1800
26		CH_3	CH_3	C_2H_5	1120
27	3-CH_3	CH_3	CH_3	C_2H_5	320
28	2-Cl	CH_3	CH_3	C_2H_5	110
29	3-Cl	CH_3	CH_3	C_2H_5	130
30	2,4-Cl	CH_3	CH_3	C_2H_5	46
31	3,4-Cl	CH_3	CH_3	C_2H_5	61
32	3,5-Cl	CH_3	CH_3	C_2H_5	34
33	2,6-Cl	CH_3	CH_3	C_2H_5	310
34	2,4,5-Cl	CH_3	CH_3	C_2H_5	48
35	3,4,5-Cl	CH_3	CH_3	C_2H_5	430
36	3,4-OCH_2O	CH_3	CH_3	C_2H_5	460
37	α-Naphthyl	CH_3	CH_3	C_2H_5	160
38	β-Naphthyl	CH_3	CH_3	C_2H_5	270
			Thioethers		
39	4-F	CH_3	CH_3	C_2H_5	~1240
40	4-Cl	CH_3	CH_3	C_2H_5	~230
			Standards		
DDT					>1400 (48 h) 14 (96 h)
Precocene II					130 (12 for 50% precocious development)
Phenylmethylsulfonyl fluoride					75

to homomevalonate (a precursor to homoterpenes); however, the compounds with dimethyl substituents at the α carbon proved to be the most active. Loss of one or both α branches caused a decrease in activity, and increasing the length of one branch likewise decreased activity (**8–15**). Polycyclic compounds generally had poor activity (**26**, **36–38**) with only the α-naphthyl (**37**) derivative showing moderate activity.

For monosubstituted compounds, the *p*-methyl and chloro ring substituents were more active than their corresponding meta or ortho analogues. For polychlorinated systems, a p-chlorine usually appeared necessary for good activity (**30**, **31**, **34**) except for the 3,5-disubstituted compound (**32**) which was the most active tested. The 3,4,5-trichloro derivative (**35**) was, however, essentially inactive.

Multiple regression analysis to search for quantitative structure–activity relationships indicated that steric parameters such as molecular refractivity, molecular volume, and E_S were poor indicators of biological activity for para-substituted clofibrate analogues. Assuming a parabolic relationship for the data, both σ and π gave a good correlation (significant at the 5% level) with the log LD_{50}. Both values indicated that the most active compound should be the one with σ or π values of *p*-chloro derivative, although its activity was greater than could be predicted

Table II. Fatty Acid Composition of Milkweed Bugs Treated with Clofibrate or Precocene II

Fatty acid	Control, %	Precocene Dose, % 22 nmol/cm²	Precocene Dose, % 63 nmol/cm²	Clofibrate Dose, % 29 nmol/cm²	Clofibrate Dose, % 83 nmol/cm²	Sunflower seeds
Percentage Total Fatty Acid (14:0-18:2)						
14:0	0.2	0.3	0.3	0.3	0.3	0.2
16:0	6.2	6.8	6.8	6.8	6.9	6.6
16:1	0.2	0.2	0.2	0.4	0.4	
18:0	5.8	6.0	5.8	5.8	5.9	4.8
18:1	14.6	15.1	14.9	15.1	15.1	11.6
18:2	73.0	71.6	72.0	71.6	71.4	76.8
Percentage Total Fatty Acid (14:0-18:1)						
14:0	0.7	1.1	1.1	1.1	1.1	0.7
16:0	23.0	23.9	24.3	25.9	24.1	28.5
16:1	0.7	0.7	0.7	1.4	1.4	
18:0	21.5	21.1	20.7	20.4	20.6	20.7
18:1	54.1	53.2	53.2	53.2	52.8	50.1

from any empirical equations. Including both σ and π terms in the same equation did not improve the correlation with biological activity, while an E_S term slightly improves the correlation of π but not σ. Molecular volumes were obtained from Exner (1967) and all other terms were obtained from compilations by Hansch et al. (1973) and Unger and Hansch (1976). The polarity term π was taken from tables of phenoxyacetic acid model compounds when possible. E_S constants were related to hydrogen rather than methyl as zero as suggested by Fahmy et al. (1973).

The symptoms of *O. fasciatus* exposed to the clofibrate analogues were very distinct from symptoms of DDT, parathion, or carbaryl poisoning. Very little mortality occurred before 24 h. After this time, *O. fasciatus* treated with high levels of precocene II (Bowers, 1976) or the clofibrate analogues began to move slowly with a large increase in mortality before 48 h. With continuous exposure through development, there was much higher mortality to *O. fasciatus* on treated filter paper than on acetone treated control paper (<5% mortality at 48 h, <10% mortality during total development). Both precocene and the clofibrate analogues caused an increase in developmental time. Half of the control insects became adults in 22 ± 5 days, while at a dose of 20 nmol/cm² over 30 days were required for half of the surviving insects exposed to clofibrate to become adults and many of the insects treated with either clofibrate or precocene remained as second or third instar larvae after all control insects had become adults. Similar delays in development due to clofibrate were observed by Levinson and Levinson (1973) for the hide beetle, *Dermestes maculatus*. Clofibrate also caused an increase in the appearance of red rather than black appendages on *O. fasciatus* and difficulty in molting. Delayed mortality and sublethal effects of phenoxyacetic acid esters were dose dependent and directly related to the acute (48 h) toxicity of all of the compounds tested.

JH I at 360, 36, and 3.6 pmol/cm² failed to reduce the 48-h mortality caused by clofibrate or high doses of precocene II. JH at 360 pmol/cm² caused no mortality for 3 weeks, but it totally prevented formation of adults. Up to 40 nmol/cm² of clofibrate failed to increase the percentage of normal adults formed when exposed to the above doses of JH. Higher doses of clofibrate slightly increased the percentage of apparently normal adults after exposure to JH during development, but the high mortality due to clofibrate alone (>90%) makes the results questionable.

When milkweed bugs were exposed to filter paper treated with two doses of clofibrate or precocene II, then analyzed qualitatively and quantitatively for their lipids, no distinct differences were found between the four groups of treated and untreated controls (Table II). Extraction of whole sunflower seeds (38% of the wet weight is lipophilic material) for 72 h in a Sohxlet with acetone, followed by drying under high vacuum, resulted in a 69% reduction in the lipid content, and the insects were still able to develop normally when these extracted seeds were their sole source of food. Alternating extraction with a chloroform–methanol azeotrope and acetone (48 h each, four cycles) resulted in an 84% reduction in the lipid content, but early instars were unable to survive on this diet. Attempts to reconstitute the sunflower seeds by dissolving extracted lipids, β-sistosterol, cholesterol, or combinations of the above components in chloroform–methanol (2:1) and soaking extracted seeds in the resulting solution while the solvent was slowly allowed to evaporate over a 2-week period failed to provide adequate diets for survival.

DISCUSSION

These compounds have negligible morphogenetic activity on *T. molitor* pupae. This study does not suggest a likely mode of action for clofibrate analogues on *O. fasciatus*, and, even in mammals, there are many potential modes of action suggested for clofibrate and its analogues. The large amount of fat in the diet and lipid reserves of *O. fasciatus* possibly overshadow changes in fatty acid composition induced by clofibrate or precocene (Table II) as might be predicted from the work of Levinson and Levinson (1973) on the lipid depressant action of clofibrate. Our inability to rear *O. fasciatus* on a lipid depleted diet thus complicates testing of a lipid depressant hypothesis. However, the studies of Levinson and Levinson (1973) on *D. maculatus* also predict that a diet high in fatty acids (such as sunflower seeds) should reduce the toxicity of clofibrate which remains surprisingly toxic in this study when compared to DDT (Table I). Disruption of fatty acid metabolism cannot be ruled out based on the preliminary studies reported here. The failure of JH to reduce either the 48-h or delayed toxicity of clofibrate to *O. fasciatus* fails to support the possibility that the toxicity is due to the disruption of hormone metabolism, but acute effects of precocene in *O. fasciatus* and lygus bugs also are not blocked by JH, and this phenomenon has been observed in other insects (Staal, 1977). The inability of many treated insects to develop past the second or third instar suggests the possibility of direct or indirect endocrine involvement.

Since steroids resulting from biosynthesis normally account for much more of the total body burden of cho-

lesterol than dietary steroids in mammals, there has been intense interest in blocking cholesterol biosynthesis as a treatment for atherosclerosis, and many compounds have been assayed as mammalian hypolipidemic and hypocholesterolemic agents. These series of compounds may hold promise as insecticides which block terpene and therefore JH, ubiquinone, or pheromone biosynthesis in insects or which act by another mechanism possibly illustrated by the acute and chronic effects of the clofibrate analogues or precocene.

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Effect of Trypsin Inhibitors on Growth and Metamorphosis of Corn Borer Larvae *Ostrinia nubilalis* (Hübner)

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To determine if the naturally occurring proteolytic enzyme inhibitors of plants are related to resistance of food plants to insects, two purified inhibitors were added to the diets of young borer larvae, *Ostrinia nubilalis* (Hübner). Soybean trypsin inhibitor (Kunitz), incorporated at levels of 2–5% in the diet, inhibited growth of the larvae and delayed pupation, but did not prevent completion of the life cycle. The trypsin inhibitors of corn (maize) were prepared by a modified method and characterized with respect to amino acid composition and interaction with trypsin. The inhibitors of corn, which are weak inhibitors of trypsin, had no effect on growth or metamorphosis of the larvae. It is suggested that the proteolytic enzyme inhibitors of legumes, which are strong stoichiometric trypsin inhibitors, may be related to the resistance of these plants to the corn borer.

The proteolytic enzyme inhibitors of plants may be a mechanism of protection of plants against insect infestation. To study this possibility, we have chosen the European corn borer, *Ostrinia nubilalis* (Hübner), an insect that does extensive damage to corn (maize) and other vegetable crops, and two purified plant inhibitors, one from soybeans and one from corn. Soybean plants suffer little damage by the corn borer, whereas corn plants are this insect's principal host.

Previous investigations on soybean inhibitors with other insects have already provided support for this hypothesis.

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