Development of Immunoassays for Type II Synthetic Pyrethroids. 1. Hapten Design and Application to Heterologous and Homologous Assays

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Immunoassays differing in selectivities for pyrethroid insecticides have been developed for the detection of type II pyrethroids, including deltamethrin, cypermethrin, and λ-cyhalothrin. Two approaches were employed in hapten synthesis to raise antibodies with different cross-reactions: (1) use of three spacer attachment points to offset different parts of molecules from the points of attachment and (2) use of linkers with and without bulky groups in the enzyme conjugate to reduce antibody affinities for the spacer arm in the immunoassay. The first approach resulted in the preparation of three series of haptens with a spacer attached (1) at the aromatic moiety of pyrethroid, (2) through the middle of the molecule, and (3) at the cyclopropane moiety. Haptens based on the derivatives of the pyrethroid metabolites were also prepared. The second approach involved the use of a linker with a bulky (cyclohexane ring) functionality for preparation of an enzyme conjugate. While most combinations of antibody and conjugate could be used in immunoassays for detection of deltamethrin in the 10−100 μg/L range, in most cases the limits of detection of the assays (for total isomers of a particular target pyrethroid) were lowered 10−50 fold by treatment of the pyrethroid standards with dilute alkali to produce a different isomer mix. Fifteen antisera prepared using 8 haptens were each screened with 14 peroxidase conjugates, and 26 antibody/conjugate combinations were selected for further study on the basis of the assay sensitivity, dynamic behavior, and specificity for deltamethrin, cypermethrin, and cyhalothrin. These immunoassays provided 50% inhibition of antibody binding (IC_{50}) values between 1.5 and 4.2 μg/L of isomerized total deltamethrin and limits of detection of 0.2−0.7 μg/L. The most sensitive immunoassay for total deltamethrin was obtained using cypermethric acid-KLH as the immunogen and a conjugate based on a derivative of cypermethrin coupled through the middle of the molecule to peroxidase. These provided an IC_{50} of 2 μg/L and a limit of detection of 0.2 μg/L of isomerized total deltamethrin. However, no particular hapten design produced antisera of clearly superior sensitivity or specificity for deltamethrin. Differing cross-reactions with the closely related pyrethroids, deltamethrin, cypermethrin, and cyhalothrin, were obtained, and for several antibodies the cross-reaction as well as the limits of detection could be altered by varying the conjugate combinations. Each of the 12 antibody/enzyme conjugate combinations that sensitively detected deltamethrin were very stereospecific, detecting the α*S*, 1*R cis*, (DM1), and α*R*, 1*R cis* (DM2) isomers only; the assay sensitivity was greater for the latter isomer.

Keywords: *Synthetic pyrethroid; deltamethrin; cypermethrin; cyhalothrin; hapten; immunoassay; agrochemical*

INTRODUCTION

Synthetic pyrethroids are used widely in domestic, public health, agricultural, forestry, and veterinary applications (Hassall, 1990). In domestic use, pyrethroids are used to control cockroaches, houseflies, and many other household insects. In public health, deltamethrin, cypermethrin, fenpropathrin, fenvalerate, and permethrin are used against mosquitoes, simulid, and tse-tse fly. In forestry, permethrin is used to control spruce budworm in the pine forest ecosystem. In agricultural situations, pyrethroids are used on a wide range of crops, including potatoes, sugar beet, cereals, coffee, tea, rice, vines, tobacco, and cotton for control of a wide range of pests, including *Helicoverpa* spp. Pyrethroids are also used in protection of stored commodities and for the control of lice, fleas, ticks, and scabies in domestic and farm animals. The increasing use of synthetic pyrethroids, compared with that of other classes of insecticides, is attributed to their remarkably high insecticidal activities and low toxicities to mammals (Elliott, 1977; Davies, 1985).

The synthetic pyrethroids and natural pyrethrins can be divided into two groups of compounds on the basis of chemical structure and mechanism of action at insect target sites. The type I compounds are simple cyclic alcohol esters of 2,2-dimethyl-3-(2-methyl-1-propenyl)-cyclopropanecarboxylic acid. The type II compounds are esters of an arylcyanohydrin. The type I pyrethroids are less photostable than the type II pyrethroids, restricting their use to indoor application (Davies, 1985),

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S0021-8561(97)00438-X CCC: $15.00
Published on Web 01/14/1998

have different toxicological effects, and may have slightly different mechanisms of action at the insect neuron (Hassall, 1990). Residues of synthetic pyrethroids can be analyzed by either high-performance liquid chromatography (HPLC) or gas−liquid chromatography (GLC). The synthetic pyrethroids exist as either a single stereoisomer (e.g. deltamethrin) or a mixture of stereoisomers and can undergo transformations to insecticidally inactive isomers in water (Takahashi et al., 1985; Maguire, 1990). Analysis of the optical isomers of a variety of pyrethroids, including λ-cyhalothrin, cypermethrin and α-cypermethrin, has been reported using normal-phase HPLC and Pirkle columns (Cayley and Simpson, 1986; Lisseter and Hambling, 1991; Perschke and Hussain, 1992). Pyrethroids containing halogen atoms, such as deltamethrin, cypermethrin, cyhalothrin, and bifenthrin, are commonly analyzed by GLC with electron capture detection. The sensitivities for these pyrethroids are comparably lower than those of other pesticides containing halogen groups, such as organochlorine insecticides, with limits of detection between 0.01 and 0.05 mg/kg (Chapman and Harris, 1978). The sensitivities for non-halogen-containing pyrethroids are even lower.

These problems have stimulated research on alternative techniques for pyrethroid residue analysis, such as immunoassay. Few immunoassays have been developed that detect type II synthetic pyrethroids. An immunoassay based on a monoclonal antibody to (1*R*)-*trans*-permethric acid coupled to protein (Bonwick et al., 1994; Pullen and Hock, 1995a,b) was able to detect the type I pyrethroids such as allethrin, bioallethrin, *S*-bioallethrin, permethrin, bioresmethrin, and pyrethrin, while an assay based on a polyclonal antibody enabled the detection of both type I (permethrin and *S*-bioallethrin) and II pyrethroids (cyfluthrin and cypermethrin), although other type II pyrethroids such as deltamethrin were not detected. The primary objective for this work was to develop two types of immunoassays, namely a class-specific assay (detecting type II pyrethroids) and compound-selective assays (detecting either deltamethrin, cypermethrin, or λ-cyhalothrin). These compounds were the targets for immunoassay development in this study as they comprise ∼81% of total pyrethroids used in the Australian cotton industry for control of *Helicoverpa* spp. (Barrett et al., 1991). Apart from having differing mixtures of isomers in their insecticidal formulations (Hassall, 1990), they differ only in the halogen atoms or groups on the vinyl side chain: deltamethrin has two bromine atoms, cypermethrin has two chlorine atoms, and cyhalothrin has a chlorine atom and a trifluoromethyl group. Pyrethroids are relatively large molecules with no obvious functional group for direct conjugation. Previous work demonstrated that a linker could be placed at the aromatic moiety (Demoute et al., 1986) and at the cyclopropane moiety (Stanker et al., 1989; Hill et al., 1993). Haptens derived from the pyrethroid metabolites also have been prepared (Pullen and Hock, 1995a,b; Wengatz et al., 1996). These immunoassays were selective for compounds containing the dimethyl or (1*R*)-*trans*-dichlorovinyl dimethylcyclopropane moiety, because they were based on one hapten derived from *trans*-permethric acid. Another possible place for coupling would be at the cyano group of the type II pyrethroid. In this study, a range of synthetic approaches to hapten production and the antibodies that resulted from these synthetic approaches were evaluated.

MATERIALS AND METHODS

Materials. Chemicals used in hapten synthesis were obtained from either Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO). Other chemicals used in preparation of buffers were purchased from either Ajax Chemicals (Sydney, Australia) or BDH-Merck (Melbourne, Australia). Silica gel 60 (70−230) mesh was purchased from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) and horseradish peroxidase (HRP) were purchased from Boeheringer Mannheim, Germany. Keyhole limpet hemocyanin (KLH) and the BCA protein assay were purchased from Pierce (Rockville, IL). Tween 20 and Freund's complete and incomplete adjuvants and 3,3′,5,5′-tetramethylbenzidine were obtained from Sigma. Econo-Pac 10DG columns were obtained from Bio-Rad (Hercules, CA). Protein A agarose was purchased from Phamacia (Uppsala, Sweden). Maxisorp polystyrene 96-well plates were obtained from Nunc (Roskilde, Denmark), and swine anti-rabbit IgG was purchased from Dako (Glostrup, Denmark). Deltamethrin isomers (DM1, DM2, DM3, DM4, DM1′, DM2′, DM3′, and DM4′) were gifts from AgrEvo Environmental Health (Berkamsted, U.K.). Deltamethrin [(*S*)-α-cyano-3-phenoxybenzyl (1*R*)-*cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate], λ-cyhalothrin [(*RS*)-α-cyano-3-phenoxybenzyl (*Z*)-(1*RS*)-*cis*-3-(2-chloro-3,3,3-trifluoropropenyl)-2,2-dimethylcyclopropanecarboxylate], and α-cypermethrin [(*RS*)-α-cyano-3-phenoxybenzyl (1*RS*)-*cis*-3-(2,2-dichlorovinyl)-1,1-dimethylcyclopropanecarboxylate] were provided by the Australian Government Analytical Laboratory (Sydney).

Chemical Analyses. ^{1}H and ^{13}C NMR spectra were recorded with a Varian Gemini II instrument, operating at 300 and 75 MHz, respectively, using deuterated chloroform ($CDCl_3$, 99.8% D) as solvent with 0.03% (v/v) tetramethylsilane (Aldrich) as internal standard. Thin-layer chromatography (TLC) was performed on 0.2 mm thick precoated silica gel 60 F_{254} on plastic sheets from Merck. TLC spots were detected by irradiation with UV light and by exposure to iodine vapor. If necessary, TLC plates were sprayed with either 0.03% $KMnO_4$ in concentrated H_2SO_4 (caution) or 1% (w/v) silver nitrate. Column chromatography was performed on silica gel (particle size 63−200 μm, 70−230 mesh ASTM), Merck). Flash chromatography (J. T. Baker, Philipsburg, NJ) was performed using silica gel 200−400 mesh (Aldrich). Radial chromatography on a Chromatotron (Harrison Research, Palo Alto, CA) was performed for small samples (<200 mg) on 2- and 4-mm-thick silica gel (gel 60 PF_{254} containing gypsum, Merck) coated plates.

Hapten Synthesis. The first series of haptens with a spacer attachment at the aromatic moiety was prepared by an esterification of 2-hydroxydeltamethrin/4-hydroxydeltamethrin with succinic anhydride (Zhang and Scott, 1994). The second series of haptens involved a conversion of an α-cyano group of the pyrethroid to a corresponding acid for a spacer attachment (Figure 1). The third series of haptens involved an oxidation of the vinyl moiety using $KIO_3/KMnO_4$ to produce an acid, followed by a reaction with *tert*-butyl 3-aminopropanoate (Figure 2). In addition, haptens were prepared that derived from pyrethroid metabolites including deltamethric acid, cypermethrin acid, cyhalothric acid, and 3-phenoxybenzaldehyde cyanohydrin. Use of a bulky functional group in a spacer in the second approach was expected to reduce the affinity of the antibodies for the spacer in immunoassays and hence increase the sensitivity of antibody for free analyte in immunoassay (Brady et al., 1989; Hill et al., 1993). The bulky spacer was prepared using a method by Hill et al. (1993) and was introduced only for those haptens for which enzyme conjugates with linear spacers exhibited moderate sensitivity (IC_{50} of 10−100 μg/L) in competition immunoassay.

Coupling at the Aromatic Moiety. *(S)-α-Cyano-[3′-(2″-hydroxyphenoxy)phenyl]methyl (1R,3R)-3-(2′,2′-Dibromoethen-*

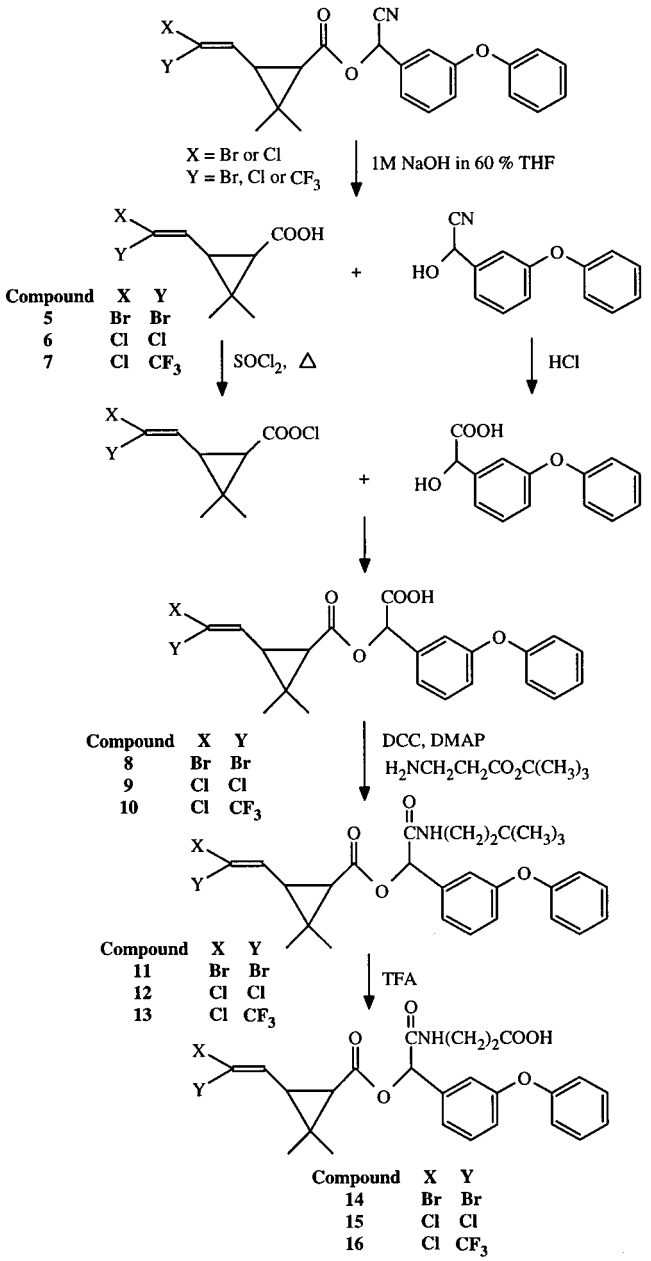

Figure 1. Reaction scheme for synthesis of haptens with spacer arm attachment at the α-position of benzylic carbon for conjugation through the middle of the molecule. Refer to Materials and Methods for compound names.

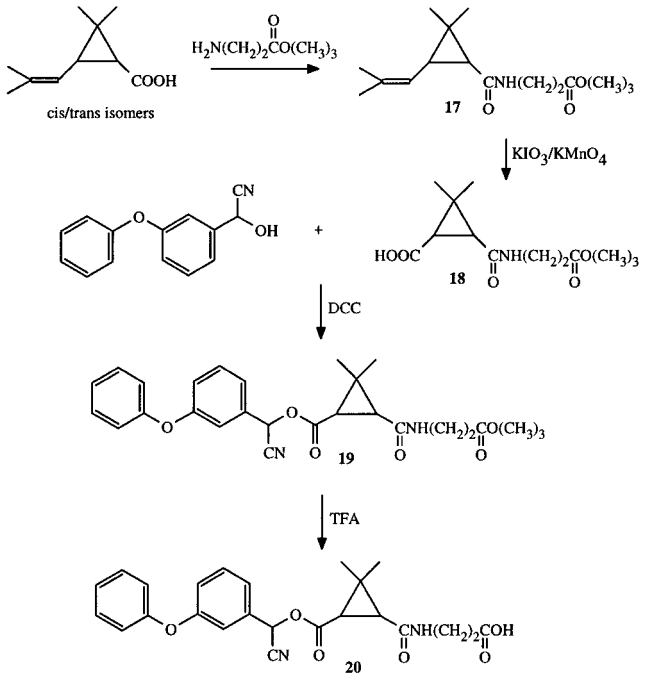

Figure 2. Reaction scheme for synthesis of haptens with a spacer attachment at the cyclopropane moiety. Other details are as for legend to Figure 1.

yl)-2,2-dimethylcyclopropanecarboxylate (2-Hydroxydeltamethrin, ***1****) and (S)-α-Cyano-[3′-(4″-hydroxyphenoxy)phenyl]methyl (1R,3R)-3-(2′,2′-Dibromoethenyl)-2,2-dimethylcyclopropanecarboxylate (4-Hydroxydeltamethrin,* ***2****).* Lead(IV) trifluoroacetic acid was prepared according to the method of Partch (1967). Lead oxide (7.5 g, 0.011 mol) was added to a flask containing trifluoroacetic acid (TFA, 17.6 mL) and trifluoroacetic acid anhydride (10 mL, caution), and the mixture was stirred under nitrogen until a white crystalline compound was formed. Deltamethrin (5.05 g, 10 mmol) was added slowly to a flask containing lead (IV) trifluoroacetic acid (5.59 g, 10 mmol) and TFA (33.3 mL) in an ice bath, and the resultant mixture stirred for 2 h. Then 10% K_2CO_3 (250 mL) was added to the flask and the solution was stirred for 15 min; the deltamethrin trifluoroacetate intermediates were not isolated. The reaction mixture was extracted three times into ethyl acetate and then washed with water and brine (saturated NaCl), and the combined organic layer was concentrated. The residue was chromatographed on silica four times [first with *n*-hexane/ethyl acetate (1:1) and three times with benzene/ethyl acetate (6:1)] to yield 223 mg of **1** (4.3%) and 510 mg of **2** (10%) as bright yellow oils. The identities of the compounds were confirmed by comparison with R_f values [for *n*-hexane/ethyl acetate (1:1)] and ^{1}H NMR [$(CD_3)_2CO$] data of Zhang and Scott (1994).

(S)-α-Cyano-[3′-(2″-(2-carboxyethylcarbonyloxy)phenoxy)-phenyl]methyl (1R,3R)-3-(2′,2′-Dibromoethenyl)-2,2-dimethylcyclopropanecarboxylate (3). Succinic anhydride (51 mg, 0.51 mmol) and dimethylaminopyridine (DMAP, 2 mg) were added to the alcohol **1** (223 mg, 0.43 mmol) in dry pyridine (2 mL), and the mixture was stirred overnight. The solution was extracted with ethyl acetate (30 mL), and the resulting organic layer was washed with 1.0 M HCl, water, and brine and then dried. The organic solution was concentrated and the residue was chromatographed on silica using a Chromatotron [ethyl acetate/petroleum ether (bp 60−80 °C, 4:6] to give the product as a pale yellow oil (41.3 mg, 28.9%): TLC R_f 0.21 [ethyl acetate/petroleum ether 4:6]; ^{1}H NMR ($CDCl_3$) δ 7.0−7.5 (m, aromatic H), 6.70 (d, =CH, J = 8.5 Hz), 6.35 (s, CHCN), 2.67 (m, 12 lines, A2B2), 2.07 (t, CHC, J = 8.5 Hz), 1.92 (d, CHCO, J = 8.5 Hz), 1.23 (s, CH_3), and 1.19 (s, CH_3).

*(S)-α-Cyano-[3′-(4″-(2-carboxyethylcarbonyloxy)phenoxy)-phenyl]methyl (1R,3R)-3-(2′,2′-Dibromoethenyl)-2,2-dimethylcyclopropanecarboxylate (****4****).* Succinic anhydride (23 mg, 0.23 mmol) followed by DMAP (2 mg) was added to the alcohol **2** (100 mg, 0.19 mmol) in dry pyridine (2 mL) and stirred overnight. The solution was extracted in the same manner as for **1**, and the residue was chromatographed on silica using a Chromatotron [ethyl acetate/petroleum ether (3:7)] to yield a pale yellow oil (42.4 mg, 13.4%): TLC R_f 0.24 [ethyl acetate/petroleum ether (3:7)]; ^{1}H NMR ($CDCl_3$) δ 7.0−7.4 (m, aromatic H), 6.73 (d, =CH, J = 8.3 Hz), 6.38 (s, CHCN), 2.88 (d, CH_2-CO, J = 6.4 Hz), 2.84 (d, CH_2CO, J = 6.4 Hz), 2.10 (t, CHC, J = 8.2 Hz), 1.94 (d, CHCO, J = 8.6 Hz), 1.27 (s, CH_3), and 1.23 (s, CH_3).

(S)-α-Cyano-[3′-(2″-(2-carboxyethylcarbonyloxy)phenoxy)-phenyl]methyl (1R,3R)-3-(2′,2′-Dibromoethenyl)-2,2-dimethylcyclopropanecarboxy Succinimidyl Ester. To the acid **3** (41.3 mg, 0.064 mmol) in dichloromethane (5 mL, 0 °C) was added dicyclohexylcarbodiimide (DCC; 14.6 mg, 0.07 mmol) and DMAP (5 mg) followed by *N*-hydroxysuccinimide (NHS; 7.4 mg, 0.07 mmol). The mixture was stirred overnight and then filtered through Celite, and the solvent was evaporated to give the active ester as a pale yellow oil.

(S)-α-*Cyano-[3′-(4′′-(2-carboxyethylcarbonyloxy)phenoxy)-phenyl]methyl (1R,3R)-3-(2′,2′-Dibromoethenyl)-2,2-dimethylcyclopropanecarboxy Succinimidyl Ester*. The acid **4** (42.4 mg, 0.068 mmol) was treated as for the acid **3** to give a pale yellow oil. The reaction scheme and structures of the compounds are shown in Skerritt and Lee (1996).

Conjugation through the Middle of the Molecule (Figure 1). *1. Hydrolysis of Pyrethroids. 3-(2′,2′-Dibromoethenyl)-2,2-dimethylcyclopropanecarboxylic Acid (**5**).* To a flask containing deltamethrin (15.4 g, 0.31 mol) was added 300 mL of 1 M NaOH in 60% tetrahydrofuran (THF), and the mixture was refluxed for 3 days. The resulting solution was acidified using concentrated HCl (caution) and extracted three times with ethyl acetate. The combined organic layers were then washed (0.1 M HCl, water, and brine), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was chromatographed on silica using flash chromatography [ethyl acetate/petroleum ether/acetic acid (10:89.5:0.5)] to give **5** as a white solid, which was recrystallized to give white needle-like crystals (5.0 g, 55%): mp 108−110 °C; TLC R_f 0.43 [ethyl acetate/petroleum ether/acetic acid (20:79.9:0.1)]; ^{1}H NMR ($CDCl_3$) δ 6.72 (d, =CH, J = 8.6 Hz), 2.04 (t, CHC, J = 8.5 Hz), 1.88 (d, CHCO, J = 8.5 Hz), 1.29 (s, CH_3), and 1.28 (s, CH_3); ^{13}C NMR δ 171.1 (COOH), 133.0 (=CH), 89.8 (=CBr_2), 36.4, 31.6 (2 × CH_3), 28.5 [$C(CH_3)$], 28.5 (CHCO), and 15.0 (CHC).

*3-(2′,2′-Dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic Acid (**6**).* To α-cypermethrin (10 g, 24 mmol) was added 300 mL of 1 M NaOH in 60% THF, and the mixture was refluxed for 36 h. The mixture was extracted in the same manner as for deltamethrin, and the residue was chromatographed on silica [ethyl acetate/petroleum ether/acetic acid (2:7.9:0.1)] to yield **6** as a white solid. After recrystallization from ethyl acetate/petroleum ether, white crystals were obtained (3.12 g, 73.4%): mp 89−91 °C; TLC R_f 0.45 [ethyl acetate/petroleum ether/acetic acid (2:7.9:0.1)]; ^{1}H NMR ($CDCl_3$) δ 6.18 (d, =CH, J = 8.7 Hz), 2.09 (t, CHC, J = 8.5 Hz), 1.84 (d, CHCO, J = 8.6 Hz), 1.26 (s, CH_3), and 1.25 (s, CH_3); ^{13}C NMR δ 177.4 (COOH), 124.4 (=CH), 121.1 (=CCl_2), 33.4, 31.7 (2 × CH_3), 28.5 (CHCO), 28.5 [C(CH3)], and 14.9 (CHC).

*3-(2′-Chloro-3′,3′,3′-trifluoropropenyl)-2,2-dimethylcyclopropanecarboxylic Acid (**7**).* λ-Cyhalothrin (10.3 g, 22.9 mmol) was treated in the same manner as for α-cypermethrin, and after recrystallization from ethyl acetate/petroleum ether yielded crystals (2.35 g, 47.9%): mp 115−117 °C; TLC R_f 0.38 [ethyl acetate/petroleum ether/acetic acid (2:7.9:0.1)]; ^{1}H NMR ($CDCl_3$) δ 6.84 (d, =CH, J = 9.3 Hz), 2.04 (t, CHC, J = 9.2 Hz), 1.88 (d, CHCO, J = 8.4 Hz), 1.30 (s, CH_3), and 1.29 (s, CH_3); ^{13}C NMR δ 177.1 (COOH), 129.6, 129.5, 129.49, 129.43 (=CH), 122.4, 122.2, 121.9 (CF3), 118.6 (=CCF_3), 32.6 (CH_3), 31.6 (CH_3), 29.6 ($C(CH_3)$), 28.4 (CHCO), and 14.8 (CHC).

*2. Generation of Esters via Acid Chlorides. (±)-1-Carboxy-(3′-phenoxyphenyl)methyl 3-(2′,2′-Dibromoethenyl)-2,2-methylcyclopropanecarboxylate (**8**).* The acid **5** (0.86 g, 3.0 mmol) was treated with thionyl chloride ($SOCl_2$, 5 mL, caution) by stirring under reflux for 1 h. The solution was concentrated under reduced pressure to remove excess $SOCl_2$, and the resulting yellow liquid was purified by bulb-to-bulb distillation. 3-Phenoxymandelic acid was prepared by treating 3-phenoxybenzaldehyde cyanohydrin (Aldrich) with concentrated HCl (caution). 3-Phenoxymandelic acid (104 mg, 0.43 mmol) was added to the flask containing 1 mL of acid chloride **8** and stirred for 6 days, after which time 1,4-dioxane (1 mL) and saturated aqueous $NaHCO_3$ (2 mL) were added and stirred for 1 h. The solution was acidified with concentrated HCl (caution) to pH 1 and extracted three times with ethyl acetate. The combined organic layers were washed with water and brine, dried over $MgSO_4$, and concentrated. The residue was chromatographed on silica [chloroform/acetic acid (99.5:0.5)] to give a white solid (109 mg, 49%): ^{1}H NMR ($CDCl_3$) δ 9.6 (bs, COOH), 7−7.4 (m, aromatic H), 6.72 (d, =CH, J = 8.6 Hz), 5.86, 5.89 (2 × s, CHCOO), 2.0 (m, CHC), 1.8 (d, CHCO, J = 8.4 Hz), and 1.27 (m, 2 × CH_3); ^{13}C NMR δ 178.7, 178.1 (COOH), 159 (C3, C1′), 134.1, 134, 133.9 (C1), 131.3, 131.0, 130.9 (C5), 125.9 (=CH), 125.2, 12.9, 12.8 (C6), 123.3 (C4′), 120.9 (C2), 120.4, 120.3, 120.2 (C2′, C4′), 119.01, 118.9 (C4), 90.9 (=CBr_2), 75.0 (CHCOO), 37.5, 37.2, 37.1 (CH_3), 32.7, 32.5, 32.4 (CH_3), 29.6, 29.57, 29.53 [$C(CH_3)$], 29.4, 29.3, 29.2 (CHCO) and 16.2, 16.1 (CHC).

*(±)-1-Carboxy-(3′-phenoxyphenyl)methyl 3-(2′,2′-Dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate (**9**).* The acid **6** (0.47 g, 2.6 mmol) was treated with 10 mL of $SOCl_2$ (caution) to yield the acid chloride as a light brown oil. 3-Phenoxymandelic acid (633 mg, 2.6 mmol) was added to a flask containing the acid chloride prepared from **6** (2.6 mmol), and the mixture was stirred for 3 days. It was extracted and concentrated in the same manner as for compound **8**, and the residue was chromatographed on silica [ethyl acetate/petroleum ether/acetic acid (20:79.9:0.1)] to yield a light brown oil (780 mg, 70%): TLC R_f 0.2 [ethyl acetate/petroleum ether/acetic acid (3:6.9:0.1)]; ^{1}H NMR ($CDCl_3$) δ 9.55 (bs, COOH), 7−7.4 (m, aromatic H), 6.24 (d, =CH, J = 8.8 Hz), 6.24 (d, =CH, J = 8.6 Hz), 5.89, 5.92 (2 × s, CHCOO), 2.01 (m, CHC, CHCO), and 1.25, 1.27, 1.28, 1.30 (4 × s, 2 × CH_3); ^{13}C NMR δ 174.5, 174.3 (COOH), 170.6, 170.5 (CO), 158.4, 157.3 (C3, C1′), 135.9, 135.7 (C1), 130.9 (C5), 130.6 (C3′, C5′), 125.2, 125.0 (C6), 124.4, 124.3 (=CH), 122.9, 122.9 (C4′), 121.9, 121.8 (C2), 119.9, 119.8 (C2′, C6′), 118.7, 118.6 (C4), 118.2 (=CCl_2), 103.6 (=CCl_2), 75.6, 74.5 (CHCOO), 33.9, 33.8 (CH_3), 32.1, 31.9 (CH_3), 29.2 [$C(CH_3)$], 29.0, 28.9 (CHCO), and 15.7, 15.6 (CHC).

*(±)-1-Carboxy-(3′-phenoxypheny)methyl 3-(2′-Chloro-3′,3′,3′-trifluoropropenyl)-2,2-dimethylcyclopropanecarboxylate (**10**).* The acid **7** (1 g, 4.66 mmol) was also treated with 10 mL of $SOCl_2$ (caution) to yield the acid chloride as a light brown oil. The acid chloride (4.7 mmol) was treated with 3-phenoxymandelic acid (379 mg, 1.55 mmol) to yield a light brown oil (610 mg, 86%): ^{1}H NMR ($CDCl_3$) δ 7−7.4 (m, aromatic H), 6.89 (d, =CH, J = 9.2), 6.86 (d, =CH, J = 9.7), 6.82 (d, =CH, J = 9.2), 6.81 (d, =CH, J = 9.1), 5.87, 5.88 (2 × s, CHCOO), 2.2 (m, CHC), 2.10 (d, CHCO, J = 8.4), 2.09 (d, CHCO, J = 5.9), and 1.27, 1.28, 1.30, 1.33 (4 × s, 2 × CH3); ^{13}C NMR δ 191.6 (COOH), 170.5, 170.3 (CO), 137.7, 135.2 (C3, C1′), 132.1, (C1), 130.4 (C5), 130.3 (C3′, C5′), 129.5 (=CH), 124.8, 124.7 (=CH, C6), 123.1 (C4′), 121.3, 121.8, 122.1, 122.2 (CF3), 120.5 (C2), 120.3 (C2′, C6′), 119.1 (C4), 118.6, 118.1 (=CCF3), 60.6, 60.8 (CHCO), 33.0 (CH_3), 30.7 (CH_3), 28.5 [$C(CH_3)$], 28.4 (CHCO), and 14.9 (CHC).

*3. Preparation of Carbamoyl Derivatives. N-2-(1′,1′-Dimethylethyloxycarbonylethyl)-carbamoyl-(3′-phenoxyphenyl)-methyl 3-(2′,2′-Dibromoethenyl)-2,2-dimethylcyclopropanecarboxylate (**11**).* The flask containing the acid **8** (46 mg, 0.09 mmol) in 2 mL of dichloromethane was cooled in an ice bath for 30 min, and then DCC (23 mg, 0.1 mmol) followed by DMAP (0.5 mg) was added and stirred for 30 min. Finally, *tert*-butyl 3-aminopropanoate (26 mg, 0.1 mmol) was added, and the mixture was stirred overnight. After filtration of the solution to remove the precipitate of dicyclohexylurea, the mixture was extracted with ethyl acetate and the organic layer was washed (0.1 M HCl, saturated $NaHCO_3$, water, and brine), dried, and concentrated. The residue was chromatographed on silica [ethyl acetate/petroleum ether (30:70)] to give the *tert*-butyl ester (21 mg, 37%): ^{1}H NMR ($CDCl_3$) δ 7−7.4 (m, aromatic H), 6.22 (d, =CH, J = 8.7), 6.18 (d, =CH, J = 8.5 Hz), 5.98, 6.00 (2 × s, CHCOO), 3.49 (m, CH_2N), 2.44 (m, CH_2-CO), 2.09 (m, CHC, CHCO), 1.44 (s, *tert*-butyl), and 1.25, 1.26 (2 × s, 2 × CH_3).

*N-2-(1′,1′-Dimethylethyloxycarbonylethyl)carbamoyl-(3′-phenoxyphenyl)methyl 3-(2′,2′-Chloroethenyl)-2,2-dimethylcyclopropanecarboxylate (**12**).* The acid **9** (260 mg, 0.6 mmol) was treated in the same manner as for compound **8** to produce a light brown oil (82 mg, 23%): ^{1}H NMR ($CDCl_3$) δ 7−7.4 (m, aromatic H), 6.22 (d, =CH, J = 8.7 Hz), 6.18 (d, =CH, J = 8.5 Hz), 5.98, 6.0 (2 × s, CHCOO), 3.49 (m, CH_2N), 2.44 (m, CH_2-CO), 2.09 (m, CHC, CHCO), 1.44 (s, *tert*-butyl), and 1.25, 1.26 (2 × s, 2 × CH_3).

*N-2-(1′,1′-Dimethylethyloxycarbonylethyl)carbamoyl-(3′-phenoxyphenyl)methyl 3-(2′-Chloro-3′,3′,3′-trifluoropropenyl)-2,2-dimethylcyclopropanecarboxylate (**13**).* The acid **10** (242 mg,0.55 mmol) was also treated in the same manner as for compound **8** to produce a light brown oil (265 mg, 87%): ^{1}H

NMR ($CDCl_3$) δ 7−7.4 (m, aromatic H), 6.82 (d, =CH, J = 9.6 Hz), 5.94, 5.97 (2 × s, CHCOO), 3.46 (m, CH_2N), 2.4 (m, CH_2-CO), 2.10 (d, CHCO, J = 9.0 Hz), 1.40, 1.43 (2 × s, *tert*-butyl), and 1.23, 1.27, 1.28 (3 × s, 2 × CH_3).

*4. Removal of tert-Butyl Protecting Group. N-2-(Carboxyethenyl)carbamoyl-(3′-phenoxyphenyl)methyl 3-(2′,2′-Dibromoethenyl)-2,2-dimethylcyclopropanecarboxylate (**14**) and Succinimidyl Ester.* TFA (1 mL, caution) was added to the ester **11** (21 mg, 0.03 mmol), and the mixture was stirred for 10 min. The excess TFA was removed under reduced pressure to yield an acid **14**, which was used in the next step without further purification. The ^{1}H NMR spectrum for **14** showed the disappearance of the *tert*-butyl peak at 1.4 ppm. DCC (8 mg, 0.04 mg) and DMAP (0.2 mg) were added to the acid in dichloromethane (2 mL), which was cooled in an ice bath and stirred for 30 min, and then NHS (7 mg, 0.035 mmol) was added, and the mixture was stirred overnight. The dicyclohexylurea precipitate was removed by filtration, and the filtrate was concentrated to yield a viscous yellow oil (16 mg, 80%).

*N-2-(Carboxyethyl)carbamoyl-(3′-phenoxyphenyl)methyl 3-(2′,2′-Chloroethenyl)-2,2-dimethylcyclopropanecarboxylate (**15**) and Succinimidyl Ester.* The *tert*-butyl ester **12** (80.7 mg, 0.16 mmol) was also treated with 1 mL of TFA (caution) to yield acid **15**, and it was treated with NHS (21.5 mg, 0.19 mmol), DCC (39.4 mg, 0.19 mmol), and DMAP (0.98 mg) to yield a light yellow oil (79.3 mg, 89%): ^{1}H NMR for **15** ($CDCl_3$) δ 7−7.4 (m, aromatic H), 6.21 (d, =CH, J = 8.8 Hz), 6.18 (d, =CH, J = 8.7 Hz), 5.99, 6.02 (2 × s, CHCOO), 4.15 (dd, CH_2N, J = 8.5 and 6.9 Hz), 3.15 (m, CH_2CO), 2.13 (d, CHCO, J = 8.6 Hz), 1.99 (d, CHCO, J = 8.4 Hz), and 1.26, 1.29 (2 × s, 2 × CH_3).

*N-2-(Carboxyethyl)carbamoyl-(3′-phenoxyphenyl)methyl 3-(2′-Chloro-3′,3′,3′-trifluoropropenyl)-2,2-dimethylcyclopropanecarboxylate (**16**) and Succinimidyl Ester.* The *tert*-butyl ester **13** (242 mg, 0.55 mmol) was also treated in the same manner as for **11** to yield **16**: the ^{1}H NMR spectrum showed the disappearance of *tert*-butyl peaks at 1.4 ppm. Then the acid was treated with DCC, DMAP, and NHS to produce an active ester as a light brown oil (265 mg, 79%).

Coupling at the Cyclopropane Moiety (Figure 2). *Preparation of Chrysanthemic Acid.* To chrysanthemum monocarboxylic acid ethyl ester (4.52 g, 23 mmol; 2,2-dimethyl-3-isobutenylcyclopropane-1-carboxylic acid, Sigma) in 80% ethanol was added KOH (2.4 g, 42.8 mmol), and the mixture was refluxed at 80 °C overnight. The acidified solution (pH 2) was extracted twice with ethyl acetate, and the combined organic layers were washed with 0.1 M HCl, water, and brine, dried over $MgSO_4$, then concentrated to give a white solid (3.82 g, 99%); TLC R_f 0.39 [ethyl acetate/petroleum ether (3:7)].

*N-2-(1′,1′-Dimethylethyloxycarbonylethyl) 2,2-Dimethyl-3-(2′-methylpropenyl)cyclopropanecarboxamine (**17**).* To chrysanthemum monocarboxylic acid (500 mg, 2.63 mmol) in dichloromethane (20 mL) cooled in an ice bath for 15 min were added DCC (308 mg, 2.63 mmol) and DMAP (20 mg), and the mixture was stirred for a further 15 min. *tert*-Butyl 3-aminopropanoate (382 mg, 2.63 mmol) was then added, and the mixture was stirred overnight. After filtration and concentration, the residue was chromatographed on silica [chloroform/acetic acid (99.9:0.1)] to yield a light yellow oil (381.7 mg, 49%): TLC R_f 0.6 [chloroform/acetic acid (99.9:0.1)] and R_f 0.7 [ethyl acetate/petroleum ether/acetic acid (30:69.9:0.1)]; ^{1}H NMR ($CDCl_3$) δ 6.17 (bs, NH), 5.36 (d, 1 H, J = 8.61 Hz), 4.9 (d, =CH, J = 7.74 Hz), 4.9 (d, =CH, J = 7.9 Hz), 3.4 (m, CH_2N), 2.4 (t, CH_2-CO, J = 6.03 Hz), 2.08 (d, CHCO, J = 7.3 Hz), 1.95 (t, CHC, J = 8.8 Hz), 1.65, 1.73 [2 × s, $((CH_3)_2C=]$ 1.4 (s, *tert*-butyl), and 1.05, 1.13, 1.15, 1.17 (4 × s, 2 × CH_3).

*N-2-(1′,1′-Dimethylethyloxycarbonylethyl)carbamoyl 2,2-Dimethylcyclopropanecarboxylate (**18**).* The ester **17** (406 mg, 1.38 mmol) in *tert*-butyl alcohol (7 mL) was added to a mixture containing 25 mL of 0.22 M $NaIO_4$, 3.64 mM $KMnO_4$, and 84 mM Na_2CO_3 and stirred overnight. The solution was acidified to pH 2 and extracted twice with ethyl acetate. The combined organic layer was washed with 0.1 M $Na_2S_2O_5$, water, and brine and then dried and concentrated to give a light brown oil (298 mg, 76%). The crude oil was used in the next step without further purification since NMR showed complete oxidation of the double bond: TLC R_f 0.6 [ethyl acetate/petroleum ether/acetic acid (30.69.9:0.1)] and R_f 0.38 [chloroform/petroleum ether/acetic acid (90:9.9:0.1)]; ^{1}H NMR ($CDCl_3$) δ 3.47 (m, CH_2N), 2.49 (m, CH_2CO), 2.08 (d, CHC, J = 7.3 Hz), 1.95 (t, CHCO, J = 8.8 Hz), 1.41, 1.42, 1.44, 1.45 (4 × s, *tert*-butyl), and 1.26, 1.27 (2 × s, CH_3).

*(±)-Cyano-(3-phenoxyphenyl)methyl N-2-(1′,1′-Dimethylethyloxycarbonylethyl)carbamoyl-2,2-dimethylcyclopropanecarboxylate (**19**).* To the monoester **18** (298 mg, 1.05 mmol) in dichloromethane cooled in ice bath was added DCC (286 mg, 1.38 mmol), and the mixture was stirred for 15 min. Phenoxybenzylcyanohydrin (311 mg, 1.38 mmol, caution) was then added to the mixture, and stirring continued overnight. After the solution was filtered and concentrated, the residue was dissolved in ethyl acetate and washed with water and brine. The ester was obtained as a yellow oil (167 mg, 28.7%) after chromatography on silica [ethyl acetate/petroleum ether (25:75)]; ^{1}H NMR ($CDCl_3$) δ 7−7.4 (m, aromatic H), 6.31 (s, CHCN), 3.43 (m, CH_2N), 2.41 (m, CH_2CO), 2.08 (d, CHC, J = 7.3 Hz), 1.42 (s, *tert*-butyl), and 1.15, 1.16 (2 × s, 2 × CH_3).

*(±)-Cyano-(3-phenoxyphenyl)methyl N-2-(Carboxyethyl)carbamoyl-2,2-dimethylcyclopropanecarboxylate (**20**) and Succinimidyl Ester.* The monoester was treated with TFA for 15 min (caution) to produce an acid, **20**. The cleavage of the *tert*-butyl group was confirmed by NMR: ^{1}H NMR ($CDCl_3$) δ 8.9 (bs, COOH), 7.0−7.4 (m, aromatic H), 6.31 (s, CHCN), 3.83 (m, CH_2N), 2.66 (m, CH_2CO), and 1.26 (m, 2 × CH_3); ^{13}C NMR δ 176.4 (COOH), 174.8 (CO), 169.5, 168.3 (COCN), 158.0, 156.0 (C3, C1′), 133.2 (C1), 130.6 (C5), 129.9 (C3′, C5′), 124.1, 124.0, 123.9 (C6, =CH), 122.2 (C4′), 120.2 (C2), 119.4 (C2′, C6′), 117.8 (C4), 35.6, 35.1 (CH_2CN), 33.6, 33.5 (CHCO), 31.9, 31.4 (CH_2-CO), 26.2, 25.4 (CHCO), 29.7 [$C(CH_3)$], 20.4 (CH_3), 19.9 (CH_3). The active ester was obtained by adding NHS (65 mg, 027 mmol) to a solution containing the acid **20** (62 mg, 0.23 mmol), DCC (57 mg, 0.27 mmol), and DMAP (5 mg) in dichloromethane (10 mL) and stirred overnight, before filtration and concentration to yield a yellow oil (80%).

Coupling Only the Cyclopropane Moiety. *N-2-(1′,1′-Dimethylethyloxycarbonylethyl) 2,2-Dimethyl-3-(2′,2′-bromoethenyl)cyclopropanecarboxyamide (**21**).* The acid **5** (200 mg, 0.67 mmol) in dichloromethane was cooled in ice bath for 15 min. DCC (94 mg, 0.81 mmol) followed by DMAP (4 mg) was added to the solution and stirred for a further 30 min in the ice bath. *tert*-Butyl 3-aminopropanoate (117 mg, 0.81 mmol) was then added to the solution and stirred overnight. The precipitates of dicyclohexylurea were removed by filtration through Celite, and the filtrate was extracted in diethyl ether twice and concentrated to a pale brown oil, which was used without further purification: ^{1}H NMR ($CDCl_3$) δ 6.75 (d, =CH, J = 8.6 Hz), 4.07 (t, CH_2N, J = 6.4 Hz), 3.67 (t, CH_2CO, J = 6.4 Hz), 2.34 (m, CHC), 1.86 (d, CHCO, J = 7.3 Hz), 1.43 (s, *tert*-butyl), and 1.23, 1.25 (2 × s, 2 × CH_3).

*N-2-(1′,1′-Dimethylethyloxycarbonylethyl) 2,2-Dimethyl-3-(2′,2′-dichloroetheyl)cyclopropanecarboxyamide (**22**).* The acid **6** (200 mg, 0.96 mmol) was treated in the same manner with DCC (135 mg, 1.15 mmol), DMAP (7 mg), and *tert*-butyl 3-aminopropanoate (167 mg, 1.15 mmol) to produce a pale yellow oil, which was used without further purification, as the TLC spots were only weakly UV active: ^{1}H NMR ($CDCl_3$) δ 6.92 (d, =CH, J = 9.4 Hz), 6.91 (d, =CH, J = 9.5 Hz), 4.1 (t, CH_2N, J = 6.4 Hz), 3.67 (t, CH_2CO, J = 6.4 Hz), 2.16 (t, CHC, J = 8.9 Hz), 2.00, (m, CHC), 1.86 (d, CHHCO, J = 8.9 Hz), 1.43 (s, *tert*-butyl), 1.30, 1.31 (2 × s, CH_3), and 1.30 (s, 3 H).

*N-2-(1′,1′-Dimethylethyloxycarbonylethyl) 2,2-Dimethyl-3-(2′-chloro-3′,3′,3′-trifluoropropenyl)cyclopropanecarboxyamide (**23**).* The *tert*-butyl ester of 3-(2-chloro-3,3,3-trifluoropropenyl)-2,2-dimethylcyclopropanecarboxylic acid (200 mg, 0.93 mmol) was prepared in a similar manner to yield an amber oil. This was used in the next step without futher purification, due to lack of sufficient UV activity: ^{1}H NMR ($CDCl_3$) δ 6.93 (d, =CH, J = 9.4 Hz), 5.7 (bs, NH), 4.1 (t, CH_2N, J = 6.5 Hz), 2.31 (t, CH_2CO, J = 7.5 Hz), 2.00, (m, CHC), 1.86 (d, CHCO, J = 8.9 Hz), 1.43 (s, *tert*-butyl), and 1.30, 1.31 (2 × s, CH_3).

*N-2-(Carboxyethyl) 2,2-Dimethyl-3-(2′,2′-dibromoetheyl)cyclopropanecarboxyamide (**24**), N-2-(Carboxyethyl) 2,2-Dimethyl-3-(2′,2′-dichloroetheyl)cyclopropanecarboxyamide (**25**), and N-2-(Carboxyethyl) 2,2-Dimethyl-3-(2′-chloro-3′,3′,3′-trifluoropropenyl)cyclopropanecarboxyamide (**26**).* The compounds **21**−**23** were treated with TFA (1 mL, caution) to yield an acid, and the excess TFA was removed under reduced pressure. The clevage of the *tert*-butyl group was confirmed by proton NMR, and the crude acids were used in the next step without further purification. The ^{1}H NMR spectral data of **24**−**26** were as follows: (**24**) ($CDCl_3$) δ 6.76 (d, =CH, J = 8.6 Hz), 4.10 (t, CH_2N, J = 6.3 Hz), 2.45 (t, CH_2CO, J = 7.4 Hz), 1.93 (t, CHC, J = 8.5 Hz), 1.82 (d, CHCO, J = 8.4 Hz), and 1.23, 1.24, 1.25, 1.27 (4 × s, CH_3); (**25**) ($CDCl_3$) δ 6.90 (d, =CH, J = 9.5 Hz), 6.89 (d, =CH, J = 9.4 Hz), 4.35 (t, CH_2N, J = 6.1 Hz), 2.47 (t, CH_2CO, J = 7.3 Hz), 2.16 (t, CHC, J = 7.4 Hz), 1.96 (d, CHCO, J = 8.9 Hz), and 1.28, 1.29 (2 × s, CH_3); (**26**) ($CDCl_3$) δ 6.89 (d, =CH, J = 11 Hz), 6.88 (d, =CH, J = 10.4 Hz), 4.12 (t, CH_2N, J = 6.3 Hz), 2.45 (t, CH_2CO, J = 7.3 Hz), 2.21 (t, CHC, J = 7.8 Hz), 1.97 (d, CHCO, J = 8.3 Hz), 1.97 (d, CHCO, J = 8.4 Hz), and 1.27, 1.28, 1.3 (3 × s, CH_3).

N-2-(Carboxyethyl) 2,2-Dimethyl-3-(2′,2′-dibromoetheyl)cyclopropanecarboxyamide Succinimidyl Ester. To a solution containing acid **21** (108.4 mg, 0.31 mmol) and dichloromethane (5 mL) cooled in an ice bath were added DCC (75.8 mg, 0.37 mmol) and DMAP (20 mg) and stirred for 30 min; NHS (42.2 mg, 0.37 mmol) was then added, and the mixture was stirred overnight. The dicyclohexylurea precipitate was removed by filtration, and the filtrate was concentrated to yield an off-white solid: ^{1}H NMR ($CDCl_3$) δ 6.78 (d, =CH, J = 8.5 Hz), 6.62 (d, =CH, J = 8.1 Hz), 4.16 (t, CH_2N, J = 6.2 Hz), 2.85 (bs, 2 × CH_2CON), 2.75 (t, CH_2CO, J = 7.2 Hz), 2.20 (t, CHC, J = 8.3 Hz), 2.11 (t, CHC, J = 8.3 Hz), 1.97 (t, CHC, J = 8.5 Hz), 1.88 (d, CHCO, J = 8.4 Hz), 1.26, 1.27 (2 × s, CH_3); ^{13}C NMR δ 171.9, 169.2, 168.2 (4 × CON), 89.4 (=CBr_2), 37.1 (CH_2N), 35.8 (CH_2CO), 33.8 (CH_3), 31.8 (CH_3), 27.9 (CHCO), 25.7 (2 × CH_2CON), and 15.2 (CHC).

N-2-(Carboxyethyl) 2,2-Dimethyl-3-(2′,2′-dichloroetheyl)cyclopropanecarboxyamide Succinimidyl Ester and N-2-(Carboxyethyl) 2,2-Dimethyl-3-(2′-chloro-3′,3′,3′-trifluoropropenyl-cyclopropanecarboxyamide Succinimidyl Ester. The acids **22** and **23** were also treated with DCC, DMAP, and NHS in a normal manner to produce white solids. The ^{1}H NMR spectral data for succinimidyl ester **22** were as follows: δ 6.92 (d, =CH, J = 9.0 Hz), 4.20 (t, CH_2N, J = 6.0 Hz), 3.73 (m, CH_2CO), 2.87 (bs, 2 × CH_2CON), 2.13 (t, CHC, J = 6.0 Hz), 2.05 (d, CHCO, J = 8.4 Hz), and 1.25, 1.26, 1.27, 1.28, 1.32, 1.34 (6 × s, 2 × CH_3); ^{13}C NMR δ 170.0, 169.0, 168.0 (4 × CON), 130.0, 129.9 (=CCl_2), 32.6 (CH_2N), 30.9 (CH_2CO), 29.3 (CHCO), 25.5 (2 × CH_2CON), and 14.8 (CHC). The ^{1}H NMR spectral data for the succinimidyl ester of **23** were as follows: δ 6.91 (d, =CH, J = 9.3 Hz), 6.73 (d, CH, J = 9.3 Hz), 6.72 (d, =CH, J = 9.0 Hz), 4.11 (t, CH_2N, J = 6.5 Hz), 2.83 (bs, 2 × CH2CON), 2.45 (t, CH_2CO, J = 7.4 Hz), 2.39 (t, CH_2CO, J = 9.0 Hz), 2.16 (t, CHC, J = 9.3 Hz), 2.15 (t, CHC, J = 8.6 Hz), 1.97 (d, CHCO, J = 8.3 Hz), and 1.27, 1.28 (2 × s, CH_3); ^{13}C NMR δ 172.4, 170.8, 169.4, 166 (4 × CON), 130.3, 130.2, 128.3, 128.2 (=CH), 122.4, 121.9 (CF_3), 118.6, 188.4, (=CCl), 33.8 (CH_2N), 32.3 (CH_3), 31.1 (CH_3), 30.5 (CH_2CO), 28.6 (CHCO), 15.1, 15.0 (CHC).

Coupling Only the Phenoxybenzyl Moiety. *(±)-Cyano-(3′-phenoxyphenyl)methyl 2-Trimethylsilylethyloxycarbonylethylcarboxylate (**27**).* (±)-3-Phenoxybenzylaldehyde cyanohydrin (1 mL, 3.1 mmol) and 2-trimethylsilylethyl oxycarbonylethylcarboxylic acid (762 mg, 3.5 mmol) in dichloromethane (30 mL) were cooled to 0 °C in an ice bath for 15 min. DCC (767 mg, 3.7 mmol) was added followed by DMAP (20 mg), and the mixture was stirred at room temperature for 6 h. After dicyclohexylurea was removed by filtration, the filtrate was extracted with ethyl acetate, water (slightly acidified), and brine. The organic layer was dried over $MgSO_4$ and concentrated to produce a red oil (unable to determine the percentage yield as product was suspected to be contaminated with dicyclohexyl urea): TLC R_f 0.57 [ethyl acetate/petroleum ether (3:7)]; ^{1}H NMR ($CDCl_3$) δ 7−7.4 (m, aromatic H), 4.2 (m, 7 lines, OCH_2), 2.65 (m, 14 lines, A2B2), 0.98 (m, CH_2Si), 0.023 [s, $Si(CH_3)_3$].

*(±)-Cyano-(3′-phenoxyphenyl)methyl 2-Carboxyethylcarboxylate (**28**) and Succimidyl Ester.* To **27** in acetonitrile (30 mL) was added tetraethylammonium fluoride (TEAF; 671 mg, 4.5 mmol), and the mixture was refluxed at 90 °C for 1 h. The solution was concentrated, and the residue was dissolved in ethyl acetate. The organic layer was washed with water and brine, dried (over $MgSO_4$), and concentrated to yield a bright yellow product (42 mg): TLC R_f 0.46 [ethyl acetate/petroleum ether (3:7)]. The acid (42 mg, 0.13 mmol) was treated with DCC (34 mg, 0.16 mmol), DMAP (0.8 mg), and NHS (19 mg, 0.16 mmol) in dichloromethane (5 mL) at 0 °C for 6 h. The byproduct dicyclohexylurea was removed by filtration through Celite and the filtrate concentrated.

Haptens Utilizing Bulky Spacers. *N-4′-(2′-Trimethylsilylethyloxycarbonyl)cyclohexylmethylcarbamoylmethyl 3-(2′,2′-Dibromoethenyl)-2,2-dimethylcyclopropanecarboxylate (**29**).* 2-(Trimethylsilyl)ethyl *trans*-4-aminomethylcyclohexanecarboxylate (250 mg, 1 mmol; Hill et al., 1993) was added to a flask containing the acid **5** (250 mg, 0.84 mmol) and DCC (207 mg, 1 mmol) in dichloromethane cooled in an ice bath and stirred overnight. The mixture was filtered through Celite to remove dicyclohexylurea, and the filtrate was partitioned between dichloromethane and water/brine and then dried over $MgSO_4$. Following the concentration, the residue was chromatographed on silica [ethyl acetate/petroleum ether (2:8)] to obtain an alkene, **29** (352 mg, 78.5%): ^{1}H NMR ($CDCl_3$) δ 6.94 (d, =CH, J = 8.8 Hz), 5.9 (bs, NH), 4.15 (m, OCH_2), 3.17 (t, NCH_2, J = 8.0), 1.0−2.3 (m, cyclohexyl), 1.99 (t, CHC, J = 7.9 Hz), 1.81 (d, CHCO, J = 8.5 Hz), 1.23, 1.24 (2 × s, CH_3), 0.92 (m, CH_2-Si), and 0.02 [s, $Si(CH_3)_3$].

*N-(4′-Cyclohexylmethyl)carbamoylmethyl 3-(2′,2′-Dibromoethenyl)-2,2-dimethylcyclopropane carboxylate (**30**) and Succinimidyl Ester.* To the alkene **29** (352 mg, 0.785 mmol) in acetonitrile was added TEAF (351.5 mg, 2.36 mmol), and the solution was refluxed for 2 h. The residue was dissolved in dichloromethane after concentration and extracted with 1 M HCl, water, and brine, and then dried ($MgSO_4$). The residue was chromatographed on silica [ethyl acetate/petroleum ether (1:1)] to yield an acid **30**. The acid (300 mg, 0.79 mmol) was then treated with DCC (178.6 mg, 0.86 mmol), DMAP (10 mg), and NHS (90.4 mg, 0.79 mmol) in dichloromethane to produce a succinimidyl ester; ^{1}H NMR for **30** ($CDCl_3$) δ 6.89 (d, =CH, J = 8.8 HZ), 5.69 (bs, NH), 3.09 (t, NCH_2, J = 8.0), 1.0−2.3 (m, cyclohexyl), and 1.15, 1.26 (2 × s, 2 × CH_3).

*N-4′-(2′-Trimethylsilylethyloxycarbonyl)cyclohexylmethylcarbamoylmethyl 3-Phenoxybenzamide (**31**).* To 3-phenoxybenzoic acid (500 mg, 2.3 mmol) and DCC (496.8 mg, 2.4 mmol) in 10 mL of dichloromethane was added 2-(trimethylsilyl)ethyl *trans*-4-aminomethylcyclohexanecarboxylate (600 mg, 2.4 mmol), and the mixture was stirred overnight in an ice bath. After the solution was extracted with water and brine and concentration following the filtration, the residue was chromatographed on silica [methanol/chloroform (1:99)] to produce **31** (206.3 mg, 20%): ^{1}H NMR ($CDCl_3$) δ 7−7.45 (m, aromatic H), 6.44 (bt, NH, J = 5.3 Hz), 4.42 (t, OCH_2, J = 8.3), 3.28 (t, NCH_2, J = 6.5 Hz), 1.0−2.3 (m, cyclohexyl), 0.98 (m, CH_2Si), and 0.02 [s, $Si(CH_3)_3$]; ^{13}C NMR δ 175.9 (COOC), 166.9 (CON), 157.4, 156.3 (C3, C1′), 136.3 (C1), 129.7 (C3′, C5′), 123.5 (C6), 121.3 (C6′), 121.2 (C2), 118.9 (C2′, C4′), 117.5 (C4), 62.3 (CH_2O), 45.7 (CH_2N), 43.2 ($CHCH_2N$), 37.2 ($CHCO_2$, cyclohexyl), 32.3 ($CHCO_2$), 29.6 (2 × CH_2CHCO), 28.2 (2 × CH_2CHCH_2), 17.0 (CH_2Si), and −1.7 [$Si(CH_3)_3$].

*N-(4′-Cyclohexylmethyl)carbamoylmethyl 3-Phenoxybenzamide (**32**) and Succinimidyl Ester.* A mixture of compound **31** (206 mg, 0.46 mmol) and TEAF (149 mg, 1 mmol) in 10 mL of acetonitrile was reacted under reflux for 2 h. The solution was concentrated, dissolved in dichloromethane, and extracted with 1 M HCl, water, and brine. After drying, the residue was chromatographed on silica [ethyl acetate/petroleum ether/acetic acid (30:69.5:0.5)] to yield an acid **32** (100 mg, 100%). The acid (100 mg, 0.46 mmol) was then treated with DCC (142.7 mg, 0.69 mmol), DMAP (20 mg), and NHS

(79.4 mg, 0.69 mmol) in dichloromethane to yield a succinimidyl ester: ^{1}H NMR for **32** ($CDCl_3$) δ 7−7.4 (m, aromatic H), 6.3 (bt, NH, J = 5.7 Hz), 3.31 (t, NCH_2, J = 6.3 Hz), 1−2.3 (m, cyclohexyl); ^{13}C NMR δ 180.7 (COOH), 167.4 (CON), 157.8, 156.8 (C3, C1′), 136.7 (C1), 130.1 (C3′, C5′), 123.9 (C6), 121.7 (C6′), 121.6 (C2), 119.3 (C2′, C4′), 117.5 (C4), 46.1 (CH_2N), 43.2 ($CHCH_2N$), 37.5 (CHCOOH), 30.0 (2 × CH_2CHCH_2N), and 28.5 (2 × CH_2CHCO). ^{1}H NMR for the succinimidyl ester of **32** ($CDCl_3$) δ 7−7.4 (m, aromatic H), 6.3 (bs, NH), 3.38 (t, NCH_2, J = 8.0 Hz), 2.85 (s, 2 × CH_2CON), and 1−2.3 (m, cyclohexyl).

*(±)-3-Phenoxyphenyl-N-4′-(2″-trimethylsilylethyloxycarbonyl)cyclohexylmethylcarbamoylmethyl 3-(2′,2′-Dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate (**33**).* To a flask containing the acid **9** (200 mg, 0.46 mmol), DCC (149 mg, 0.72 mmol), and DMAP (20 mg) was added 2-(trimethylsilyl)ethyl *trans*-4-aminomethylcyclohexanecarboxylate (208.4 mg, 0.46 mmol), and the mixture was stirred overnight in an ice bath. Following the filtration and concentration, the residue was chromatographed on silica [ethyl acetate/petroleum ether (3:7)] to obtain compound **33** (59.3 mg, 14.8%): ^{1}H NMR ($CDCl_3$) δ 7−7.4 (m, aromatic H), 6.88 (d, =CH, J = 8.0 Hz), 6.84 (d, =CH, J = 8.0 Hz), 5.7 (bs, NH), 4.3 (t, OCH_2, J = 6.1 Hz), 3.26 (t, NCH_2, J = 6.1 Hz), 1−2.5 (m, cyclohexyl), 1.18, 1.24 (2 × s, 2 × CH_3), 0.96 (m, CH_2Si), and 0.02 [s, $Si(CH_3)_3$].

*(±)-3-Phenoxyphenyl-N-(4′-carboxycyclohexylmethyl)carbamoylmethyl 3-(2′,2′-Dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate (**34**).* TEAF was added to a flask containing compound **33** (59.3 mg, 0.068 mmol) in acetonitrile (10 mL) and was reacted under reflux for 2 h. The solution was concentrated, and the residue was dissolved in 10 mL of dichloromethane and extracted with 20 mL of 1 M HCl, water, and brine. The extractant was then dried over $MgSO_4$, concentrated, and chromatographed on silica [ethyl acetate/petroleum ether/acetic acid (30:69.5:0.5)] to yield acid **34** (17.4 mg, 54%). The acid was treated with DCC, DMAP, and NHS in dichloromethane to produce a succinimidyl ester: ^{1}H NMR for **34**: ($CDCl_3$) δ 7−4.4 (m, aromatic H), 6.23 (d, =CH, J = 8.9 Hz), 5.0 (s, NH), 3.7 (m, CH_2N), 2.1 (t, CHC, J = 8.9 Hz), 1.88 (d, CHCO, J = 8.4 Hz), 1.0−2.2 (m, cyclohexyl), and 1.27, 1.28, 1.29 (3 × s, 2 × CH_3).

Protein and Enzyme Conjugation. The conjugation of NHS esters to ovalbumin (OA), keyhole limpet hemocyanin (KLH), and horseradish peroxidase (HRP) was performed as follows. A 20 mole excess of NHS ester was added to OA or KLH or a 13 or 40 mole excess to HRP, with the protein being dissolved at 5 mg/mL in 0.2 M K_2HPO_4, pH 9.1. The solution was allowed to stand at 4 °C overnight, and the uncoupled hapten was removed by desalting on a Sephadex G-25 column (Pharmacia). Three enzyme conjugates based upon three different haptens coupled via a spacer arm containing an aromatic group were synthesized: a "bulky" spacer arm was placed on haptens based on (1) cypermethrin and conjugated through the middle of this molecule, (2) deltamethric acid, and (3) the phenoxybenzyl moiety. The concentration of coupled proteins was detetermined using the bicinchoninic acid assay (Smith et al., 1985). The coupling ratio was measured by using a method according to Plapp et al. (1971). Trinitrobenzenesulfonate (0.72 mg in 0.1 mL) was added to triplicate standards containing 0, 62.5, 125, 250, 500 μg/mL and samples in 0.05 M sodium borate buffer, and the absorbance was measured at 367 nm after 2 h.

Immunizations. New Zealand white rabbits were immunized with DEL, CYP, CYH, LHDEL, LHCYP, LHCYH, PBCY, FULPYR, and 4′OHDEL coupled to either OA or KLH, according to the procedure described in Lee et al. (1995). The OA and KLH conjugates of each immunogen (1 mg/mL) were emulsified with an equal volume of Freund's complete adjuvant (Sigma) and injected half-subcutaneously, half-intramuscularly, into New Zealand White rabbits. Following two booster injections 4 weeks apart, each containing 0.5 mg/mL immunogen in Freund's incomplete adjuvant (Sigma), blood was collected from the marginal ear vein 8−10 days after the last injection and clotted to form serum. IgG from the antisera were purified by protein G−agarose affinity chromatography (Bjorck et al., 1984).

Pyrethroid Standard Preparation. Pyrethroid standards at four to seven concentrations ranging from 0.1 to 1000 μg/L were prepared in 10% methanol from a 1000 mg/mL stock solution in methanol. The standards were 10× concentrations in methanol, diluted 1:10 with purified water. For a comparison, a 1000 μg/L standard was prepared in either 10% methanol or purified water and serially diluted to appropriate concentrations. Isomerized deltamethrin (see Results and Discussion) was obtained by adding 5 mM NaOH to 1000 mg/mL deltamethrin in methanol and allowed to stand at room temperature for about 6 h. The solution was then stored at −20 °C for use up to 4 weeks without an obvious loss of sensitivity; however, routinely 1000 mg/L isomerized deltamethrin in methanol was freshly prepared each week. Cypermethrin and λ-cyhalothrin were isomerized immediately prior to use as these compounds were shown to hydrolyze more rapidly than deltamethrin (see Results and Discussion). To avoid chemical and photochemical isomerization of pyrethroids (Ruzo et al., 1977; Maguire, 1990; Perschke and Hussain, 1992), standard curves were obtained by use of a freshly prepared 1.0 mg/mL stock solution in methanol.

Immunoassays. Immunoassays were performed using an immobilized antibody competitive immunoassay format. All steps were performed at room temperature (18−23 °C). Microwell plates (Maxisorp, Nunc) were coated for 16 h with antibody (100 μL/well) after dilution to 10 μg/mL in 50 mM sodium carbonate buffer, pH 9.6. Microwells were washed three times in 50 mM sodium phosphate/0.9% NaCl, pH 7.2 (PBS)/0.05% Tween 20 (PBS/T) and then nonspecific antibody binding blocked with 150 μL per well of 1% BSA in PBS for 1 h. The assay was performed by the addition of 100 μL/well diluted pyrethroid standard or sample (diluted initially in methanol and then 1:10 in water), and 100 μL/well enzyme conjugate (diluted in 1% BSA in PBS) and incubation for 1 h. After the microwells were washed five times with distilled water, color was developed by the addition of 150 μL/well 3,3′,5′-tetramethylbenzidine (Sigma)/peroxide-based substrate (Hill et al., 1991) for 30 min. Color development was stopped by addition of 50 μL/well 1.25 M sulfuric acid, and absorbance values were determined at 450 nm using a microplate reader, interfaced with a personal computer; data were fitted to a four-parameter logistic plot using SOFTmax (Molecular Devices, Menlo Park, CA). The optimal concentration of enzyme conjugate was determined for each assay as the lowest concentration required to produce assay color of 1.0−1.2 OD units. Initial characterizations of all the combinations were conducted by determining the percentage inhibitions for deltamethrin, cypermethrin, and λ-cyhalothrin at 10 μg/L. Those combinations providing >50% inhibition and utilizing a low concentration of enzyme conjugate were selected for further characterization.

RESULTS AND DISCUSSION

Hapten Synthesis Considerations. Two approaches were used in hapten synthesis. The first approach (using haptens derived from deltamethrin, cypermethrin, and λ-cyhalothrin) utilized various spacer attachment points to obtain antibodies with different selectivities (i.e., attachment site heterology), and the second approach was to utilize different linking groups in the conjugation to an enzyme to reduce the affinity of the antibodies for the spacer arm in immunoassays. Three series of haptens were synthesized with spacer attachment (1) at the aromatic moiety, (2) through the middle of the molecule, and (3) at the cyclopropane moiety. This approach was attempted to offset different regions of the molecules from the coupling point; it is likely that different conformations were also presented to the immune system since the target pyrethroids are not planar molecules. In addition, the reaction conditions used would possibly lead to isomerization during the synthesis; therefore, syntheses were carried out

using racemic mixtures without identification or separation of each stereoisomer. The active esters were relatively insoluble in the *N*,*N*′-dimethylformamide-buffer coupling solution, and the measured coupling densities with carrier proteins and peroxidase were relatively low compared with those of other conjugates [e.g. McAdam et al. (1992)], even using active esters at a 40 molar excess. The hydrophobic nature of these haptens probably provides less interaction of the active ester and the amino groups on lysine residues of the carrier protein.

Coupling at the Aromatic Moiety. Initial attempts to introduce a spacer arm at the aromatic moiety of the pyrethroid were through a nitro group introduced on the para position of the phenoxybenzyl group. This approach would provide a hapten with the cyclopropane moiety distal to the point of coupling, retaining the full pyrethroid structure. The synthetic route approached involved a multiple-step reaction, similar to that of Demoute et al. (1984), and was initiated by a reaction of *p*-nitrophenol and *m*-bromophenyl-1,3-dioxalane to form *p*-nitrophenoxybenzyl-1,3-dioxalane. A succinyl linker was formed by esterification of the reduced 1,3-dioxalane derivative with succinic anhydride. The deprotection of the 1,3-dioxalane group was achieved following the protection of the carboxylic acid of the succinyl linker with a *tert*-butyl group, and the resulting aldehyde was reacted with NaCN and water to form a cyanohydrin derivative. However, the esterification of the cyanohydrin derivative with deltamethric acid chloride to form a succinyl derivative of deltamethrin could not be achieved. Thus, various approaches were attempted, including a formation of a succinyl derivative after the esterification of deltamethric acid chloride with *p*-nitrophenoxybenzyl derivative. The reaction of the amino group with succinic anhydride, however, could not be achieved without a simultaneous removal of the cyano group. Use of 2-(trimethylsilyl)ethyl succinate instead of succinic anhydride resulted in a loss of a spacer arm through hydrolysis when esterification was performed with deltamethric acid. The next attempt was to form α-cyano-3-benzyl(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate, and a spacer arm was to attach at the meta position of the aromatic moiety via reduction to produce hydroxy derivative. This approach, however, produced multiple products with very low yields, while a reaction of 4-(benzyloxy)phenol and 2-(3-bromophenyl)-1,3-dioxalane using Ullmann synthesis resulted in varying degrees of success. A simple two-step reaction was, therefore, used to introduce a spacer on the aromatic moiety. Deltamethrin hemisuccinate was prepared by the esterification of deltamethrin alcohol with succinic anhydride. The deltamethrin alcohols (at the 2- and 4-positions) were prepared using lead(IV) trifluoroacetic acid (LTTFA) and have been assumed to maintain the original stereochemistry (Zhang and Scott, 1994). As noted by these authors, the yields of the alcohols produced in this manner were very poor but, in our hands, were sufficient to develop haptens for immunization and enzyme conjugation.

Conjugation through the Middle of the Molecule. This approach produced haptens retaining most of the structure of the original pesticide, except for the cyano group (Figure 1). Antibodies raised against these haptens would be expected to recognize both the cyclopropane moiety and the phenoxybenzyl moiety. The displacement of a cyano group with the carboxylic acid group may influence the affinity of the antibody. The cyano group of a cyanazine hapten was shown to be essential to raise antibodies specific for cyanazine, suggesting this group was a strong haptenic determinant (Lawruk et al., 1993). Hydrolysis of deltamethrin and α-cypermethrin (single isomer pyrethroids) and λ-cyhalothrin (four isomers) produced acids with one (for deltamethrin and α-cypermethrin) and four (for λ-cyhalothrin) stereoisomers, according to proton and carbon NMR data, presumably similar to the original compounds. Esterification of 3-phenoxymandelic acid with pyrethric acid chloride produced a pyrethroid analogue containing a carboxylic acid instead of a cyano substituent, allowing the formation of an amide derivative with *tert*-butyl-β-alanine. Three different pyrethric acids, deltamethric acid, permethric acid, and cyhalothric acid, were employed here to obtain structurally different haptens on the cyclopropane moiety. The esterification resulted in a number of isomers (according to NMR data), and the combined isomers were used in the succeeding step without an attempt to identify the stereochemistry of these isomers.

Coupling at the Cyclopropane Moiety. Coupling via the cyclopropane moiety was achieved by oxidation of the dimethylvinyl group. This approach enabled the aromatic moiety of pyrethroid to be offset from the coupling point but still retained the cyclopropane ring. An immunoassay developed using this synthetic principle provided sensitive detection of bioresmethrin in grain (Hill et al., 1993). In the present synthetic scheme, a different protecting group (*tert*-butyl ester) and a racemic mixture of chrysanthemic acid were used (Figure 2). The use of 2-(trimethylsilyl)ethanol was initially attempted, but deprotection of the trimethylsilyl group with tetraethylammonium fluoride could not be achieved without loss of the cyano group.

Synthesis of Metabolite Analogues. Haptens using the pyrethric acids of deltamethrin, cypermethrin, and λ-cyhalothrin and the phenoxybenzyl moieties, namely 3-phenoxybenzoic acid and 3-phenoxybenzaldehyde cyanohydrin, were also synthesized. The reaction of each pyrethric acid (deltamethric, cypermethric, and cyhalothric acids) with *tert*-butyl 3-aminopropanoate yielded a *tert*-butyl ester. The resulting product was a racemic mixture even though a single isomer of each deltamethric and cypermethric acid was used, indicating the acid had been isomerized.

Initial Screening of Antibody/Enzyme Conjugate Combinations. Each of the haptens was successfully coupled to carrier proteins and HRP, with coupling ratios as follows: (1) for DEL (compound **14**), 6.0 mol of hapten/mol of OA, 14.3 mol of hapten/mol of KLH, and 3.8 mol of hapten/mol of HRP; (2) for CYP (compound **15**), 3.3 mol of hapten/mol of OA, 4.5 mol of hapten/mol of KLH, and 2.3 mol of hapten/mol of HRP; (3) for CYH (compound **16**), 4.2 mol of hapten/mol of OA, 3.9 mol of hapten/mol of KLH, and 3.6 mol of hapten/mol of HRP; (4) for LHDEL (compound **24**), 5.7 mol of hapten/mol of OA, 8.7 mol of hapten/mol of KLH, and 4.7 mol of hapten/mol of HRP; (5) for LHCYP (compound **25**), 5.3 mol of hapten/mol of OA, 7.6 mol of hapten/mol of KLH, and 6.2 mol of hapten/mol of HRP; (6) for LHCYH (compound **26**), 5.8 mol of hapten/mol of OA, 3.2 mol of hapten/mol of KLH, and 1.5 mol of hapten/mol of HRP; (7) for FULPYR (compound **20**), 3.9 mol of hapten/mol of OA and 2.7 mol of hapten/mol of

Table 1. Inhibition by Unisomerized Deltamethrin (10 μg/L) of Various Antibody/Conjugate Combinations[a]

	antibody							
enzyme conjugate	DEL	CYP	CYH	PBA	LHDEL	LHCYP	LHCYH	4-OHDEL
DEL	*/*	−/*	*/*	*/*	*/−	−/*	−	*/−
CYP	*/*	−/*	*/*	−/*	*/−	*/−	−	*/−
CYH	*/**	−/*	−/*	−/*	−/−	−/−	−	−/−
PBA	−/*	*/**	*/−	*/*	*/−	−/−	−	−/−
PBCY	−/**	−/−	−/−	−/*	*/−	−/−	−	*/−
LHDEL	*/*	*/*	*/−	−/*	−/−	−/−	−	−/−
LHCYP	*/*	−/*	*/−	−/−	−/−	**/−	−	−/−
LHCYH	*/−	−/*	−/*	−/*	−/−	**/*	−	−/−
FULPYR	*/*	*/*	*/−	*/**	−/−	−/*	−	−/−
4OHDEL	−/*	**/*	−/*	*/*	−/−	−/*	−	*/−
2OHDEL	*/*	−/*	*/*	−/−	−/*	*/*	*	**/−
LHDEL-BULKY	**/**	−/*	*/*	*/*	*/*	*/*	*	*/*
PBA-BULKY	*/***	*/*	*/*	−/−	*/−	−/*	−	−/−
CYP-BULKY	*/*	*/*	*/*	*/*	*/*	*/*	−	−/−

[a] − denotes <10% inhibition, * denotes 10−25% inhibition, ** denotes 26−50% inhibition, *** denotes 51−75% inhibition, and **** denotes 76−100% inhibition of antibody binding. Asterisks on the left-hand side of the slash represent inhibition values for antibody raised against KLH conjugates and on the right-hand side for OA conjugates. The concentrations of enzyme conjugates used were optimized in preliminary experiments to provide an OD of 0.8−1.2. Data shown are means of quadruplicate determinations.

Table 2. Inhibition by Isomerized Deltamethrin (10 μg/L) of Various Antibody/Conjugate Combinations[a]

	antibody							
enzyme conjugate	DEL	CYP	CYH	PBA	LHDEL	LHCYP	LHCYH	4-OHDEL
DEL	*/*	****/***	***/*	**/*	***/**	*/**	*	*/**
CYP	**/***	***/**	****/*	*/*	**/**	***/***	**	*/*
CYH	*/**	**/**	*/*	*/**	*/*	*/*	*	−/*
PBA	***/−	**/**	**/*	**/*	*/*	−/−	−	−/−
PBCY	*/***	**/−	−/**	−/*	*/*	*/−	−	*/−
LHDEL	*/**	**/**	***/*	*/**	**/−	−/*	−	*/−
LHCYP	*/**	**/**	***/−	*/**	***/*	−/*	−	−/−
LHCYH	*/*	*/*	*/*	*/**	**/*	−/*	−	−/−
FULPYR	**/**	**/*	*/*	**/**	**/*	−/*	*	−/−
4OHDEL	−/*	***/**	**/**	*/*	*/**	−/**	*	*/−
2OHDEL	−/**	**/**	**/*	**/*	−/**	**/**	*	**/−
LHDEL-BULKY	*/**	*/**	**/**	**/**	*/*	*/*	*	*/*
PBA-BULKY	**/***	***/***	***/**	*/*	**/**	−/**	−	−/−
CYP-BULKY	*/*	*/*	*/*	*/*	*/*	*/*	−	−/−

[a] Other details as for Table 1.

KLH (FULPYR-HRP precipitated); (8) for 4OHDEL (compound **4**), 4.5 mol of hapten/mol of OA, 4.7 mol of hapten/mol of KLH, and 6.6 mol of hapten/mol of HRP; (9) for PBA, 4.4 mol of hapten/mol of OA, 3.1 mol of hapten/mol of KLH, and 7.5 mol of hapten/mol of HRP; (10) for PBCY (compound **28**), 5.9 mol of hapten/mol of OA, 9.1 mol of hapten/mol of KLH, and 6.2 mol of hapten/mol of HRP; (11) for 2OHDEL (compound **3**), 8.2 mol of hapten/mol of HRP; (12) for LHDEL-BULKY (compound **30**), 1.5 mol of hapten/mol of HRP; (13) for CYP-BULKY (compound **34**), 2.6 mol of hapten/mol of HRP; and (14) PBA-BULKY (compound **32**), 1.4 mol of hapten/mol of OA, 2.2 mol of hapten/mol of KLH, and 1.7 mol of hapten/mol of HRP.

The concentrations of enzyme conjugates were optimized in initial experiments to provide assay color development between 0.8 and 1.2 units. Fifteen antisera prepared using 8 haptens were each screened with 14 peroxidase conjugates, providing 210 assays for evaluation (Tables 1−4). To select antibody/enzyme conjugate combinations that provided relatively high sensitivity for one or more of the target compounds, inhibition values for deltamethrin at 10 μg/L (Table 1) and 1 mg/L were initially established, using the lowest conjugate dilutions that provided an OD of 0.8. Although a large proportion of antibody/conjugate combinations exhibited 30−70% inhibition for deltamethrin at 1 mg/L, only a few combinations were inhibited by >25% at 10 μg/L. Isomerization of pyrethroids has been noted as a part of their chemical and photochemical transformations (Ruzo et al., 1977; Maguire, 1990; Perschke and Hussain, 1992). The isomerization of deltamethrin in solvents such as methanol, acetone, and acetonitrile is thought to occur through a proton exchange with the solvent at the α-carbon of the benzyl group (Ruzo et al., 1977; Perschke and Hussain, 1992). It occurs only with the type II pyrethroids, such as deltamethrin, cyfluthrin, cypermethrin, and λ-cyhalothrin (Perschke and Hussain, 1992). Isomerization can be slowed by acidification and occurs only in solvents that hydrogen bond the proton at the α-carbon (Perschke and Hussain, 1992). Since it was anticipated that hydroxide ions would accelerate proton exchange with solvent, the effects of addition of NaOH to pyrethroid standards dissolved in methanol were investigated. The isomerization of pyrethroids in methanol was initially examined by adding different concentrations of NaOH in water [to 0.5% (v/v), final] in methanol containing a 1 g/L stock of deltamethrin and incubating for 30 min prior to preparation of deltamethrin standards in water (Figure 3A). The sensitivity of the assay increased with base concentration; presumably the extent of deltamethin isomerization in 30 min increased with base concentration (Figure 3A). The isomerization reached maximum at 5 mM NaOH. The isomerization of deltamethrin occurred almost immediately upon the addition of NaOH (Figure 3B). The rates of isomerization of cypermethrin and λ-cyhalothrin were somewhat

Table 3. Inhibition by Isomerized Cypermethrin (10 μg/L) of Various Antibody/Conjugate Combinations[a]

	antibody							
enzyme conjugate	DEL	CYP	CYH	PBA	LHDEL	LHCYP	LHCYH	4-OHDEL
DEL	*/*	*/**	*/–	**/*	*/–	–/*	–	–/–
CYP	**/**	*/–	**/–	**/***	*/–	**/*	*	–/–
CYH	*/**	**/*	*/–	*/**	–/*	–/–	–	–/–
PBA	***/**	****/****	**/**	**/**	*/–	–/–	–	–/–
PBCY	***/****	****/*	–/**	*/*	–/*	–/–	–	*/–
LHDEL	–/*	–/*	**/*	**/**	–/–	–/–	–	*/–
LHCYP	*/*	*/*	**/*	**/**	–/–	–/–	–	*/–
LHCYH	*/*	–/–	–/–	**/***	–/–	–/*	–	–/*
FULPYR	***/**	**/**	*/*	**/**	–/–	–/*	–	–/–
4OHDEL	–/*	****/***	**/***	**/***	–/*	–/*	–	*/–
2OHDEL	–/*	**/**	***/***	***/***	*/**	–/*	–	**/*
LHDEL-BULKY	**/**	***/***	****/****	***/**	*/*	*/*	*	*/*
PBA-BULKY	**/****	****/****	***/****	*/*	*/*	–/*	–	–/–
CYP-BULKY	*/*	*/*	*/*	*/*	*/*	*/*	–	–/–

[a] Details as for Table 1.

Table 4. Inhibition by Isomerized Cyhalothrin (10 μg/L) of Various Antibody/ Conjugate Combinations[a]

	antibody							
enzyme conjugate	DEL	CYP	CYH	PBA	LHDEL	LHCYP	LHCYH	4-OHDEL
DEL	*/*	*/*	*/*	**/*	*/*	*/**	*	–/–
CYP	**/*	*/–	**/*	**/***	*/**	***/**	*	*/*
CYH	*/**	*/–	–/*	**/***	–/**	*/*	*	**/**
PBA	***/**	***/***	**/**	**/**	*/–	–/–	–	–/–
PBCY	****/****	***/–	*/***	*/**	–/*	*/–	–	*/–
LHDEL	–/–	–/–	**/–	**/**	–/**	–/*	–	*/–
LHCYP	–/*	–/*	**/*	**/***	*/*	*/*	–	*/–
LHCYH	*/*	–/*	*/–	**/***	*/*	–/**	–	–/*
FULPYR	****/**	***/*	*/*	**/***	*/**	–/*	*	–/–
4OHDEL	*/**	****/***	**/**	***/***	–/–	–/–	*	*/–
2OHDEL	*/*	****/***	***/**	***/***	*/**	*/*	*	**/–
LHDEL-BULKY	***/**	***/****	***/***	***/***	*/*	*/*	*	*/*
PBA-BULKY	**/****	***/****	**/***	*/*	*/**	–/**	–	–/–
CYP-BULKY	*/*	*/*	*/*	*/*	*/*	*/*	–	–/–

[a] Details as for Table 1.

slower, with maximum sensitivities obtained after 15 and 30 min, respectively, from the addition of NaOH (data not shown). Using the antibody/conjugate combination described above, there was at least a 20-fold increase in assay sensitivity after the alkali treatment. There was a gradual loss of sensitivity on extended exposure to alkali, probably due to hydrolysis; the loss of sensitivity was greater for λ-cyhalothrin than cypermethrin. Isomerization of 1 g/L stock pyrethroid standard in methanol was achieved routinely by adding 5 mM NaOH, and isomerized deltamethrin was stored at −20 °C for up to 4 weeks without an obvious loss of assay sensitivity, while the other pyrethroids were treated immediately before use. The increases in assay sensitivity with alkali treatment were quite marked; for example, with antibody DEL-OA and conjugate CYP-HRP (Figure 3) a 40-fold increase in sensitivity was noted.

Inhibition data for isomerized deltamethrin, cypermethrin, and λ-cyhalothrin at 10 μg/L are shown in Tables 2−4. This screening method enabled the selection of antibody/enzyme conjugate combinations that provide high (>40%) inhibition at this concentration of one or more of these pyrethroids; combinations that exhibited poorer inhibition were not investigated further. Twenty-six antibody/conjugate combinations were selected for further study on the basis of the assay sensitivity, dynamic behavior, and specificity for deltamethrin, cypermethrin, and cyhalothrin. The concentrations of conjugate used routinely in selected immunoassays to provide color development between 0.8 and 1.2 OD units were as follows: 0.31 μg/mL in the CYP-OA/LHCYP-HRP combination, 0.01 μg/mL in CYH-KLH/LHCYP-HRP, 1.6 μg/mL in DEL-OA/LHCYP, 1.6 μg/mL in PBA-OA/LHCYP, 0.03 μg/mL in LHDEL-KLH/LHCYP-HRP, 0.9 μg/mL in PBA-OA/LHCYH-HRP, 0.05 μg/mL in LHDEL-KLH/LHCYH-HRP, 0.06 μg/mL in LHDEL-KLH/LHDEL-HRP, 0.06 μg/mL in DEL-OA/LHDEL-HRP, 0.005 μg/mL in CYH-KLH/LHDEL-HRP, 0.06 μg/mL in CYP-OA/LHDEL-HRP, 0.06 μg/mL in CYP-KLH/LHDEL-HRP, 0.4 μg/mL in CYP-KLH/CYP-HRP, 0.04 μg/mL in CYH-KLH/CYP-HRP, 0.4 μg/mL in DEL-KLH/CYP-HRP, 0.4 μg/mL in DEL-OA/CYP-HRP, 2 μg/mL in PBA-OA/CYP-HRP, 0.8 μg/mL in LHCYP-KLH/CYP-HRP, 4 μg/mL in CYP-KLH/DEL-HRP, 0.2 μg/mL in CYP-OA/DEL-HRP, 0.05 μg/mL in CYH-KLH/DEL-HRP, 0.04 μg/mL in LHDEL-KLH/DEL-HRP, 1.2 μg/mL in PBA-OA/CYH-HRP, 4.9 μg/mL in FULPYR-KLH/4OHDEL, 4.9 μg/mL in DEL-OA/4OHDEL-HRP, and 0.5 μg/mL in PBA-BULKY-HRP. In general, most of the combinations showing high inhibition were heterologous immunoassays (i.e. different haptens used for immunization and conjugation to the reporter enzyme). A few exceptions, such as the LHDEL-KLH/LHDEL-HRP and CYP-KLH/CYP-HRP combinations, were observed.

An antibody was raised against the 3-phenoxybenzyl alcohol hapten to investigate its potential use in a generic method for detection of pyrethroids with a phenoxybenzyl moiety. Of all the antisera studied in this paper, these antisera gave the highest titer with the corresponding haptens in indirect ELISA. They detected phenoxybenzyl metabolites at relatively low micrograms per liter concentrations. However, the

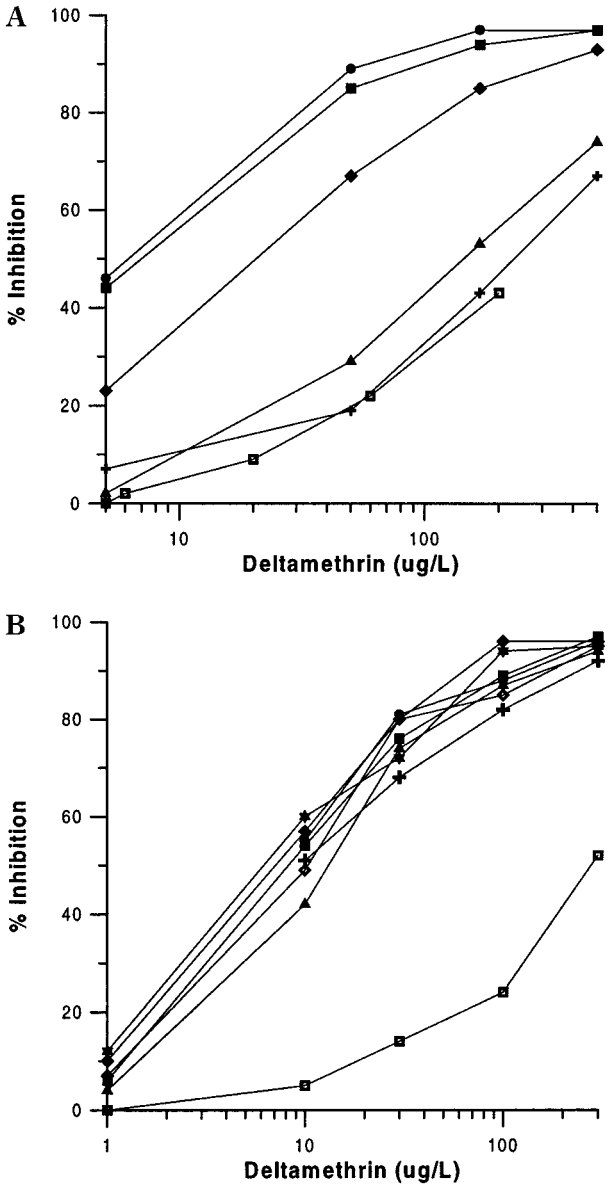

Figure 3. Effects of addition of NaOH on sensitivity for deltamethrin determined after (A) isomerization for 30 min with NaOH at 0 (□), 0.05 (+), 0.1 (▲), 1 (◆), 5 (■), and 10 mM (●) (data shown are final concentrations of NaOH in methanol containing 0.5% water) and (B) isomerization using 5 mM NaOH (final) after 0 min (◇), 10 min (●), 30 min (■), 1 h (◆), 2 h (▲), 5 h (+), and 24 h (★). The unisomerized deltamethrin is indicated by □. Data were generated using antibody DEL−OA and CYP−enzyme conjugate and are the means of two determinations in triplicate. Standard deviations of percent inhibition did not exceed 10%; error bars are omitted for clarity.

antibodies did not provide sufficient sensitivity for the detection of pyrethroid parent compounds containing the phenoxybenzyl moiety. Wraith et al. (1986) raised antibody against 3-phenoxybenzoic acid and found that the antibody exhibited a low sensitivity for both phenoxybenzoic acid and cypermethrin (IC_{50} of 1 mg/L). However, the phenoxybenzyl derivative coupled to protein was successfully used as an immobilized antigen in a sensitive immunoassay for permethrin and phenothrin, providing a lower detection limit of 1.5 μg/L permethrin (Skerritt et al., 1992).

Enzyme conjugates with haptens containing the phenoxybenzyl moiety, such as PBA, PBCY, and PBA-BULKY, usually showed >40% inhibition for both isomerized (Tables 3 and 4) and unisomerized forms (data not shown) of cypermethrin and cyhalothrin, when used in combinations with antibodies to DEL, CYP, CYH, and PBA. However, the concentrations of the conjugates used in these immunoassays were extremely high (23−35 μg/mL) and the stability of the conjugates was poor. Poor stability of conjugates was defined as an irreversible loss in assay color when the conjugate is stored for >1 month at 2 mg/mL at 4−6 °C. Poor stability was also observed for 2OHDEL-HRP and 4OHDEL-HRP. Conjugates based on CYP-BULKY did not show a sufficient inhibition of color development for deltamethrin, cypermethrin, and cyhalothrin (unisomerized and isomerized), possibly due to the low coupling density. On the other hand, the LHDEL-BULKY-HRP conjugate showed a high inhibition at 10 μg/L in conjunction with several antibodies but exhibited poor stability. For this reason some rather sensitive antibody/enzyme conjugate combinations, such as the CYP or CYH antibodies with either 2OHDEL-HRP, 4OHDEL-HRP, or LHDEL-BULKY-HRP, were not studied in detail. Only the PBA-BULKY enzyme conjugate appeared to be sufficiently stable to extended storage (6 months at 4 °C). Most of the combinations with the unstable enzyme conjugates were eliminated from the selection, except for CYH-KLH/PBA-BULKY, DEL-OA/4OHDEL, and FULPYR-KLH/4OHDEL combinations; these three combinations gave reasonably reproducible results. Other combinations with inhibition values >40% did not show these effects.

Assay Sensitivities for Deltamethrin, Cypermethrin, and Cyhalothrin. Initially, the potential adsorption of deltamethrin, cypermethrin, and λ-cyhalothrin was examined by preparing standard curves in both water and 10% methanol and incubating in untreated glass tubes and in glass tubes treated with 5% (w/v) poly(ethylene glycol) (PEG; molecular weight 20 000), using the method described by Helmuth et al. (1983). Standards of each of the three pyrethroids were diluted to 1 to 500 mg/L and sat in glass tubes for various times up to 1 h before addition to the antibody-coated microwell plate and analysis using an antibody to CYH-KLH and the LHCYP-HRP conjugate. The adsorption was determined by reduction in the assay sensitivity obtained as IC_{50} and IC_{15} of the standard curves. No noticeable adsorption was observed for deltamethrin or cypermethrin, but up to 30% of the λ-cyhalothrin in both water and 10% methanol was adsorbed onto the untreated and 15% onto the treated glass tubes after 60 min of incubation. Thus, although the PEG treatment reduced the adsorption, the assay could be performed without this treatment if the pyrethroid standards were used within 30 min of preparation, to minimize significant loss of pyrethroid in standard solutions.

The water solubilities of pyrethroids are in the very low micrograms per liter range (Lefebvre et al., 1993); thus, erroneous data could be obtained if pyrethroid was not fully soluble under the assay conditions. To determine the greatest deltamethrin concentration at which standards could be prepared, a series of stocks at different concentrations in methanol were diluted 1:10 in water to produce standards and assayed in the ELISA. Stocks of 200 μg/L and below provided the most sensitive assays, suggesting that deltamethrin remained in true solution after dilution. These data emphasize that the solubilities of pyrethroids in aqueous solution

Table 5. IC_{50} Values of Deltamethrin, Cypermethrin, and Cyhalothrin for Different Antibody/Enzyme Conjugate Combinations

		IC_{50} (μg/L)					
antibody	enzyme conjugate	DEL[a]	DEL[b]	CYP[a]	CYP[b]	CYH[a]	CYH[b]
DEL-OA	LHDEL	–	33	–	–	–	–
DEL-OA	LHCYP	–	25	–	–	–	–
DEL-OA	CYP	130	34	–	80	–	7
DEL-OA	4′OHDEL	–	16	–	–	–	–
FULPYR-KLH	4′OHDEL	210	4	160	82	650	200
LHDEL-KLH	DEL	70	2	1000	10	–	–
LHDEL-KLH	LHCYH	–	2	–	45	–	–
LHDEL-KLH	LHDEL	–	3	–	20	–	–
LHDEL-KLH	LHCYP	–	4	–	18	–	–
CYP-KLH	DEL	–	9	–	–	–	–
CYP-KLH	LHDEL	–	4	–	–	–	–
CYP-KLH	CYP	150	2	–	8	–	–
DEL-KLH	CYP	50	5	60	200	–	–
CYH-KLH	PBA-BULKY	220	4	320	31	–	–
CYH-KLH	DEL	230	9	–	40	1000	65
CYH-KLH	LHDEL	–	4	–	60	–	50
CYH-KLH	LHCYP	250	2	85	13	–	12
CYH-KLH	CYP	50	4	70	22	1000	11
LHCYP-KLH	CYP	60	2	320	15	–	70
CYP-OA	DEL	180	2	1000	30	–	–
CYP-OA	LHDEL	–	6	–	86	–	–
CYP-OA	LHCYP	–	9	–	–	–	–
PBA-OA	LHCYH	–	8	–	7	–	16
PBA-OA	LHCYP	600	4	–	4	1000	5
PBA-OA	CYH	–	120	–	–	–	48
PBA-OA	CYP	–	5	1000	3	1000	10

[a] Unisomerized compounds. [b] Isomerized compounds. DEL, deltamethrin; CYP, cypermethrin; CYH, cyhalothrin. Dash indicates IC_{50} >1000 μg/L.

and the adsorption of pesticide onto the container wall could be important factors affecting the performance of the immunoassay.

Twenty-six combinations of antibody and enzyme conjugate were selected on the basis of the inhibition data for deltamethrin, cypermethrin, λ-cyhalothrin, and the isomerized forms of these compounds at 10 μg/L. The results obtained are summarized in Table 5. Each immunoassay was further characterized by determining the sensitivity (determined as IC_{50}), limit of detection (determined as IC_{15}), and dynamic behavior (difference in percentage inhibition between 1 and 10 μg/L) for isomerized and unisomerized deltamethrin, cypermethrin and λ-cyhalothrin. The most sensitive immunoassay for deltamethrin was obtained using antibody to LHCYP-KLH and CYP-HRP conjugate, providing an IC_{50} of 2 μg/L and a limit of detection of 0.2 μg/L of the isomerized deltamethrin, and several other assays had a similar IC_{50} but slightly higher limits of detection. Antibodies raised and conjugates prepared to haptens containing dihalovinylcyclopropane moieties provided sensitive immunoassays, suggesting that the antibody specificity may be directed toward this portion of the molecule rather than the aromatic groups. One possible explanation for this would be the greater distance between the 2′-vinyl carbon of the cyclopropane moiety to the coupling point than the C3′ of phenoxybenzyl group as determined from molecular modeling of the hapten coupled through the middle of the molecule, providing a greater distance in the former haptens from the carrier protein, facilitating antibody recognition. On the other hand, the aromatic part of the pyrethroid plays an important role in establishing sensitive assays. First, the antibodies generated to haptens that contained only the dihalovinyl cyclopropane moieties usually detected the three pyrethroids less sensitively than those raised to the full synthetic pyrethroid or to phenoxybenzoic acid. Second, some of the assays using enzyme conjugates derived from phenoxybenzoic acid were highly sensitive (Tables 1−4). Most of the selected immunoassays had moderately steep standard curves, with a >30% difference in inhibition for 10-fold difference in deltamethrin concentration; only three antibody/enzyme conjugate combinations showed poorer dynamic behavior, including FULPYR-KLH/4OHDEL-HRP (22% difference in inhibition), CYH-KLH/DEL-HRP (23%), and PBA-OA/LHCYP (28%).

While several of these immunoassays have high sensitivity for isomerized deltamethrin, different selectivities were obtained by using different enzyme conjugates. For example, when the antibody to DEL-OA was used with enzyme conjugates based upon LHDEL, LHCYP, and 4OHDEL, immunoassays were selective for isomerized deltamethrin, which largely contains insecticidally inactive isomers. However, when CYP-HRP was used with this antibody, the assay detected both isomerized deltamethrin and cyhalothrin but only relatively poorly detected cypermethrin, even though a derivative of this compound was used in the conjugate. The poor ability of cypermethrin to displace antibody binding may relate to the halogen substituents being smaller that those on the other pyrethroids. Another example is the antibody raised against PBA-OA. The immunoassays using this antibody typically provided a broad selectivity, but the degree of the cross-reaction for each compound varied with different enzyme conjugates. The PBA-OA antisera/LHCYP (cyclopropane moiety of cyhalothrin)-HRP combination enabled the detection of deltamethrin, cypermethrin, and λ-cyhalothrin (in the isomerized forms) at similar sensitivities. The cross-reaction of cyhalothrin decreased to 50% (compared with the other conjugates) when LHCYH-HRP was used. When an enzyme conjugate using a hapten derived from the entire pyrethroid molecule was used in a combination with antibody to PBA-OA, the assay sensitivity increased significantly. In addition,

Table 6. IC_{50} Values for Deltamethrin Isomers

		IC_{50} (μg/L)							
antibody	enzyme conjugate	DM1	DM2	DM3	DM4	DM1′	DM2′	DM3′	DM4′
DEL-OA	CYP	52	11	–	420	–	–	–	–
DEL-KLH	CYP	60	5	–	–	–	–	–	–
CYP-OA	DEL	480	10	–	–	–	–	–	–
CYH-KLH	LHDEL	140	16	–	–	–	–	–	–
CYH-KLH	LHCYP	73	16	–	700	–	–	–	–
CYH-KLH	CYP	120	3	–	190	–	–	–	–
CYH-KLH	PBA-BULKY	175	29	–	950	–	–	–	–
LHDEL-KLH	DEL	130	40	–	825	–	–	–	–
LHDEL-KLH	LHDEL	170	25	–	–	–	–	–	–
LHDEL-KLH	LHCYP	150	21	–	–	–	–	–	–
LHCYP-KLH	CYP	160	4	–	510	–	–	–	–
FULPYR-KLH	4′OHDEL	155	55	–	–	–	–	–	–

[a] Deltamethrin isomers are as follows: DM1, 1*R cis* α*S*; DM1′, 1*S cis* α*R*; DM2, 1*R cis* α*R*; DM2′, 1*S cis* α*S*; DM3, 1*R trans* α*S*; DM3′, 1*S trans* α*R*; DM4, 1*R trans* α*R*; DM4′, 1*S trans* α*S*. The stereochemistry of isomers is indicated in order of geometry of the double bond of the 3-substituent in the cyclopropane ring, configuration at the 1- and 3-positions in the cyclopropane ring, and configuration of the cyano-bearing carbon of the alcohol component. Dash indicates IC_{50} >1000 μg/L.

with the use of CYP-HRP the cross-reaction for cypermethrin increased, while cross-reaction for cyhalothrin decreased. The cross-reaction for cyhalothrin increased when CYH-HRP was used in the immunoassay. Thus, both the specificity and the sensitivity of the immunoassay can be manipulated by the use of different enzyme conjugates for a given antiserum.

Stereoselectivity of Antibodies to Deltamethrin Isomers. The stereoselectivity of the antibodies (using 12 different antibody/enzyme conjugate combinations) was determined by comparing standard inhibition curves for eight deltamethrin isomers (Table 6). Only two of the eight deltamethrin isomers, DM1 (the insecticidally active isomer of deltamethrin, the α*S*, 1*R cis* isomer) and DM2 (the α*R*, 1*R cis* isomer), were detected, with DM2 (the α*R*, 1*R trans* isomer) being detected more sensitively than DM1. Some antibodies were also capable of detecting DM4, but at very high concentrations. These three isomers have in common the *R* configuration at C1 and C3 of the cyclopropane ring, suggesting that the antibodies may be specific for 1*R*,3*R* configuration at the cyclopropane moiety. This degree of isomer specificity was unexpected as antibodies were raised against mixtures of isomers. A possible explanation is that isomerization of the hapten in the immunogen occurred in vivo and/or the biodegradation rates of each isomer differ in vivo. Indeed, the α*S*, 1*S cis* configuration is unstable at room temperature (Roussel Uclaf, unpublished data), and the antibody response to freshly prepared dilutions of the isomers with the *S* configuration at the C1 and C3 of cyclopropane ring was minimal.

GENERAL DISCUSSION

In this study, we have attempted to evaluate systematically strategies for the development of haptens for the type II pyrethroids, utilizing a range of hapten coupling positions. The hapten synthesis approaches combined both novel strategies and adapted concepts that had been successful for development of pyrethroid haptens in previously reported studies. The development of haptens based on an entire pyrethroid molecule for immunogens has been reported for the type I pyrethroids, phenothrin/permethrin (Stanker et al., 1989), and bioresmethrin (Hill et al., 1993). In contrast to their approach, we found only the full pyrethroid hapten (coupled through the vinyl group) in the HRP conjugate to be useful in the assays. The synthesis described in Figure 1 used the same general approach as the bioresmethrin hapten synthesis, although chrysanthemic acid was used for re-esterification with the aromatic moiety. Even though ozonolysis of the vinylic side chain of the pyrethroid to produce a carboxylic acid, followed by the direct coupling of the acid to a carrier protein, was successful for synthesis of phenothrin haptens (Stanker et al., 1989), this approach was not successful for dihalovinyl pyrethroids.

Demoute et al. (1986), in a patent application, were the first to attempt hapten synthesis for a cyanopyrethroid, deltamethrin, but no immunoassay was described. The approach was to introduce a linker on the 3-phenoxybenzyl end of the molecule. Their synthesis involved six steps. We utilized a much simpler approach based on direct derivatization of deltamethrin with lead(IV) trifluoroacetate to produce a phenol derivative for subsequent coupling. In developing the immunoassay for the human metabolites of fenvalerate and fenpropathrin, Wengatz et al. (1994) also synthesized the pyrethroid analogues with an amine substitution on the aromatic group. Attempts to synthesize haptens using the pyrethroid metabolites were first reported by Wraith et al. (1986). The antibody was produced to a protein conjugate prepared via the three-carbon chain length spacer arm with 3-phenoxybenzoic acid and dichlorovinyldimethylcyclopropanecarboxylic acid. The antibody recognized 3-phenoxybenzoic acid and cypermethrin but not dichlorovinylcyclopropanecarboxylic acid. The assay was applied to soil and water even though the limit of detection was very high, with an IC_{50} of 1 mg/L. Direct coupling of (1*R*)-*cis*-permethric acid and (1*R*)-*trans*-permethric acid to the carrier proteins via carbodiimide activation of the carboxylic acids was reported by Pullen and Hock (1995a,b) in the course of our study. Both polyclonal and monoclonal antibodies were generated, showing different cross-reactions. The monoclonal antibody (Pullen and Hock, 1995a) detected the type I pyrethroids allethrin, bioallethrin, *S*-bioallethrin, permethrin, bioresmethrin, and pyrethrin, suggesting the dimethylcyclopropane moiety was a haptenic determinant group. Allethrin was detected with the most sensitivity and a limit of detection of 1 μg/L. The polyclonal antibody (Pullen and Hock, 1995b) detected permethrin, cyfluthrin, cypermethrin, *S*-bioallethrin, and *trans*-permethric acid with a detection limit of ~0.4 μg/L. The haptenic determinant group appeared to be (1*R*)-*trans*-dichlorovinyldi-

methylcyclopropane moiety. Both antibodies, however, were unable to detect deltamethrin or other type II pyrethroids containing different halogen atoms, such as λ-cyhalothrin. Bonwick et al. (1994) have also reported the synthesis of haptens based on permethrin hydrolysis products, but in this case using either 6-aminohexanoic acid or 4-aminobutanoic acid spacer arms to link the permethric and phenoxybenzoic acids to protein carriers. The best assay using, the latter hapten, was not particularly sensitive (IC_{50} of 1 mg/L).

The syntheses we undertook provided a series of immunogens and enzyme conjugates differing in the structures of the haptens and the spacer arms, and in the coupling point, thus exposing different portions of pyrethroid for antibody recognition. The resulting antibodies provided more than two dozen useful immunoassays with different selectivities for deltamethrin, cypermethrin, and λ-cyhalothrin in both unisomerized and isomerized forms. Isomerization of deltamethrin, cypermethrin, and λ-cyhalothrin was achieved by simply adding 5 mM NaOH to the stock standard in methanol, and the rate of isomerization was in the order deltamethrin > cypermethrin > λ-cyhalothrin, with deltamethrin being the most tolerant to hydrolysis. In general, the sensitivity was greater for isomerized compounds than for unisomerized compounds. The most sensitive immunoassay enabled detection of deltamethrin as low as 0.2 μg/L (isomerized). Assay specificity was able to be manipulated by varying the haptens coupled to the detection enzyme. The antibody specificities were usually determined by the portion distal to the carrier protein. That is, antibodies raised to the aromatic end of pyrethroids were able to detect compounds with this group, such as cypermethrin, deltamethrin, λ-cyhalothrin, permethrin, esfenvalerate, fluvalinate, and phenothrin, and antibodies generated from the cyclopropane end generally detected compounds with the cyclopropane moiety, such as deltamethrin, cypermethrin, λ-cyhalothrin, permethrin, tetramethrin, bioallethrin, allethrin, *S*-bioallethrin, cyfluthrin, and bifenthrin [data in the accompanying paper, Lee et al. (1998)]. This initial development provides a basis for further selection of the most useful immunoassays and a complete characterization of assay sensitivity, specificity, and precision. The specificity properties and validation of the performance of these assays using spiked water, soil, and grain samples and relationships between immunoassay data with GC/MS analyses for spiked and field samples are described in the following paper (Lee et al., 1998).

ABBREVIATIONS USED

BSA, bovine serum albumin; DCC, dicyclohexylcarbodiimide; DMAP, dimethylaminopyridine; GLC, gas−liquid chromatography; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; IC_{50}, concentration producing 50% inhibition of antibody binding; KLH, keyhole limpet hemocyanin; NHS, *N*-hydroxysuccinimide; OA, ovalbumin; TEAF, tetraethylammonium fluoride; TFA, trifluoroacetic acid; THF, tetrahydrofuran; (abbreviations for haptens) DEL, compound **14**; CYP, compound **15**; CYH, compound **16**; LHDEL, compound **24**; LHCYP, compound **25**; LHCYH, compound **26**; FULPYR, compound **20**; 4OHDEL, compound **4**; PBA, phenoxybenzioc acid; PBCY, compound **28**; 2OHDEL, compound **3**; LHDEL-BULKY, compound **30**; CYP-BULKY, compound **34**; PBA-BULKY, compound **32**.

ACKNOWLEDGMENT

We thank Mr. A. Rigg for assistance with the synthesis of haptens DEL and PBCY. We are grateful to Dr. M. H. Black, Director of Research and Development, AgrEvo Environmental Health Ltd., Berkamsted, Herts, U.K., for provision of deltamethrin isomers. We acknowledge the advice and assistance of R. Mayadunne and Professors L. Mander and J. Elix (Australian National University) and Dr. J. E. Nemorin (University of Sydney).

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Received for review May 23, 1997. Revised manuscript received November 3, 1997. Accepted November 10, 1997. We are grateful to the Australian Cotton Research and Development Corporation for financial support.

JF970438R