DEVELOPMENT OF PYRETHROID ANALOGS FOR DIAGNOSIS OF RESISTANCE MECHANISMS IN THETOBACCO BUDWORM, *HELIOTHIS VIRESCENS* (F.)

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Abstract

The alcohol moiety of conventional pyrethroids is a major site of oxidative metabolism in resistant tobacco budworms, *Heliothis virescens* (F.). In this study, the phenoxybenzyl moiety found in conventional pyrethroids was replaced with known P450 monooxygenase inhibiting or oxidatively blocked groups. Isomers (*1R/1S, cis/trans*) were tested as insecticides or synergists against insecticide-susceptible tobacco budworms, or insects that expressed metabolic resistance to cypermethrin. A number of compounds with pentafluorophenyl (PFP), methylenedioxyphenyl (MDP), and propargyloxyphenyl (PP) groups had insecticidal activity that was dependent on both geometric and stereochemical configuration of the permethric acid moiety. Both *trans* and *cis* isomers of *1R*-fenfluthrin, which contains a pentafluorophenyl group, suppressed insecticide resistance in Pyr-R insects suggesting that oxidative metabolism of the phenoxybenzyl alcohol is the major mechanism of resistance in this strain. In contrast, the *1S, trans*- isomer of fenfluthrin was inactive. Of the methylenedioxyphenyl compounds, *1R,trans* and *cis* isomers were toxic and partially suppressed resistance in Pyr-R larvae. Again, the *1S,trans* isomer of this compound was not active either as a toxin or synergist of cypermethrin toxicity. Similarly, *αS,1R, trans* and *cis* isomers of propargyloxyphenyl compounds were insecticidal but *αR,1R,*analogs were inactive. Finally, of the nontoxic isomers, *αR,1R,cis* isomers of methylenedioxyphenyl- and propargyloxyphenyl- containing compounds significantly enhanced toxicity of cypermethrin.

Introduction

The tobacco budworm, *Heliothis virescens* (F.) was first recognized as a pest of cotton in 1934 (Folsom, 1936) and has become one of the most important insects attacking cotton in the United States (Sparks, 1981). This pest, combined with the cotton bollworm, *Helicoverpa zea* (Boddie), has caused almost one third of all insect damage to U. S. cotton during the 1990s (Head, 1992; 1993; Williams, 1994; 1995; 1996). Insecticide resistance is a major factor responsible for our inability to manage populations of *H. virescens* on cotton (Sparks, 1981; Sparks et al., 1993).

Insecticide resistance is due to the expression of one or more of three major mechanisms: reduced cuticular penetration, enhanced metabolic detoxication and reduced target site sensitivity (Oppenoorth, 1985). Monitoring insecticide susceptibility in the tobacco budworm and identifying actual or potential resistance mechanisms expressed during the cotton growing season are essential to maximize the likelihood of success of insecticide resistance management (IRM) strategies (ffrench-Constant and Roush, 1990).

Studies with *H. virescens* suggest that resistance to pyrethroids, which are widely used against cotton pests in the U.S., is associated with all three mechanisms (Gladwell et al., 1990; McCaffery et al., 1991; Ottea et al., 1995). Biochemical and pharmacokinetic studies have shown the importance of cytochrome P450 monooxygenases to pyrethroid resistance in laboratory and field-collected strains of *H. virescens*. (Little et al., 1989; McCaffery et al., 1991; Ottea et al., 1995). Further, oxidative metabolism of pyrethroids by these enzymes occurs predominantly at the 2' and 4' carbons of the phenoxybenzyl group that is prevalent in commercial pyrethroids. In addition, pyrethroid toxicity is enhanced in biological assays with field-collected tobacco budworms by cytochrome P450 monooxygenase inhibitors such as piperonyl butoxide (PBO) (Graves et al., 1991; Elzen et al., 1993) and propynyl ethers (Payne, 1987).

Current methods for characterizing metabolic resistance in field populations of the tobacco budworm include bioassays with synergists (Raffa and Priester, 1985) and biochemical assays that measure activities of enzymes associated with insecticide metabolism. However, multiple forms of these enzyme exists with differing substrate specificities and suceptibilities to inhibition by synergists (Feyereisen et al., 1991; Brown et al., 1996); thus, relevance of results from these assays is limited by the reliability of model substrates and synergists as indicators of toxicologically significant enzyme activities (Sawicki, 1987; Kirby et al., 1994; Ibrahim and Ottea, 1995). Further, limitations in the utility of results from bioassays with synergists are exacerbated by nonmetabolic effects of these compounds and the lack of structural similarity between conventional synergists (such as PBO and propynyl ethers) and pyrethroid insecticides.

The purpose of this research was to develop and test novel pyrethroid analogs as diagnostic compounds for detection of P450 monooxygenase-associated pyrethroid resistance. Biological activity of pyrethroids that contained enzyme inhibiting side chains or groups blocking potential sites of oxidative metabolism was evaluated, and the significance of these compounds as diagnostic tools is discussed.

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Materials and Methods

Chemicals

Cypermethrin (technical grade) was obtained from FMC Corporation (Princeton, NJ). Piperonyl butoxide (PBO) was purchased from ChemService (West Chester, PA). Trichlorophenyl propynyl ether (TCPPE) and novel pyrethroids (Table 1) were synthesized using established methods (Shan et al., submitted). All other chemicals were of analytical quality and purchased from commercial suppliers.

Insects

Pyrethroid-susceptible and -resistant laboratory strains of *H. virescens* were studied. The susceptible strain (LSU) was established in 1977 (Leonard et al., 1988) and has been reared in the laboratory since that time without exposure to insecticides. The resistant Pyr-R strain was derived from a field collection made in August 1995 from the Red River Research Station (Bossier City, LA). Field insects were reared and selected repeatedly as fifth stadium larvae with cypermethrin (1.75 μg/larva) for three generations. Survivor were crossed with LSU insects and progeny were selected as third stadium larvae with 1 μg cypermethrin/larva, a dose corresponding to 21 times the LD_{50} for LSU larvae. Preliminary results using molecular genetic markers suggest that reduced neuronal sensitivity is not a major resistance mechanism in this strain (Park and Taylor, unpublished).

Biological Assays

Fifth stadium larvae (day 1; 180$\pm$20 mg) were treated on the mid-thoracic dorsum with 1 μl of chemical solutions (in acetone) or with acetone alone (control). Dose-mortality relationships for each compound were assessed using triplicate assays with five doses and 10 insects per dose. After treatment, larvae were maintained at 27 °C and mortality was recorded after 72 h. The criterion for mortality was absence of coordinated movement within 30 s after being prodded by a pencil. Median lethal dose (LD_{50}) values were computed by probit analysis using SAS (1985, SAS Institute Co., Cary, North Carolina, USA) and toxicity ratios (RRs, relative to the LSU susceptible strain) were calculated at the LD_{50} level.

Non-toxic isomers of diagnostic compounds, as well as PBO and TCPPE, were applied to the dorsal surface of the mid-abdomen thirty minutes prior to application of cypermethrin, which was applied to the mid-thoracic dorsum. Control larvae were treated with the appropriate concentration of synergist only or acetone alone. Mortality was recorded after 72 h. and LD_{50} values were computed by probit analysis. Synergistic ratios (SRs = mortality following application of cypermethrin + synergist/cypermethrin alone) were calculated at the LD_{50} level.

Results

Biological activity of pyrethroids

Variable levels of susceptibility to pyrethroids were measured in bioassays with insecticide-susceptible LSU larvae (Table 2). Fenfluthrin isomers and pyrethroid analogs were less toxic than cypermethrin, with LD_{50}s ranging from 1.06 (fenfluthrin 3) to 181 μg/larva (for compound **5**) as compared to 0.05 μg/larva for cypermethrin. For 8 of the 15 compounds, no toxicity (NT) was measured following treatment with 100 μg/larva.

The biological activity of compounds was dependent upon chemical conformation. Stereochemistry about C-1 was a major determinant of toxicity: *1R* enantiomers were toxic whereas *1S* enantiomers were not. Similarly, for some α-cyano containing compounds (**5**-**12**), *1RαS*-enantiomers were toxic whereas *1RαR*- and *1SαR*- compounds were not. In addition, susceptibility was always greater for *1R, cis*- than *1R, trans*-isomers of toxic compounds. For the non-cyano, methylenedioxyphenyl (MDP)-containing compounds, biological activity of the *cis*- isomer (**3**) was almost twice as high as the *trans*- isomer (**2**). Likewise, for toxic propargyl-oxyphenyl (PP) compounds, the *cis*-isomer (**12**) was over 50 times more toxic than the corresponding *trans*-isomer (**7**). In contrast, for toxic fenfluthrin isomers, there were no significant differences between LD_{50}s of the *trans*- and *cis*-isomers (1.46 and 1.06 μg/larva for fenfluthrin 2 and 3, respectively).

In tests with Pyr-R insects, resistance was greater to cypermethrin than to the other toxic pyrethroids tested (Table 2). Whereas the LD_{50} for cypermethrin in this strain (2.91 μg/larva) was 58-fold higher than that measured for LSU-S larvae, resistance ratios (RRs) were low for fenfluthrin isomers (2.85 and 2.75 for fenfluthrin 2 and 3, respectively), intermediate for the *1R,cis*- MDP compound (**3**; RR=24), and high for *1R,trans*- MDP (**2**; RR= 41) and *1RαS* propargyloxyphenyl (**12**; RR= 45) compounds. As in tests with LSU larvae, susceptibility of Pyr-R insects was greater to *cis* than *trans* isomers of toxic compounds (Table 2).

Synergism of pyrethroid toxicity

Nontoxic isomers of synthesized pyrethroids were tested as synergists of cypermethrin toxicity. Whereas there was no significant synergism measured with LSU-S larvae, all compounds tested increased the susceptibility of Pyr-R larvae to cypermethrin (Table 3). The propynyl ether, TCPPE, was the most effective synergist with a synergism ratio (SR) of 4.69. Of the newly-synthesized propynyl ethers, only compound **11** (*1RαR-cis* isomer) significantly enhanced toxicity of cypermethrin (SR= 2.55). Coapplication of cypermethrin and **6**, the *1RαR-cis* MDP compound, increased cypermethrin toxicity by 2.69-fold, which was greater than synergism with PBO (SR= 1.97).

Synergism of cypermethrin toxicity in Pyr-R larvae varied depending on the isomer (Table 3). Among the 4 propargyloxyphenyl compounds evaluated, synergism was statistically significant with compound **11** (*1RαR-cis*-PP; SR= 2.55), but was insignificant with the corresponding *trans* isomer (**8**). In addition, the structurally related PP compounds, **9** (*1SαR trans*-) and **10** (*1SαS trans*-), were inactive as synergists. For MDP compounds, synergism of cypermethrin toxicity was greater with **6** (*1RαR cis*- SR = 2.69) than PBO (SR = 1.97), and no statistically significant synergism was measured with compound **1** (*1S,trans*, no αCN).

Discussion

Enhanced metabolism is a major mechanism of resistance to pyrethroids. Oxidative metabolism in a number of insects has been shown to occur at the 2', 4' and 6 positions of the phenoxybenzyl alcohol moiety, or the geminal dimethyl groups of the acid moiety (Shono et al., 1979; Casida and Ruzo, 1980). In studies with *H. virescens*, Lee et al. (1989) and Little et al. (1989) suggested that the 2' and 4' positions on the alcohol moiety of cypermethrin are the main sites of metabolism, whereas oxidation at geminal dimethyl groups is less important. In addition, oxidative diphenyl ether cleavage also was observed in a study with fenvalerate and the Colorado potato beetle, *Leptinotarsa decemlineata* (Soderlund et al., 1987). The objective of this study was to evaluate effects of structural modifications at some metabolically sensitive sites of the pyrethroid molecule on toxicity and resistance.

The insecticidal activity and synergism of cypermethrin toxicity measured in bioassays with fenfluthrin and structurally-modified pyrethroids confirm that P450 monooxygenases are associated with pyrethroid resistance in the Pyr-R strain. Isomers of fenfluthrin, in which oxidative sites of metabolism in the alcohol moiety have been blocked, were highly toxic to pyrethroid-resistant *H. virescens*. These results are consistent with those reported by Scott and Georghiou (1986) in the house fly, *Musca domestica*, and Forrester et al. (1993) in *Helicoverpa armigera*. In addition, resistance ratios were lower in bioassays with compounds in which the metabolically-labile phenoxybenzyl group was replaced with MDP- or propargyloxyphenyl- groups than with cypermethrin.

Involvement of P450 monooxygenases in resistance was further supported by studies with synergists. Cypermethrin toxicity was increased by coapplication of compounds containing propynyl ether and MDP side chains, and synergism of toxicity was greatest with the propynyl ether, TCPPE. These results agree with those from previous studies demonstrating the activity of this compound against pyrethroid-resistant *H. armigera* (Forrester et al. 1993) and *H. virescens* (Brown et al., 1996). However, synergism was lower in tests with PBO than TCPPE, which supports the conclusion of Brown et al. (1996) that different classes of P450 monooxygenase are involved in resistance-associated metabolism of pyrethroids. Taken together, these results suggest that fenfluthrin, which lacks the metabolically-sensitive sites of phenoxybenzyl-containing pyrethroids, may be a useful diagnostic compound to monitor P450 monooxygenase-associated resistance.

Insecticidal and synergistic activity of pyrethroids was dependent on chemical configuration about C-1 and the α carbon. No significant toxicity or synergism was measured with *1S* isomers, and *1R,cis* isomers were always more toxic than corresponding *trans* compounds. In addition, toxicity of *1R*-fenfluthrin was slightly higher with *cis* than *trans* isomers in both pyrethroid-susceptible and -resistant insects. Finally, only *1R,cis* (but not *trans*) chrysanthemates containing MDP (**6**) or propargyloxyphenyl (**11**) groups significantly synergized cypermethrin toxicity. At the α carbon, only *S* enantiomers were toxic (**5, 7,12**) whereas the corresponding *αR,cis*- enantiomers were synergists (**6** and **11**). Assuming that synergism results from P450-monooxygenase inhibition (Feyereisen et al., 1991), these results suggest that interaction between these enzymes and synergists is stereospecific.

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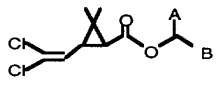
Table 1. Structure of pyrethroids



Compound	A	B	Stereochemistry in Alcohol	Acid
Fenfluthrin1	H	PFP	---	*trans*, 1S
Fenfluthrin2	H	PFP	---	*trans*, 1R
Fenfluthrin3	H	PFP	---	*cis*, 1R
1	H	MDP	---	*trans*, 1S
2	H	MDP	---	*trans*, 1R
3	H	MDP	---	*cis*, 1R
4	CN	MDP	αRS	*trans*, 1RS
5	CN	MDP	αS	*cis*, 1R
6	CN	MDP	αR	*cis*, 1R
7	CN	PP	αS	*trans*, 1R
8	CN	PP	αR	*trans*, 1R
9	CN	PP	αR	*trans*, 1S
10	CN	PP	αS	*trans*, 1S
11	CN	PP	αR	*cis*, 1R
12	CN	PP	αS	*cis*, 1R

Table 2. Toxicity of pyrethroids in topical bioassays with pyrethroid-susceptible and -resistant *H. virescens*

	LSU		PYR-R		
Compounds	LD_{50} (µg/larva)	slope	LD_{50} (µg/larva)	slope	RR
Cypermethrin	0.05	2.52	2.91	2.20	58
Fenfluthrin1	NT	---	NT	---	---
Fenfluthrin2	1.46	2.34	4.16	3.40	2.85
Fenfluthrin3	1.06	3.55	2.92	4.67	2.75
1	NT	---	NT	---	---
2	9.44	2.36	386	2.17	41
3	4.85	2.17	117	2.65	24
4	NT	---	NT	---	---
5	181	2.14	NT	---	---
6	NT	---	NT	---	---
7	57.5	1.44	NT	---	---
8	NT	---	NT	---	---
9	NT	---	NT	---	---
10	NT	---	NT	---	---
11	NT	---	NT	---	---
12	1.14	2.32	51.3	1.69	45

Table 3. Synergism of pyrethroid toxicity in pyrethroid-susceptible and -resistant *H. virescens*

	LSU		Pyr-R	
	LD_{50} (µg/larva)	SR	LD_{50} (µg/larva)	SR
Cypermethrin +				
PBO	0.04	1.25	1.48	1.97
TCPPE	0.04	1.25	0.62	4.69
Fenfluthrin1	0.05	1.00	2.07	1.41
1	0.05	1.00	1.74	1.67
6	0.05	1.00	1.08	2.69
8	0.06	0.83	1.84	1.58
9	0.04	1.25	1.80	1.62
10	0.04	1.25	2.55	1.14
11	0.04	1.25	1.14	2.55