Hapten Synthesis for a Monoclonal Antibody Based ELISA for Deltamethrin

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An enzyme-linked immunosorbent assay (ELISA) was developed for the insecticide deltamethrin. Two haptens were synthesized: the first one was a derivative of the whole molecule with a spacer arm bound to the aromatic ring; the second one was a derivative of deltamethric acid. Twelve monoclonal antibodies were obtained. A competitive ELISA using monoclonal antibody Del 01 yielded the most sensitive assay. The IC_{50} value for deltamethrin was estimated to be 0.5 μg mL^{-1} with a detection limit of 0.08 μg mL^{-1}. Monoclonal antibody Del 01 seems to be deltamethrin specific since <1% cross-reactivity with other pyrethroid analogues was observed.

Keywords: *Deltamethrin; ELISA; hapten; monoclonal antibodies*

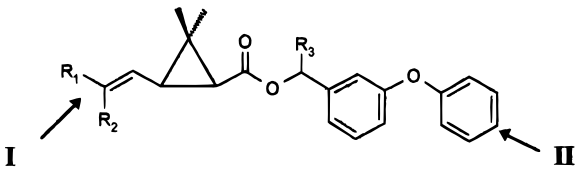
INTRODUCTION

Deltamethrin, 1(*R*)-*cis*-α(*S*)-3-(2,2-dibromoethenyl)-2,2-dimethylcyclopropane carboxylic acid cyano(3-phenoxyphenyl)methyl ester, is one of the most potent insecticides known. It is a synthetic pyrethroid developed by Elliot et al. (1974). Deltamethrin is widely used because of its excellent insecticidal properties (Elliot, 1976) and low mammalian toxicity (Mestres and Mestres, 1992). Classical methods for pyrethroid analysis (Papadopoulou-Mourkidou, 1983) include gas chromatography (Sharp et al., 1988) with electron capture detection for halogenated derivatives (Okadu et al., 1983) or gas chromatography/mass spectrometry (Mestres et al., 1979). These procedures are time-consuming and require expensive equipment.

To respond to the increasing number of analyzed samples, it appeared necessary to develop fast and economic techniques. Since Hammock and Mumma (1980) pointed out that immunoassays could be used to detect pesticide residues, it has been demonstrated that immunoassays enable quick screening of samples (Hall et al., 1990; Jung et al., 1989; Newsome, 1986).

Several mono/polyclonal-based immunoassays dealing with pyrethroids have been reported; they included allethrin (Pullen and Hock, 1995a,b), *S*-bioallethrin (Wing et al., 1978), cypermethrin (Wraith et al., 1986), deltamethrin (Demoute et al., 1986), bioresmethrin (Hill et al., 1993), permethrin/1(*R*)-phenothrin (Stanker et al., 1989; Skerritt et al., 1992), and permethrin (Bonwick et al., 1994). Because of its low molecular weight, deltamethrin, like most of pesticides, is not immunogenic. It must be conjugated to a carrier protein to induce an immune response. To be bound to the carrier, the pesticide must contain a functional group. Since deltamethrin has none, it was necessary to synthesize an analogue that mimicked deltamethrin. Since an antibody's specificity depends on both the hapten nature, that is, the entire molecule or part of it, and the position of the binding site on the carrier protein, different approaches could be considered.

Table 1. Structure of Several Pyrethroids



name	R_1	R_2	R_3
deltamethrin	Br	Br	CN
phenotrin	CH_3	CH_3	H
permethrin	Cl	Cl	H
cypermethrin	Cl	Cl	CN
λ-cyalothrin	CF_3	Cl	CN

The first one is the use of the whole molecule. Pyrethroids (Table 1) exhibit the same chemical structure, that is, a phenoxybenzyl moiety esterified by a molecule of chrysanthemic acid of which the ethylenic chain is generally substituted by a halogen (Cl, Br, F). Pyrethroid structure suggests at least two possible positions for the binding of the spacer arm as illustrated for deltamethrin in Table 1 (position I or II).

(i) Spacer arm binding on position I, that is, on the ethylenic chain, leaves the aromatic ring free. Stanker et al. (1989) used this method to obtain antibodies against permethrin. They synthesized the required hapten by ozonolysis of phenothrin. Monoclonal antibodies produced by Stanker et al. (1989) were specific to phenothrin and permethrin and presented cross-reactivities of 0.5−10% with pyrethroids carrying a cyano group. To produce antibodies against bioresmethrin, that is, a pyrethroid with a benzylfuran moiety instead of phenoxybenzyl, Hill et al. (1993) used position

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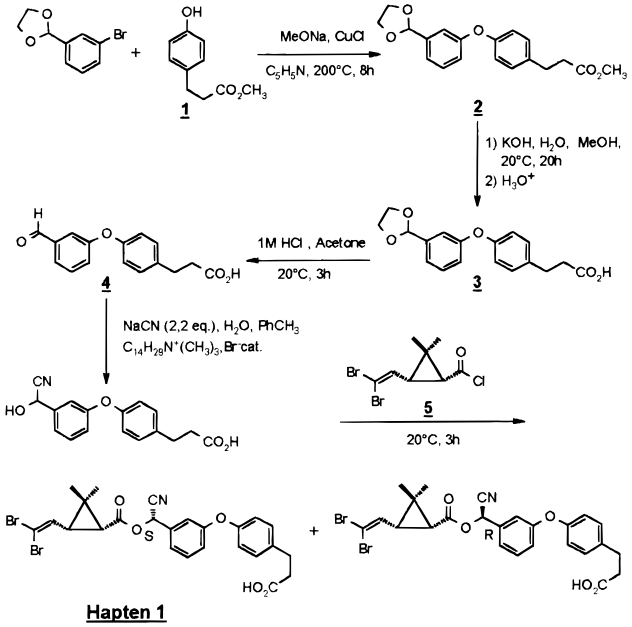

Figure 1. Hapten **1** synthesis pathway.

I to bind the spacer arm. Polyclonal antibodies obtained were specific to the 1*R* trans isomer (bioresmethrin) and presented a cross-reactivity with the 1*R*,*S* cis,trans isomer (resmethrin).

(ii) When the spacer arm is on position II (linked to the aromatic ring), most of haptenic determinant groups remain free, preserving the dibromovinylcyclopropane part of the molecule, which is characteristic of deltamethrin. Demoute et al. (1986) used this method to synthesize deltamethrin hapten, which was used in a radioimmunoassay. The spacer arm was linked on the aromatic part of deltamethrin. There were no results on the sensitivity and specificity of the radioimmunoassay.

The second approach is the use of pyrethric acid to produce antibodies. The functional group −COOH allowed a direct coupling of the hapten to the carrier protein. Pullen and Hock (1995a) conjugated 1(*R*)-*trans*-permethric acid to BSA to produce monoclonal antibodies for allethrin. The assay was more sensitive for *S*-bioallethrin and showed a 43% cross-reactivity with natural pyrethrins. Bonwick et al. (1994) coupled permethric acid to the carrier protein through a 6-C spacer arm to generate polyclonal antibodies against permethrin. Cross-reactivities were observed for phenothrin, cyfluthrin, and deltamethrin.

In the present work these two approaches were used to prepare two haptens. We simplified the synthesis of Demoute et al. (1986) to prepare an analogue of deltamethrin (hapten **1**; Figure 1). Another hapten was obtained from deltamethric acid (hapten **2**; Figure 2). These two haptens were used to produce monoclonal antibodies that offer a more definite specificity than polyclonal antibodies and an unlimited production. These monoclonal antibodies were used to develop an immunoassay for the detection of deltamethrin.

MATERIALS AND METHODS

Preparation of Deltamethrin Haptens. Thin-layer chromatography (TLC) and column chromatography were carried out using Merck silica gel 60. Products **1**−**5** were identified by proton NMR spectroscopy with a 100 MHz (JEOL-FX) instrument. The structures of haptens **1** and **2** were confirmed by proton NMR spectroscopy with a 400 MHz (Bruker DRX)

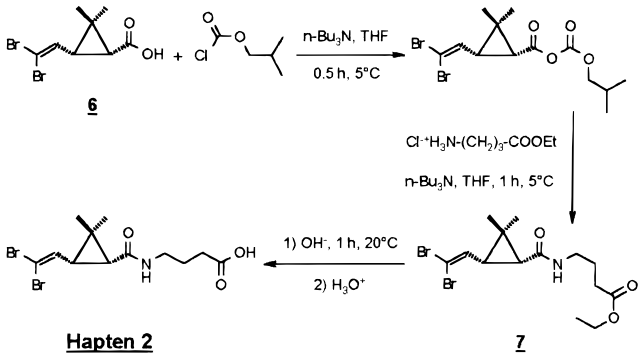

Figure 2. Hapten **2** synthesis pathway.

instrument. Preparative HPLC separations were performed with an HPLC system (Spectra SP 8 800) equipped with a UV detector (Spectra 1 000) and a preparative column containing silica (10 μm, 10 × 250). The flow rate was 5 mL min^{-1}.

*3-(4-Hydroxy-1-phenyl)propanoic Acid Methyl Ester (**1**).* Fourteen grams (77.69 mmol) of 3-(4-methoxy-1-phenyl)-propanoic acid was stirred with 75 mL of bromohydric acid 48%. After hydrolysis, the reaction mixture was extracted with dichloromethane. The organic layer was dried over $MgSO_4$ and concentrated. The residue was recrystallized from petroleum ether and esterified by methanol in the presence of sulfuric acid to give the methyl ester (**1**) (5.6 g; 40% yield): ^{1}H NMR ($CDCl_3$, 100.3 MHz) δ 2.57 (t, 2H, ArCH_2), 2.84 (t, 2H, CH_2CO_2), 3.65 (s, 3H, OCH_3), 6.75−7.00 (m, 4H, Ar*H*), 7.2 (s, 1H, O*H*).

*3-[4-{3-(1,3-Dioxolan-2-yl-phenoxy)-1-phenyl}]propanoic Acid Methyl Ester (**2**).* A mixture composed of 4.64 g (25,75 mmol) of compound **1**, 10 mL of pyridine, and 1.4 g (25.91 mmol) of sodium methylate was mixed and warmed to evaporate 2 mL of pyridine, after which 0.64 g (6.46 mmol) of cuprous chloride and 9.80 g (42.78 mmol) of 2-(3-bromophenyl)-1,3-dioxolane were added. The mixture was vigorously stirred and warmed to 200 °C (external temperature), while pyridine was eliminated. This temperature was maintained for 8 h. The residue was dissolved with ether and washed with an aqueous solution of 1 M HCl and then with water. The organic layer was dried over $MgSO_4$ and concentrated. The residue was purified on a silica gel column using ethyl acetate/hexane (1:6) as eluent. The solution was washed with 1 M NaOH and extracted twice with ether to give compound **2** (3.91 g; 46% yield): ^{1}H NMR ($CDCl_3$, 100.3 MHz) δ 2.6 (t, 2H, CH_2Ar), 2.9 (t, 2H, CH_2CO), 3.62 (s, 3H, CH_3O), 4.00 (m, 4H, CH_2 dioxolane), 5.7 (s, 1H, C*H* dioxolane), 6.7−7.5 (m, 8H, Ar*H*).

*3-[4-{3-(1,3-Dioxolan-2-yl-phenoxy)-1-phenyl}]propanoic Acid (**3**).* Fifteen milliliters of an aqueous solution of potassium hydroxide (0.78 g, 13.90 mmol) was added to a solution of **2** (3.84 g, 11.70 mmol) in 30 mL of methanol. The reaction mixture was stirred for 20 h at room temperature, acidified with 1 M HCl, and extracted twice with 30 mL of ether. The organic phase was dried ($MgSO_4$) and concentrated to yield compound **3** (3.67 g; 100% yield): ^{1}H NMR ($CDCl_3$, 100.3 MHz) δ 2.62 (t, 2H, CH_2Ar), 2.89 (t, 2H, CH_2CO), 3.97 (m, 4H, CH_2 dioxolane), 5.71 (s, 1H, CH dioxolane), 6.7−7.6 (m, 8H, Ar*H*), 11.0 (s, 1H, COO*H*).

*3-[4-(3-Formylphenoxy)-1-phenyl]propanoic Acid (**4**).* Five milliliters of 1 M HCl was added to a solution of compound **3** (0.786 g, 2.5 mmol) in 10 mL of acetone. The mixture was stirred for 3 h at room temperature and concentrated before its extraction with ether (2 × 30 mL). The organic layer was dried over $MgSO_4$ and concentrated to yield **4** (0.675 g; 100% yield). This compound was pure enough (^{1}H NMR) to be used directly in the next reaction: ^{1}H NMR ($CDCl_3$, 100.3 MHz) δ 2.69 (t, 2H, CH_2−Ar), 2.95 (t, 2H, CH_2CO), 6.8−7.6 (m, 8H, Ar*H*), 11.1 (s, 1H, COO*H*).

*(1R-cis)-3-(2,2-Dibromoethenyl)-2,2-dimethylcyclopropane Carboxylic Acid Chloride (**5**).* One drop of DMF and 0.32 mL (3.67 mmol) of oxalyl chloride were added to a solution of deltamethric acid (**6**) (0.745 g, 2.5 mmol) in 20 mL of dichlo-

romethane. The reaction mixture was stirred at room temperature for 3 h and dried under vacuum to give the crude acyl chloride **5**, which was pure enough (^{1}H NMR) to be used directly in the next reaction: ^{1}H NMR ($CDCl_3$, 100.3 MHz) δ 1.27 (s, 3H, CH_3), 1.34 (s, 3H, CH_3), 2.2 (d, 7.5,1H, C*H*CO), 2.28 (t, 7.5, 1H, C*H*C═), 6.51 (d, 7.5, 1H, C*H*═CBr_2).

*[1R,1α(R),2α] and [1R,1α(S),2α]-4-[3-{Cyano[(2-(2,2-dibromoethenyl)-2,2-dimethylcyclopropyl)carbonyloxy]methyl}phenoxy]-phenylpropanoic Acid (Hapten **1**).* Aldehyde **4** (0.675 g, 2.5 mmol) dissolved in 1 mL of toluene was added to a solution of sodium cyanide (0.265 g, 5.4 mmol) and tetradecyltrimethylammonium bromide (0.0074 g, 0.02 mmol) in 2.3 mL of water at room temperature under vigorous stirring. The reaction mixture was stirred for 10 min at room temperature before acyl chloride **5** (0.79 g, 2.5 mmol) was added. The stirring was continued for 3 h before a few drops of 1 M HCl was added to bring the pH to 4. After HCN was removed under vacuum, partition between ether and water was realized. The organic layer was dried over $MgSO_4$ and concentrated. The residue was chromatographed on a silica gel column using ethyl acetate/hexane 1:3 as eluent to yield a 1-to-1 mixture of diastereoisomers (0.7 g, 48%). The products were separated by HPLC using as eluent a mixture of ethyl acetate/hexane/acetic acid (800:9200:15) with a flow rate of 5 mL min^{-1}. The retention times of the products were measured: (1*R*) t_R = 19.13 min, (1*S*) t_R = 22.44 min, respectively. The absolute configuration of CHCN was related to deltamethrin.

1*R*: ^{1}H NMR (CD_3OD, 400.1 MHz) δ 1.29−1.31 (2s, 2CH_3), 1.91 (d, 8.3, 1H, C*H*CO), 2.08 (dd, 8.3, 8.4, 1H, C*H*−C═), 2.70 (t, 7.5, 2H, CH_2COOH), 2.97 (t, 7.5, 2H, CH_2Ar), 6.32 (s, 1H, C*H*CN), 6.69 (d, 8.4, 1H, C*H*═CBr_2), 6.96−7.4 (8H, Ar*H*).

1*S*: ^{1}H NMR (CD_3OD, 400.1 MHz) δ 1.20−1.25 (2s, 2CH_3), 1.91 (d, 8.3, 1H, C*H*CO), 2.08 (dd, 8.3, 8.4, 1H, C*H*C═), 2.70 (t, 7.5, 2H, CH_2COOH), 2.97 (t, 7.5, 2H, CH_2Ar), 6.37 (s, 1H, C*H*CN), 6.70 (d, 8.4, 1H, C*H*═CBr_2), 6.96−7.4 (8H, Ar*H*).

Elementary analysis: %C_{calcd} 43.31, %C_{found} 42.56; %H_{calcd} 3.98, %H_{found} 4.05; %Br_{calcd} 28.06, %Br_{found} 27.85.

Preparation of Hapten 2 Derived from Deltamethric Acid. Freshly distilled isobutyl chloroformate (0.5 mL, 3.85 mmol) in 3 mL of THF was added at 5 °C to a mixture of deltamethric acid **6** (0.745 g, 2.5 mmol) and 1.2 mL of tributylamine in 6 mL of THF. The mixture was stirred for 40 min at 5 °C before 4-aminobutanoic acid ethyl ester hydrochloride (0.7 g, 4.2 mmol) was added; the stirring was continued for 1 h at room temperature. THF was removed under vacuum, and the residue was partitioned between CH_2Cl_2 and H_2O. The organic layer was dried over $MgSO_4$, and after evaporation of the solvent, a crude mixture was obtained. It was purified on silica gel column chromatography using CH_2Cl_2/EtOAc/AcOH (40:60:1) as eluent to obtain **7** in an oily form. Compound **7** was diluted with methanol (3 mL) and saponified with 2.5 mL of 10 M aqueous NaOH while being stirred at room temperature for 1 h. Twenty milliliters of water and 20 mL of CH_2Cl_2 were added. The organic layer was discarded. The aqueous layer was acidified with 3 M aqueous HCl and extracted twice with CH_2Cl_2 (2 × 20 mL). The organic extract was dried over $MgSO_4$ and concentrated under vacuum to yield hapten **2** as an oil (0.72 g, 75% yield): ^{1}H NMR (CD_3OD, 400.1 MHz) δ 1.18, 1.2 (2s, 6H, 2CH_3), 1.55 (d, 8.5, 1H, C*H*CO), 1.79 (t, 8.5, 1H, C*H*C═), 1.83 (q, 7.1, 2H, CH_2N), 2.36 (t, 7.1, 2H, CH_2), 3.25 (q, 7.1, 2H, CH_2COOH), 6.5 (t, 7.1, 1H, N*H*), 6.84 (d, 8.5, 1H, C*H*═CBr_2).

Preparation of Hapten−Protein Conjugates. The mixed anhydride method (Erlanger et al., 1959) was used for the condensation of hapten carboxylic group with carrier amino group. Isobutyl chloroformate (4.2 μL, 0.032 mmol) was added dropwise to a cooled solution (12−14 °C) of hapten (0.032 mmol) and tributylamine (9.8 μL, 0.041 mmol) in 0.5 mL of dry dioxane. The mixture was stirred at the same temperature for 45 min, and the resulting mixed anhydride solution was added dropwise to the solution of protein [BSA, 54 mg to prepare the immunoconjugate or ovalbumin (Ova), 108 mg for the coating antigen] in 1.5 mL of water/dioxane (5:1, v/v) at pH 7.5. The mixture was stirred at 4 °C for 5 h while the pH was maintained at 7.5 with 1 M NaOH. Conjugates were purified by ultrafiltration (Micro-thin-channel ultrafiltration system TCF2, Amicon) using a PM 10 000 membrane (Diaflo ultrafilters, Amicon) and then lyophilized.

Characterization of Hapten−Protein Conjugates. The coupling was assessed according to two methods:

(i) Br elementary analyses were performed by the CNRS (Service Central d'Analyses) and/or Roussel-UCLAF Co. Hapten density was calculated according to the following formulas, where n is the number of bromine atoms contained in the molecule, 560.3 is the deltamethrin contribution to the conjugate molecular weight, and 364.8 is the deltamethric acid contribution:

BSA-Del

$$\%\ \mathrm{Br} = 100(n \times 79.9)/(\mathrm{BSA\ weight} + n \times 0.5 \times 560.3)$$

Ova-Del

$$\%\ \mathrm{Br} = 100(n \times 79.9)/(\mathrm{ovalbumin\ weight} + n \times 0.5 \times 560.3)$$

Ova-DA

$$\%\ \mathrm{Br} = 100(n \times 79.9)/(\mathrm{ovalbumin\ weight} + n \times 0.5 \times 364.8)$$

(ii) The level of hapten substitution was estimated by measuring the loss of free amino groups on the protein. Protein concentration in conjugate solutions was first measured according to the method of Bradford (1976). The number of free amino groups was then determined by a reaction with orthophthaldialdehyde (OPDA) (Svedas et al., 1980). These two values were then correlated to estimate the hapten-to-carrier ratio. OPDA reagent was prepared as follows: 0.5 mL of OPDA in ethanol (10 mg mL^{-1}) and 0.5 mL of a mercaptoethanol/ethanol mixture (0.5:99.5) were added to 30 mL of borate buffer (0.1 M, pH 9.7). The conjugates were dissolved in the borate buffer. The conjugate and the reagent were mixed in a 1-to-1 ratio, and the absorbance of the solution was measured at 340 nm.

Polyclonal Antibody Production. Female New Zealand white rabbits were injected subcutaneously four times with 1 mg of conjugate BSA−hapten at 2-week intervals. The first injection consisted of 0.5 mL of conjugate in a saline solution (NaCl 8.5‰) emulsified with 0.5 mL of complete Freund's adjuvant. For the second injection, incomplete adjuvant was used instead of complete. Following injections were performed without adjuvant. The rabbits were bled from the ear vein 2 weeks after each inoculation.

Monoclonal Antibody Production. Five-week-old female BALB/c mice were injected intraperitoneally (ip) with 100 μg of conjugate BSA−hapten at 2-week intervals. The first injection consisted of 0.25 mL of conjugate in a saline solution (NaCl 8.5‰) emulsified in 0.25 mL of complete Freund's adjuvant. For the second inoculation, incomplete adjuvant was used instead of complete. The last injections were performed without adjuvant. Ten days after each inoculation, the mice were bled from the retroorbital plexus. Three days before the fusion, 100 μg of immunogen in 500 μL of a saline solution was injected into the mouse with the highest antiserum titer. Mouse spleen cells were fused with the Sp2/0-Ag 14 murine myeloma cells line. The cell mixture was centrifuged (200*g*, 10 min), and the cell pellet was suspended in poly(ethylene glycol) warmed at 37 °C (1 mL for 10^8 spleen cells). After 90 s, 10 mL of RPMI 1640 medium (Gibco) was added dropwise to dilute the poly(ethylene glycol). The cell suspension was centrifuged (100*g*, 10 min), the supernatant was discarded, and the cell pellet was resuspended in RPMI medium supplemented with 10% fœtal calf serum (Myoclone plus, Gibco) and 1% HAT. The cell suspension was dispensed (1 mL/well) into 30 sterile 24-well culture plates. The plates were incubated at 37 °C in a 5% CO_2 atmosphere. Ten to 15 days after the fusion, the wells containing growing hybridomas in HAT medium were tested for specific Mabs by ELISA. The HAT

medium was replaced with HT medium. Hybridoma cells from wells showing a positive response were subcloned by limiting dilution (Goding, 1980), and clones were further selected by ELISA.

Monoclonal Antibody Isotype. Isotype was determined by ELISA using affinity-purified goat anti-mouse isotype antibodies conjugated to alkaline phosphatase (Southern Biotechnology Associates).

Hapten-Immobilized Immunoassay. Microtitration plates (Nunc Maxisorp C96) were coated with 100 μL of the Ova-DA (5 μg mL^{-1}) or Ova-Del (12.5 μg mL^{-1}) conjugate dissolved in phosphate-buffered saline (pH 7.4) (PBS: NaCl, 0.137 M; KCl, 0.0027 M; KH_2PO_4, 0.0015 M; $Na_2HPO_4 \cdot 12H_2O$, 0.008 M), which was equivalent to 1 μg of Ova-DA or 2.5 μg of Ova-Del coating conjugate per well. The plates were incubated overnight at 4 °C. Unbound antigen was removed by washing three times with PBS supplemented with 0.05% Tween 20 (PBS-T). Unspecified binding sites were blocked by adding 250 μL of 0.5% (w/v) fish gelatin solution (Sigma, g-7765) in PBS (PBS-G) and incubated for 2 h at 25 °C. The blocking solution was removed, and various concentrations of the supernatants from hybridoma cultures or serum were screened for the presence of specific antibodies. Antibodies were diluted in a PBS-G solution. After incubation at 25 °C for 2 h, the plates were washed as previously described. The goat anti-mouse IgG-alkaline phosphatase conjugate (Jackson ImmunoResearch) diluted in PBS-G (1/2000) was added and incubated at 25 °C for 1 h. After the plates were washed, 100 μL of the *p*-nitrophenyl phosphate substrate (1 mg/mL) in diethanolamine buffer (diethanolamine, 1 M; $MgCl_2$, 5×10^{-7} M; pH 9.8) was added and color developed for 1 h. Absorbance of each well was measured at 405 nm with a microtiter plate scanner (Multiscan MCC 340 MK, Flow Laboratories).

Competitive Enzyme-Linked Immunosorbent Assay. A precise amount of Ova-Da or Ova-Del was deposed in the well. After incubation overnight at 4 °C, plates were washed and blocked with PBS-G for 2 h at 25 °C. After washing, 50 μL/well of anti-deltamethrin antibody and 50 μL/well of competitor were added to the wells and incubated for 2 h under stirring. The competitors, dissolved in methanol, were diluted in PBS-G buffer. The final concentration of methanol in the well was 3%. After washing, coloration was performed as described above. The results are expressed in percent inhibition as follows: % inhibition $= 100 \times [1 - (A/A_{control})]$.

RESULTS AND DISCUSSION

Hapten Synthesis. Deltamethrin has eight possible stereoisomers, but the *cis*-1*R*,3*R* configuration about the cyclopropane ring and the *S* configuration for the cyano group at the benzylic carbon are essential for insecticidal activity (Elliot, 1976). Since the specificity of an immunoassay depends on the stereochemistry of the hapten (Skerritt and Lee, 1996), we have chosen to maintain hapten stereochemistry during the synthesis. Demoute et al. (1986) first described the preparation of deltamethrin hapten in conserving the stereochemistry. The spacer arm was introduced on the phenoxybenzyl part of the molecule by using a dialdehyde derivative and a Horner−Emmons olefination under the conditions proposed by Masamune (Blanchette et al., 1984). The subsequent hydrogenation of the activated double bond yielded compound **4**. In the present work we developed a simplified approach to obtain compound **4** (Figure 1). The spacer arm was introduced before Ulmann condensation (Lindley, 1984). 3-Bromobenzaldehyde, protected by a dioxolane, was conjugated to a phenol that bears the spacer arm 3-(4-hydroxy-1-phenyl)propanoic. Ulmann condensation was then achieved under the conditions of Demoute et al. (1986). The conditions of the reaction (8 h, 200 °C) explained the low yield (46%). Saponification and deprotection of the aldehyde yielded compound **4**. The cyanhydrine was obtained by a phase transfer reaction using sodium cyanide. This cyanhydrine was acylated by deltamethric acid chloride to yield a 1-to-1 mixture of 1α(*S*) and 1α(*R*) isomers. The isomers were separated by semipreparative HPLC to yield hapten **1**.

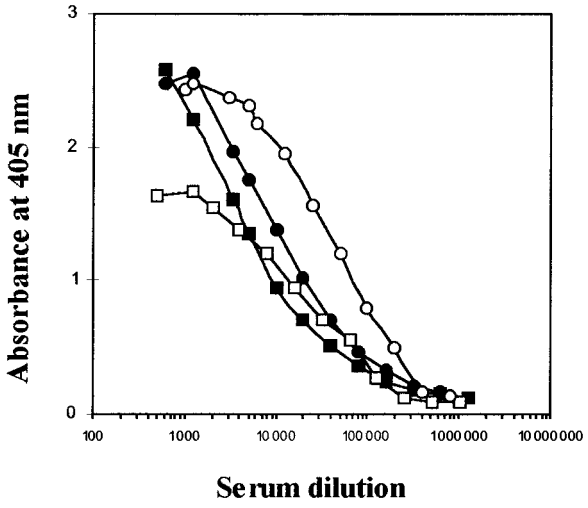

Figure 3. Determination of serum titer of rabbits immunized with BSA-DA (open symbols) or BSA-Del (solid symbols) with two coating antigens, Ova-DA (circles) and Ova-Del (squares).

Another preparation of deltamethrin was developed by Skerritt and Lee (1996). They used a simple two-step reaction in conserving the stereochemistry; deltamethrin alcohol, prepared as described by Zhang and Scott (1994), was esterified with succinic acid. The limitation of this synthesis was the poor yield (4%) of the deltamethrin alcohol.

Another hapten was synthesized by using a deltamethrin metabolite, that is, the deltamethric acid (Figure 2). To increase hapten determinant exposure (Jung et al., 1989), deltamethric acid hapten was obtained by adding a 4-C spacer arm. This was done with 4-aminobutanoic acid ethyl ester using isobutyl chloroformate according to the mixed anhydride method. The resulting ester was then saponified to yield hapten **2**.

Characterization of Conjugates and Immunization. Haptens **1** and **2** were coupled to BSA (immunoconjugates) or ovalbumin (coating antigen) by using the mixed anhydride method (Erlanger et al., 1959). Assessing the hapten-to-carrier protein molar ratio was required before immunization was carried out. Good antibody titers are generally obtained by using conjugates consisting of 8−25 haptens per mole of carrier (Erlanger, 1980; Wittmann and Hock, 1991). Hapten protein conjugation was verified by Br elementary analyses and by measuring the number of free amino group on the protein by OPDA reaction. The two methods gave nearly similar results. Hapten densities calculated from OPDA reaction and from elementary analysis were, respectively, BSA-Del, 21 and 19; BSA-DA, 31 and 29; Ova-Del, 10 and 14; and Ova-DA, 15 and 15. These hapten densities were enough, and the two immunoconjugates (BSA-Del and BSA-DA) were used for rabbit or mouse immunization.

Polyclonal Antibody Titration. Polyclonal rabbit antibodies raised against BSA-DA (antiserum 1) and BSA-Del (antiserum 2) were tested using an indirect ELISA with the two conjugates Ova-Del and Ova-DA (Figure 3) (ovalbumin prevents the detection of anti-BSA antibodies). Antibodies from antiserum 1 bound

more to Ova-DA than to Ova-Del. The serum titer, that is, the serum dilution that gave a 3 times the background absorbance, was 1:100 000 with Ova-Del and 1:250 000 with Ova-DA. Deltamethric acid was the immunizing hapten, so antibodies were produced against this molecule. The lower response against Ova-Del, which still contains the deltamethric acid epitope, could be explained by the steric bulk of the whole molecule in which deltamethric acid would be less accessible to antibodies. On the other hand, antibodies from antiserum 2 gave a nearly similar response with the two conjugates tested: the serum titer was 1:100 000 with Ova-Del and 1:150 000 with Ova-DA. The greatest response with Ova-DA indicates that more antibodies were produced against deltamethric acid epitope than against the other parts of the whole molecule. This is in agreement with immunoconjugate structure (BSA-Del) in which deltamethric acid is the most exposed part (Figure 1). Skerritt and Lee (1996) observed also that serum titer was higher when they used the half molecule to synthesize the hapten rather than the whole molecule. They explained these results by esterase hydrolysis of the entire molecule before recognition by the immune system of the animal. Since the obtained polyclonal antibodies presented a correct response, the same immunoconjugates were also used for mice immunization. Polyclonal antibodies were also used to optimizate an ELISA for monoclonal antibody selection.

Hybridoma Production. Spleen cells, from mice exhibiting the highest antiserum titer after immunization with BSA-Del or BSA-DA, were fused with the myeloma cell line Sp2/O-Ag/14. The resulting hybridomas were cultured in 24-well microculture plates. Ten days after the fusion, growing hybridomas, which were observed in 90% of the wells, were screened for pyrethroids antibodies. The culture supernatants were tested by indirect ELISA against Ova-Del and Ova-DA. Hybridomas obtained from mice immunized with BSA-DA presented low responses with the two coating antigens. They were not used for further investigations. Among the 594 wells with growing hybridomas obtained from a mouse immunized with BSA-Del, 30 wells gave a positive response against Ova-Del and/or Ova-DA. They were expanded and tested against Ova-Del and/or Ova-DA, ovalbumin, and BSA. None of them recognized BSA itself. Eighteen wells were eliminated since they presented cross-reactivity with ovalbumin, so they were not specific for the hapten only. Twelve wells contained an antibody that recognized only the conjugate but not ovalbumin. Hybridomas from these 12 wells were cloned by limiting dilution. Wells presenting growing monoclonal hybridomas were selected by ELISA, as described above, allowing the isolation of 12 monoclonal hybridoma cultures.

Monoclonal Antibody Specificity. The 12 monoclonal antibodies, named Del 01−Del 12 were IgG_1 with kappa light chain. From their response in indirect ELISA against Ova-Del and Ova-DA, they were assumed to be distributed into three groups. The first one (Del 05, Del 08, Del 11) gave an identical response with Ova-Del and Ova-DA (Figure 4A); this showed that these antibodies were able to recognize the dibromovinylcyclopropane moiety. The second group (Del 04, Del 07, Del 09, Del 10, Del 12) was more specific to Ova-DA than to Ova-Del (Figure 4B). The epitope would contain a common part of deltamethrin and deltamethric acid. The difference observed in the intensity of the response

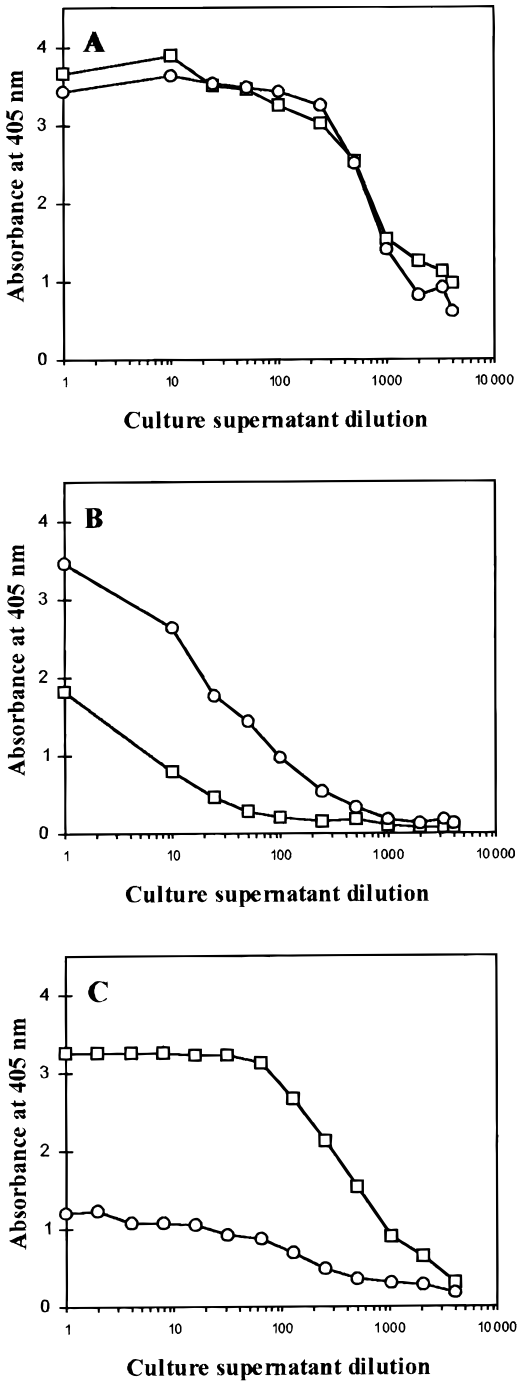

Figure 4. Determination of monoclonal antibodies specificity with two coating antigens, Ova-DA (circles) and Ova-Del (squares).

could be due to deltamethrin steric bulk. The last group (Del 01, Del 02, Del 03, Del 06) recognized more effectively Ova-Del than it did Ova-DA (Figure 4C), indicating that the epitope may include the cyclopropane ring and the phenoxybenzyl moiety.

Competitive ELISA was performed to evaluate the ability of these 12 antibodies to detect unconjugated deltamethrin. Nine monoclonal antibodies presented no competition with free deltamethrin in the conditions of the assay; the three others, Del 01, Del 02, and Del 03, showed a significant competition in the presence of free deltamethrin.

Assay Development. The sensitivity of the assay depends on both the coating antigen amount and the

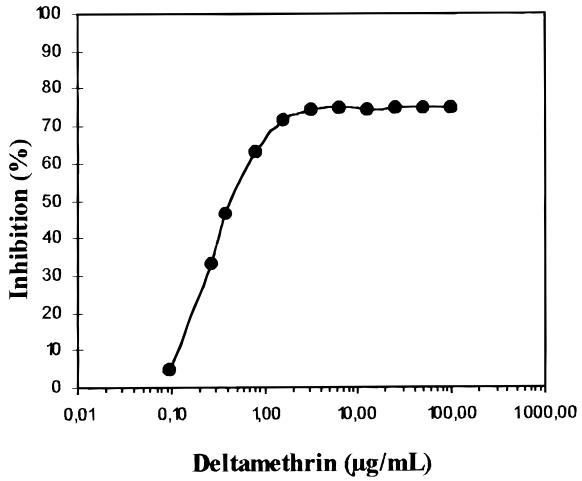

Figure 5. Competitive ELISA for deltamethrin analysis with the monoclonal antibody Del 01. The assay consisted of 50 μL of competitor solution and 50 μL of antibody solution.

antibody concentration. These parameters were optimized for the monoclonal antibody Del 01, which presented the strongest competition in the presence of free deltamethrin. The optimal ELISA for Del 01 was performed with 0.133 μg mL^{-1} monoclonal antibody, purified by chromatography on protein A (Ey et al., 1978), and 0.3 μg mL^{-1} coating antigen Ova-Del. Deltamethrin solutions were prepared before each assay by diluting in PBS-G a freshly prepared methanolic solution of deltamethrin. The final methanol concentration in the wells was 3%. Figure 5 shows deltamethrin standard curve inhibition obtained by the competitive ELISA. The IC_{50} value for deltamethrin was estimated to be 0.5 μg mL^{-1}. The linear range of the assay system was extended from 0.08 to 2 μg mL^{-1}. Assay sensitivity was lower than those developed with monoclonal antibodies against other pyrethroids such as permethrin (Stanker et al., 1989) or allethrin (Pullen and Hock, 1995a). Concerning deltamethrin, results of immunoassays developed by Skerritt and Lee (1996) and Demoute et al. (1986) were unpublished. Skerritt and Lee (1996) reported only that antibodies, prepared using deltamethrin hemisuccinate, yielded relatively low sensitivity assays. Their most sensitive assay used a heterologous system with antibodies produced from a deltamethrin hapten coupled on the halogenovinyl group and a deltamethrin hemisuccinate hapten coupled to an enzyme. They obtained a detection limit of ~0.02 μg mL^{-1} with freshly prepared deltamethrin.

Cross-Reactivity. To determine the specificity of monoclonal antibody Del 01, a competitive ELISA was performed with several pyrethroids (alphamethrin, bifenthrin, λ-cyalothrin, permethrin, fenvalerate, bioresmethrin). No cross-reactivity was observed, and each IC_{50} value was greater than the highest concentration of pyrethroid used (50 μg mL^{-1}); this corresponded to <1% cross-reactivity. Therefore, our immunoassay with the monoclonal antibody Del 01 seems to be specific to deltamethrin. These results give information about the epitope recognized by the monoclonal antibody Del 01. If the specificity of Del 01 was based only on the α-cyanophenoxybenzyl part of the molecule, λ-cyalothrin, fenvalerate, and cypermethrin would present cross-reactivity. Since fenvalerate showed no cross-reactivity, the presence of the halogenovinylcyclopropane part of the molecule is important in antibody recognition. These observations suggest that the monoclonal antibody would recognize the cyclopropane moiety, the halogen on the bound ethylenic, and the cyano group of deltamethrin.

Conclusion. To develop an immunoassay for deltamethrin detection, two haptens were synthesized and monoclonal antibodies were produced. Hapten **1** had the same stereochemistry as deltamethrin: 1*R cis* αS. It was obtained by introducing a spacer arm on the phenoxybenzyl group of the molecule. Hapten **2** was synthesized using deltamethric acid. Although the two haptens allowed the production of anti-deltamethrin polyclonal antibodies with high titer, only the BSA-Del immunoconjugate led to the production of monoclonal antibodies in the present work. According to their recognition on Ova-Del and Ova-DA, monoclonal antibodies were classified into three groups. Only those presenting a better recognition of Ova-Del than of Ova-DA were effective for the detection of free deltamethrin in a competitive ELISA. Among these monoclonal antibodies, Del 01 appeared to be the most sensitive in competitive ELISA: it allows the detection of deltamethrin, dissolved in buffer with 3% methanol, in a range from 0.080 to 2 μg mL^{-1}. Although this detection limit has to be lowered in an optimized assay, the use of monoclonal antibody allows the development of a specific ELISA for deltamethrin detection. Indeed, the most interesting aspect of monoclonal antibody Del 01 is its specificity to deltamethrin; it has no cross-reactivity with other pyrethroid molecules, so it could be used to develop a specific ELISA for deltamethrin.

ABBREVIATIONS USED

BSA, bovine serum albumin; BSA-DA, BSA conjugated to hapten **2**; BSA-Del, BSA coupled to hapten **1**; DMF, dimethylformamide; ELISA, enzyme-linked immunosorbent assay; HAT, hypoxanthine (5×10^{-3} M), aminopterin (2×10^{-5} M), thymidine (8×10^{-4} M); HT, hypoxanthine (5×10^{-3} M), thymidine (8×10^{-4} M); IC_{50}, value of the concentration inhibiting 50% of an ELISA absorbance value; Mab, monoclonal antibody; ^{1}H NMR, proton nuclear magnetic resonance; Ova-DA, ovalbumin coupled to hapten **2**; Ova-Del, BSA coupled to hapten **1**; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline plus 0.05% Tween 20; PBS-G, phosphate-buffered saline plus 0.5% fish gelatin; THF, tetrahydrofuran.

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