Development of Enzyme-Linked Immunosorbent Assays for the Insecticide Chlorpyrifos. 1. Monoclonal Antibody Production and Immunoassay Design

Juan J. Manclús,† Jaime Primo,‡ and Angel Montoya*,†

Laboratorio Integrado de Bioingeniería and Instituto de Tecnología Química, UPV-CSIC, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain

The present work describes the production and characterization of monoclonal antibodies (MAbs) to the insecticide chlorpyrifos and their incorporation into several ELISA configurations. With this aim, a collection of chlorpyrifos haptens was synthesized by introducing appropriate spacers in opposite sites of the analyte molecular structure. From mice immunized with protein conjugates of these haptens, several hybridomas secreting MAbs with the ability to sensitively bind the analyte were obtained. MAbs showing the highest affinity to chlorpyrifos in homologous assays (I_{50} values in the 20−220 nM range) were selected. Hapten heterology involving modifications of the moiety closer to the attachment site provided the highest improvement in sensitivity. MAbs displayed striking differences in their cross-reactivity pattern with structurally related compounds. One MAb (I_{50} around 10 nM) was incorporated into other ELISA formats. No remarkable changes of assay characteristics, other than immunoreagent consumption and immunoassay procedure, were found. These ELISAs are potentially very valuable analytical tools for the rapid and sensitive determination of this insecticide.

Keywords: *Insecticide; chlorpyrifos; hapten design; monoclonal antibodies; ELISA; analysis*

INTRODUCTION

Chlorpyrifos [*O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] is a broad spectrum organophosphorus insecticide that is widely used in agriculture and indoor disinfestation (Worthing and Hance, 1991; Racke, 1993). It is moderately toxic to mammalian species (Tafuri and Roberts, 1987; Gallo and Lawryk, 1991) but extremely toxic to a wide range of nontarget aquatic biota (Raven and George, 1989; Ward *et al.*, 1995). When toxicity and ecotoxicity, along with field use ratings, have been combined to establish a ranking of pesticides according to their environmental impact, chlorpyrifos is placed high in the ranking (Newman, 1995). Due to its widespread use in agriculture, a high chlorpyrifos residue occurrence in food has been reported (Neidert *et al.*, 1994; U.S. Food and Drug Administration, 1994; KAN-DO, 1995), which poses potential health hazards (Cochran *et al.*, 1995). Moreover, chlorpyrifos findings in environmental compartments have also been reported (Hallberg, 1989; Thoma and Nicholson, 1989; Nico *et al.*, 1994). Therefore, there is a growing concern about toxicological and environmental risks associated with chlorpyrifos residues, which is demanding more comprehensive monitoring programs.

Current chlorpyrifos analysis is carried out by multiresidue methods using gas chromatography (U.S. Food and Drug Administration, 1979; Association of Official Analytical Chemists, 1995). Chromatographic methods are laborious and time-consuming and require sophisticated equipment available in only well-equipped centralized laboratories. Immunoassay technology is being demonstrated as a simple, rapid, and cost-effective alternative to traditional methods when high sample throughput and/or on-site screening analyses are required (Sherry, 1992; Meulenberg *et al.*, 1995).

The application of immunoassay technology requires the production of antibodies to the analyte. With this aim, chlorpyrifos derivatives, namely haptens, must be synthesized and coupled to immunogenic proteins to raise the appropriate antibody response. Highly sensitive rabbit polyclonal antibodies to chlorpyrifos have been recently obtained using a derivative of chlorpyrifos-methyl to prepare the immunogen (Hill *et al.*, 1994). However, attempts to produce monoclonal antibodies (MAbs) with the appropriate properties from the same hapten were unsuccessful, in spite of the high number of hybridomas screened (Skerritt *et al.*, 1992; Edward *et al.*, 1993). Using a different hapten prepared according to a simple strategy, MAbs with acceptable affinity and specificity to chlorpyrifos have been obtained (Manclús *et al.*, 1994). Irrespective of whether polyclonal or monoclonal antibodies are contemplated to raise antibodies against small molecules, there is agreement on the difficulty of predicting which hapten is theoretically the most appropriate for a particular analyte and, furthermore, whether this hapten will actually elicit the adequate antibody response. If monoclonal antibody technology is going to be undertaken, this should be preceded by careful hapten synthesis work, to take advantage of the striking differences in the properties of the antibodies eventually obtained (Hammock *et al.*, 1990). Hence, the synthesis of diverse haptens is recommended, taking into account the ease of synthesis and some generally accepted rules about hapten design (Harrison *et al.*, 1990; Goodrow *et al.*, 1995).

With the aim of producing MAbs and developing enzyme-linked immunosorbent assays (ELISA) to chlorpyrifos, herein we describe the synthesis of a collection of chlorpyrifos haptens covering different approaches

* Author to whom correspondence should be addressed (telephone and fax 34-6-3877093; e-mail amontoya@upvnet.upv.es).

† Laboratorio Integrado de Bioingeniería.

‡ Instituto de Tecnología Química.

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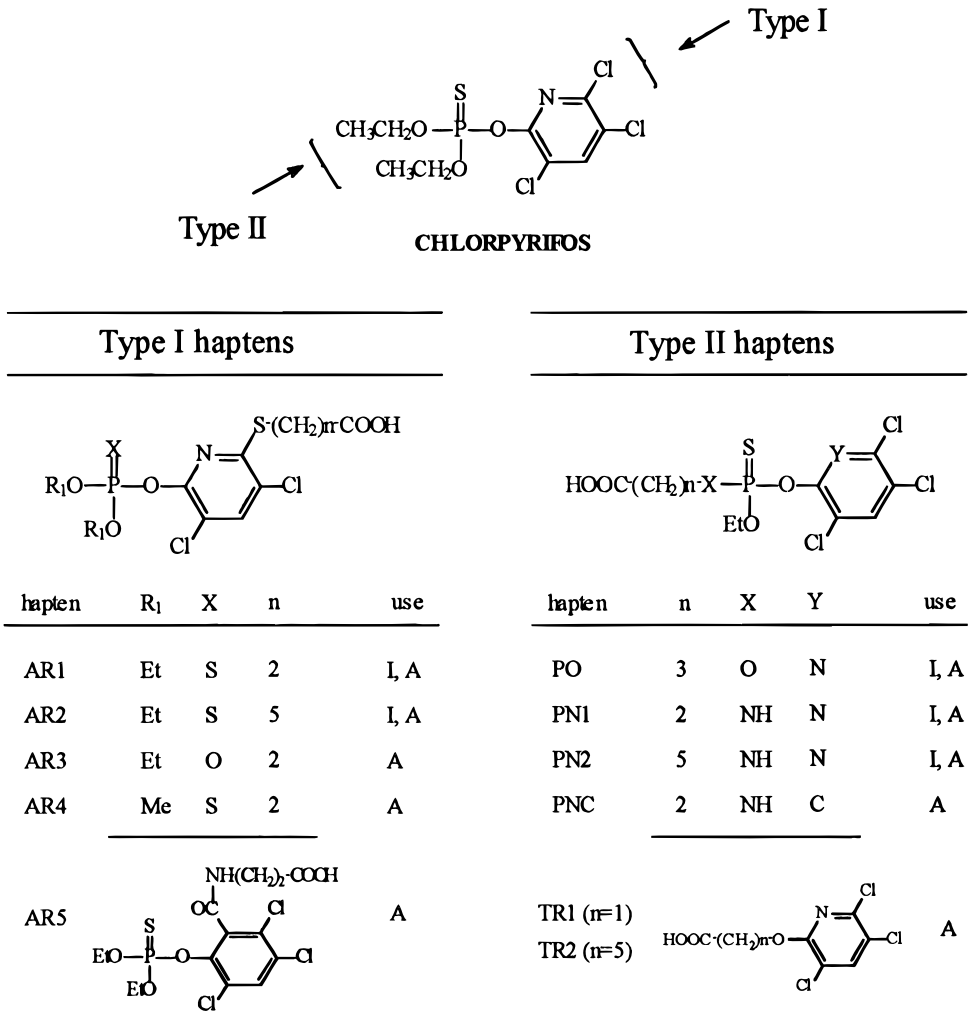


hapten	R_1	X	n	use
AR1	Et	S	2	I, A
AR2	Et	S	5	I, A
AR3	Et	O	2	A
AR4	Me	S	2	A
AR5				A

hapten	n	X	Y	use
PO	3	O	N	I, A
PN1	2	NH	N	I, A
PN2	5	NH	N	I, A
PNC	2	NH	C	A
TR1 (n=1) / TR2 (n=5)				A

Figure 1. Structures of chlorpyrifos and of the haptens used for immunization (I) and in assay conjugates (A). Arrows indicate the sites for spacer attachment.

(type, attachment site, and length of the spacer). The immunological response of protein conjugates of these haptens is characterized for sensitivity and specificity to chlorpyrifos, at both the polyclonal and monoclonal levels. For further sensitivity improvement based on hapten heterology, the usefulness of other haptens with different types of chemical modifications is examined. Finally, these immunoreagents are incorporated into different ELISA formats to perform the detection of chlorpyrifos.

MATERIALS AND METHODS

Reagents and Instruments. Most of the compounds used in this study present only minor safety concerns. However, it is advisable to work in a well-ventilated fume hood. Chlorpyrifos, chlorpyrifos-methyl, and 3,5,6-trichloro-2-pyridinol (TCP) standards, as well as technical grade chlorpyrifos, were generously provided by DowElanco (Midland, MI). Other pesticide standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Starting products for hapten synthesis and hapten−protein coupling reagents were from Fluka-Aldrich Química (Madrid, Spain). Analytical grade solvents were from Scharlau (Barcelona, Spain). Thin-layer chromatography (TLC) was performed on 0.2 mm precoated silica gel 60 F_{254} on aluminum sheets from Merck (Darmstadt, Germany). Column chromatography was carried out on silica gel (0.063−0.2 mm particle size, 70−230 mesh), also from Merck.

Ovalbumin (OVA), Freund's adjuvants, Pristane, and *o*-phenylenediamine (OPD) were obtained from Sigma Química (Madrid, Spain). Bovine serum albumin (BSA) fraction V, enzyme immunoassay grade horseradish peroxidase (HRP), and poly(ethylene glycol) (PEG) 1500 were from Boehringer Mannheim (Germany). Culture media and supplements were from GibcoBRL (Paisley, Scotland).

^{1}H nuclear magnetic resonance (NMR) spectra were obtained with a Varian VR-400S spectrometer (Sunnyvale, CA), operating at 400 MHz. Chemical shifts are given relative to tetramethylsilane. Electron impact (EI) (at 70 eV) and liquid secondary ion (LSI) (3-nitrobenzyl alcohol as matrix) mass spectra (MS) were obtained on a VG Autospec mass spectrometer (Fisons). Ultraviolet−visible (UV−vis) spectra were recorded on a UV-160A Shimadzu spectrophotometer (Kyoto, Japan). ELISA plates were washed with an Ultrawash II plate washer, and well absorbances were read with an MR 700 plate reader, both from Dynatech (Sussex, U.K.).

Hapten Synthesis. Two types of haptens were prepared depending on the site of the chlorpyrifos structure (Figure 1) through which the spacer arm was attached: the aromatic ring (type I) or the thiophosphate group (type II).

Type I Haptens. These haptens were prepared by introduction of alkyl mercaptoacids of different length through the aromatic ring of chlorpyrifos (**AR1** and **AR2**), chlorpyrifos-oxon (**AR3**), or chorpyrifos-methyl (**AR4**) or by introduction of an amino acid spacer arm (**AR5**). The syntheses of **AR1** and **AR5** are described elsewhere (Manclús *et al.*, 1994).

*O,O-Diethyl O-[3,5-Dichloro-6-[(5-carboxypentyl)thio]-2-pyridyl] Phosphorothioate (**AR2**, Figure 2).* 6-Mercaptohexanoic acid was prepared according to the method of Gee *et al.* (1988). To a solution of this acid (1 g, 6.75 mmol) in absolute ethanol (30 mL) was added KOH (0.756 g, 13.5 mmol) and heated until dissolved. Then, technical grade chlorpyrifos (2.37 g, 6.75 mmol) dissolved in absolute ethanol (30 mL) was added. After

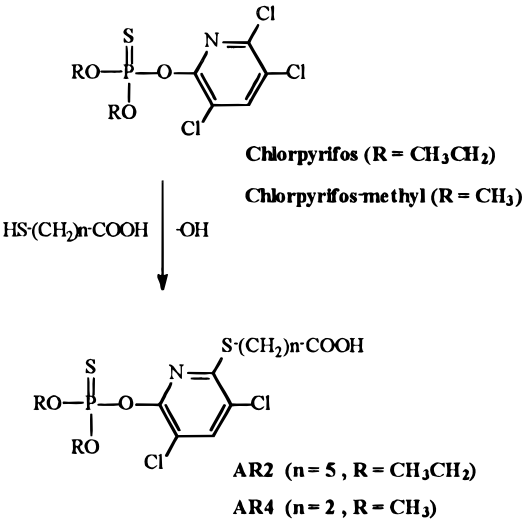


Figure 2. Synthesis of haptens **AR2** and **AR4**.

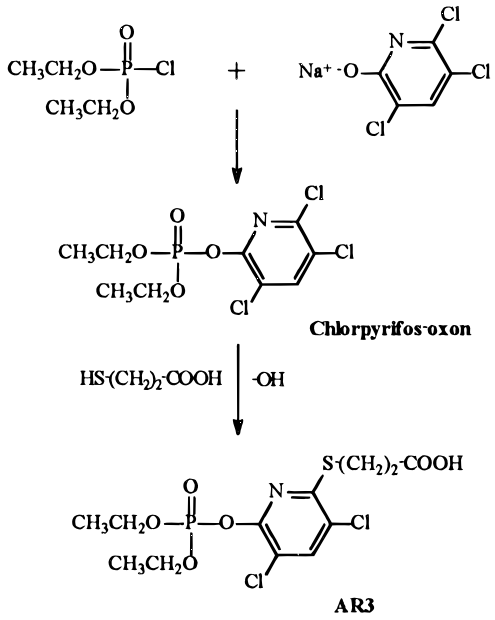


Figure 3. Synthesis of hapten **AR3**.

reflux for 1 h, the reaction mixture was filtered and the solvent was removed under reduced pressure. To the residue was added 5% $NaHCO_3$ (50 mL), followed by washing with hexane (Hx, 2 × 50 mL). The aqueous layer was acidified to pH 3 and extracted with dichloromethane (DCM, 3 × 50 mL). The extract was dried over Na_2SO_4 and concentrated, and the residue was subjected to column chromatography (Hx/tetrahydrofuran (THF)/acetic acid, 75:25:1). Combination of the fractions showing only one spot on TLC (R_f 0.37, same solvent) provided **AR2** (220 mg, 17%): ^{1}H NMR ($CDCl_3$) δ 7.63 (s, 1H, ArH), 4.33 (q+q, 4H, 2 CH_2O), 3.18 (t, 2H, SCH_2), 2.38 (t, 2H, CH_2COO), 1.6 (m, 6H, 3 CH_2), 1.41 (t, 6H, 2 CH_3); EI-MS, *m/z* (relative intensity) 463 (27, $M^+ + 2$), 461 (37, M^+), 428 (70), 426 (91), 401 (35), 380 (84), 361 (94), 347 (100), 312 (68), 256 (42), 222 (52), 211 (71), 195 (80), 153 (83), 125 (81), 97 (99).

*O,O-Diethyl O-[3,5-Dichloro-6-[(2-carboxyethyl)thio]-2-pyridyl] Phosphate (**AR3**, Figure 3).* A mixture of sodium 3,5,6-trichloro-2-pyridinolate (220 mg, 1.13 mmol) and diethyl chlorophosphate (236 mg, 1.36 mmol) in acetonitrile (5 mL) was refluxed for 1 h. The mixture was filtered and the solvent evaporated under reduced pressure. The residue was chromatographed (Hx/ethyl acetate (EtAc)/acetic acid, 80:20:1) to give *O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphate (chlorpyrifos-oxon, 360 mg, 95%): TLC R_f 0.31 (same solvent); ^{1}H NMR ($CDCl_3$) δ 7.86 (s, 1H, ArH), 4.41 (q+q, 4H, 2 CH_2O), 1.43 (t, 6H, 2 CH_3). From chlorpyrifos-oxon, **AR3** was prepared by the same reaction and molar quantities as for **AR2**, using 3-mercaptopropanoic acid instead of 6-mercaptohexanoic acid. Finally, the residue was subjected to column chromatography (Hx/EtAc/THF/acetic acid, 30:60:10:1), and fractions showing one spot on TLC (R_f 0.5, same solvent) were combined. The solvent was then evaporated and the residue further purified by preparative TLC in the same solvent to give **AR3** (37 mg, 8%): ^{1}H NMR (acetone-d_6) δ 8.05 (s, 1H, ArH), 4.36 (q+q, 4H, 2 CH_2O), 3.41 (t, 2H, SCH_2), 2.88 (t, 2H, CH_2COO), 1.42 (t, 6H, 2 CH_3); EI-MS, *m/z* (relative intensity) 407 (17, $M^+ + 4$), 405 (57, $M^+ + 2$), 403 (63, M^+), 387 (37), 385 (44), 352 (59), 331 (100), 303 (68), 275 (44), 222 (67), 197 (89), 195 (99), 163 (53).

*O,O-Dimethyl O-[3,5-Dichloro-6-[(2-carboxyethyl)thio]-2-pyridyl] Phosphorothioate (**AR4**, Figure 2).* This hapten was synthesized the same as **AR2** but starting from chlorpyrifos-methyl (190 mg, 0.58 mmol) and 3-mercaptopropanoic acid. Column chromatography (Hx/EtAc/acetic acid, 80:20:1) of the residue gave the crude product (28 mg, TLC R_f 0.67, same solvent), which was purified by preparative TLC (Hx/EtAc/THF/acetic acid, 30:60:10:1) to render **AR4** (8 mg, 3.5%): TLC R_f 0.67, same solvent; ^{1}H NMR ($CDCl_3$) δ 8.01 (s, 1H, ArH), 3.98 (s, 3H, CH_3O), 3.43 (t, 2H, SCH_2), 2.8 (t, 2H, CH_2COO); LSI-MS, *m/z* 397 (35, $M + H^+ + 4$), 395 (100, $M + H^+ + 2$), 393 (99, $M + H^+$); EI-MS, *m/z* (relative intensity) 379 (4), 377 (4), 359 (74), 357 (80), 311 (51), 283 (67), 252 (85), 222 (79), 196 (34), 166 (93), 126 (95), 97 (100).

Type II Haptens. These haptens were prepared by introduction of alkyl ω-hydroxy or ω-amino acids of different length as ester (**PO**) or amide (**PN1**, **PN2**, **PNC**) linkage of suitable thiophosphate reagents. Hapten **PNC**, with phenyl instead of pyridyl aromatic ring, and haptens **TR1** and **TR2**, without the thiophosphate group and with spacer attachment by O-alkylation, were used as heterologous haptens for immunoassay design. The respective structures of these haptens are depicted in Figure 1.

*O-Ethyl O-(3,5,6-Trichloro-2-pyridyl) O-(3-Carboxypropyl) Phosphorothioate (**PO**).* The synthetic pathway of this hapten is shown in Figure 4. To a well-stirred solution of ethyl dichlorothiophosphate (1.8 g, 10 mmol) in dry THF (6 mL) cooled in an ice bath was added, over 15 min, a solution of 3,5,6-trichloro-2-pyridinol (1 g, 5 mmol) and triethylamine (800 μL, 5.75 mmol) in dry THF (6 mL). After 2 h, the mixture was filtered and the filtrate concentrated. Column chromatography (Hx/DCM, 9:1) of the residue gave the product *O*-ethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorochloridothioate (**PO-a**) as an oil (502 mg, 30%): TLC R_f 0.4 (same solvent); ^{1}H NMR ($CDCl_3$) δ 8.03 (s, 1H, ArH), 4.51 (q+q, 2H, CH_2O), 1.51 (t, 3H, CH_3). The spacer arm, benzyl 4-hydroxybutanoate, was synthesized as follows. To a solution of benzyl bromide (1.71, 10 mmol) in DCM (25 mL) was added sodium 4-hydroxybutanoate (2.52 g, 20 mmol) in distilled water (10 mL) and tetrabutylammonium hydrogen sulfate (300 mg) as phase-transfer catalyst. After 4 days of vigorous stirring, the organic phase was separated, dried over anhydrous Na_2SO_4, and concentrated. Column chromatography (Hx/EtAc, 1:1) of the residue gave benzyl 4-hydroxybutanoate as an oil (941 mg, 49%): TLC R_f 0.35 (same solvent); ^{1}H NMR ($CDCl_3$) δ 7.37 (s, 5H, ArH), 5.13 (s, 2H, CH_2O), 3.64 (t, 2H, CH_2OH), 2.6 (s, 1H, OH), 2.46 (t, 2H, CH_2COO), 1.91 (q, 2H, CH_2). Next, to a solution of **PO-a** (500 mg, 1.47 mmol) in dry THF (3 mL) were added benzyl 4-hydroxybutanoate (427 mg, 2.2 mmol) and triethylamine (223 mg, 2.2 mmol) dissolved in dry THF (3 mL), and the mixture was stirred at room temperature for 24 h. Filtration, concentration of the filtrate, and chromatography (Hx/EtAc/acetic acid, 80:20:1) of the residue gave the triester **PO-b** as an oil (88 mg, 12%): TLC R_f 0.71 (same solvent); ^{1}H NMR ($CDCl_3$) δ 7.84 (s, 1H, ArH), 7.35 (s, 5H, ArH), 5.11 (s, 2H, CH_2O), 4.4 (q+q, 4H, CH_2OP), 2.58 (t, 2H, CH_2COO), 2.12 (m, 2H, CH_2), 1.41 (t, 3H, CH_3). The carboxylic group was deprotected by treatment with HBr-saturated acetic acid (500 μL) for 1 h. The mixture was concentrated with a flow of N_2, and column chromatography (Hx/EtAc/acetic acid, 70:30:1) of the residue gave **PO** (51 mg, 71%): TLC R_f 0.43 (same solvent); ^{1}H NMR ($CDCl_3$) δ 7.87 (s, 1H, ArH), 4.43 (q+q, 4H, CH_2O), 2.59 (t, 2H, CH_2COO), 2.11 (m, 2H, CH_2), 1.43 (t, 3H, CH_3);

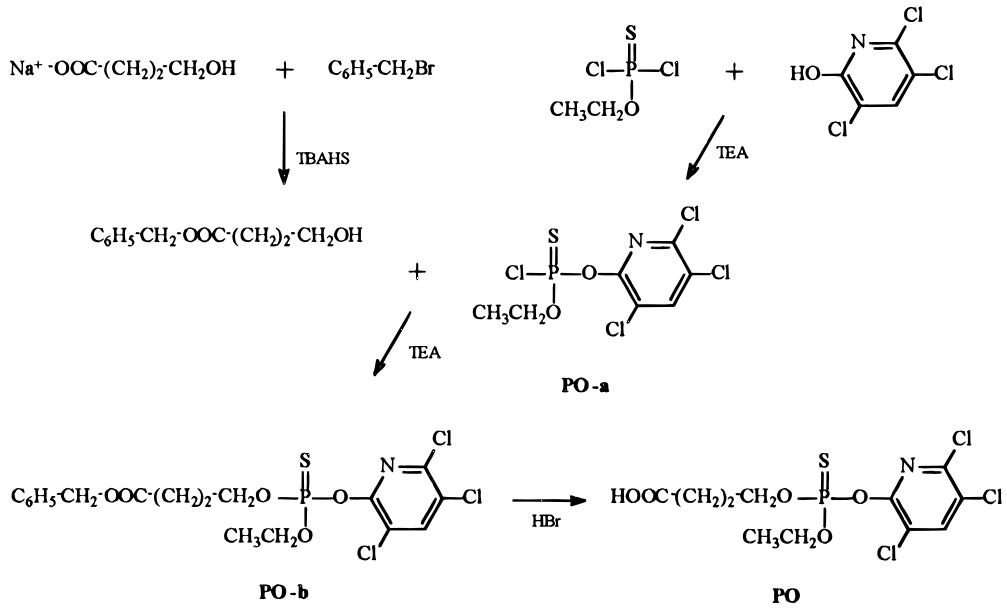


Figure 4. Synthesis pathway of hapten **PO**. TEA, triethylamine; TBAHS, tetrabutylammonium hydrogen sulfate.

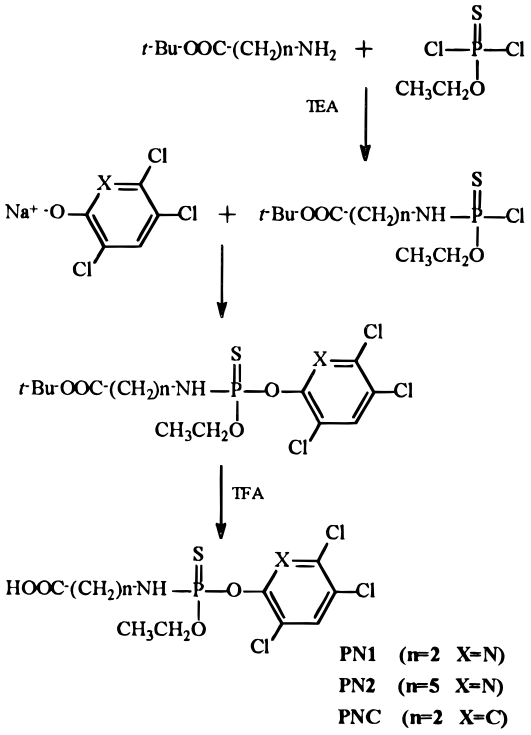


Figure 5. Synthesis of haptens **PN1**, **PN2**, and **PNC**. TEA, triethylamine; TFA, trifluoroacetic acid.

EI-MS, *m/z* (relative intensity) 409 (4, $M^+ + 2$), 407 (4, M^+), 372 (9), 370 (6), 324 (28), 322 (28), 288 (53), 286 (57), 260 (31), 258 (36), 199 (88), 197 (88), 171 (85), 69 (100).

*O-Ethyl O-(3,5,6-Trichloro-2-pyridyl) N-(5-Carboxyethyl)-phosphoramidothioate (**PN1**, Figure 5).* This hapten was synthesized according to the method of McAdam and Skerritt (1993). Briefly, ethyl dichlorothiophosphate was reacted successively with the spacer arm *tert*-butyl 6-aminopropanoate and sodium 3,5,6-trichloro-2-pyridinolate, and the resulting product was deprotected by treatment with trifluoroacetic acid (TFA) to give **PN1**. Product structure was confirmed by spectral data: ^{1}H NMR ($CDCl_3$) δ 7.85 (s, 1H, ArH), 4.29 (m, 2H, CH_2O), 4.17 (m, 1H, NH), 3.33 (m, 2H, CH_2N), 2.52 (t, 2H, $OOCCH_2$), 1.39 (t, 3H, CH_3); EI-MS, *m/z* (relative intensity) 394 (1, $M^+ + 2$), 392 (1, M^+), 359 (10), 357 (15), 199 (87), 197 (90), 167 (100).

*O-Ethyl O-(3,5,6-Trichloro-2-pyridyl) N-(5-Carboxypentyl) phosphoramidothioate (**PN2**, Figure 5).* The spacer arm *tert*-butyl 6-aminohexanoate was prepared as described by McAdam and Skerritt (1993) but starting from 6-aminohexanoic acid. To a stirred solution of *O*-ethyl dichlorothiophosphate (895 mg, 5 mmol) in dry THF (5 mL) cooled in an ice bath was added, over 15 min, a solution of *tert*-butyl 6-aminohexanoate (468 mg, 2.5 mmol) and triethylamine (400 μL, 3 mmol) in dry THF (5 mL). After 1 h, the mixture was filtered and the filtrate concentrated under reduced pressure. The residue was chromatographed (Hx/EtAc, 85:15) to give *O*-ethyl *N*-(5-carboxypentyl)phosphoramidochloridothioate (498 mg, 60%), TLC R_f 0.53 (same solvent). To a solution of this compound (200 mg, 0.6 mmol) in acetonitrile (5 mL) was added sodium 3,5,6-trichloro-2-pyridinolate (160 mg, 0.73 mmol) and refluxed for 1 h. The mixture was filtered and the filtrate concentrated. Without further purification, the residue was treated with TFA (500 μL) in DCM (1 mL) for 1 h. Column chromatography (Hx/EtAc/acetic acid, 70:30:1) gave **PN2** (231 mg, 88%): TLC R_f 0.43 (same solvent); ^{1}H NMR ($CDCl_3$) δ 7.85 (s, 1H, ArH), 4.33 (q+q, 2H, CH_2O), 3.46 (m, 1H, NH), 3.16 (m, 2H, CH_2N), 2.38 (t, 2H, $OOCCH_2$), 1.63 (m, 6H, 3 CH_2), 1.40 (t, 3H, CH_3); LSI-MS, *m/z* 439 (32, $M + H^+ + 4$), 437 (98, $M + H^+ + 2$), 435 (100, $M + H^+$); EI-MS, *m/z* (relative intensity) 403 (13), 401 (44), 399 (54), 355 (3), 353 (4), 335 (25), 333 (25), 287 (64), 285 (75), 199 (61), 197 (61), 130 (62), 114 (100).

*O-Ethyl O-(2,4,5-Trichlorophenyl) N-(2-Carboxyethyl)phosphoramidothioate (**PNC**, Figure 5).* To a solution of *O*-ethyl *N*-(5-carboxyethyl) phosphoramidochloridothioate (an intermediate of the synthesis of **PN1**) (288 mg, 1 mmol) in acetonitrile (10 mL) was added sodium 2,4,5-trichlorophenolate (220 mg, 1 mmol), and the mixture was refluxed for 1 h. The mixture was filtered and the filtrate concentrated. Chromatography (Hx/EtAc, 85:15) of the residue gave **PNC** (276 mg, 62%): TLC R_f 0.55 (same solvent); ^{1}H NMR ($CDCl_3$) δ 7.63 (s, 1H, ArH2), 7.52 (s, 1H, ArH5), 4.22 (q+q, 2H, CH_2O), 3.97 (m, 1H, NH), 3.43 (m, 2H, CH_2N), 2.66 (t, 2H, $OOCCH_2$), 1.38 (t, 3H, CH_3); LSI-MS, *m/z* 396 (35, $M + H^+ + 4$), 394 (98, $M + H^+ + 2$), (392 (100, $M + H^+$); EI-MS, *m/z* (relative intensity) 360 (29), 358 (68), 356 (75), 312 (14), 310 (21), 270 (18), 268 (26), 200 (48), 198 (78), 196 (100), 167 (82), 154 (86).

*[(3,5,6-Trichloro-2-pyridyl)oxy]acetic Acid (**TR1**).* This hapten is the herbicide triclopyr and was purchased from Riedel-de Haën (Germany).

*6-[(3,5,6-Trichloro-2-pyridyl)oxy]hexanoic Acid (**TR2**, Figure 6).* A mixture of ethyl 6-bromohexanoate (1020 mg, 4.6 mmol) and sodium 3,5,6-trichloro-2-pyridinolate (750 mg, 3.4 mmol) in acetonitrile (12 mL) was refluxed for 5 h. Filtration of the mixture, concentration of the filtrate, and column chromatography (Hx/EtAc, 80:20) gave the ethyl ester of **TR2** (1030 mg, 88%), TLC R_f 0.73 (same solvent). The ester was dissolved in

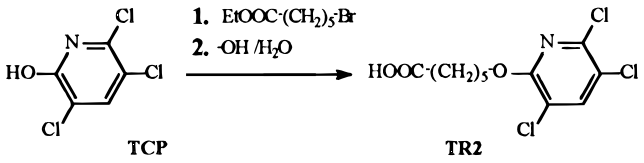


Figure 6. Synthesis of hapten **TR2**.

THF (2 mL) and 2 M NaOH (25 mL) was added. After reflux for 1 h, the mixture was acidified to pH 3 and extracted with DCM (2 × 50 mL). The extract was dried over anhydrous Na_2SO_4 and concentrated. Column chromatography (Hx/EtAc/acetic acid, 65:35:1) gave **TR2** (561 mg, 60%) as a white solid: mp 145−147 °C; TLC R_f 0.63 (same solvent); ^{1}H NMR ($CDCl_3$) δ 8.11 (s, 1H, ArH), 4.42 (t, 2H, CH_2O), 2.38 (t, 2H, CH_2COO), 1.72 (3m, 6H, 2 CH_2); EI-MS, *m*/*z* (relative intensity) 315 (13, $M^+ + 4$), 313 (38, $M^+ + 2$), 311 (39, M^+), 278 (4), 276 (6), 226 (5), 224 (8), 212 (27), 210 (29), 201 (75), 199 (99), 197 (100), 169 (49), 115 (87), 97 (71), 69 (85).

Preparation of Protein−Hapten Conjugates. All haptens used in this study contained a free carboxylic group suitable to react with amine groups of proteins. Hapten−protein conjugations were carried out by the *N*-hydroxysuccinimide (NHS)-active ester method according to Langone and Van Vunakis (1975), with slight modifications.

Immunogenic and Coating Conjugates. Typically, haptens [*ca.* 25 μmol in the appropriate volume of *N*,*N*-dimethylformamide (DMF) to bring the final concentration of hapten to 100−200 mM] were activated during 2 h at room temperature with a 50% molar excess of NHS and dicyclohexylcarbodiimide. Next, the mixture was centrifuged and the supernatant collected. To a solution of 10 mg/mL protein (BSA for immunogens, OVA for coating conjugates) in 0.2 M borate buffer (pH 9.0) was added, over 10 min and with vigorous stirring, the activated ester mixture diluted in the volume of DMF necessary to bring the solution to 20% DMF. The initial hapten to protein molar ratio in the mixture was 50:1 for immunogens and 30:1 for coating conjugates. The mixture was stirred at room temperature for 2 h. Finally, conjugates were separated from uncoupled haptens by gel filtration on Sephadex G-25, using PBS (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4) as eluant. Conjugate formation was confirmed spectrophotometrically. UV−vis spectra showed qualitative differences between carrier proteins and conjugates in the region of maximum absorbance of haptens. The hapten to protein molar ratio of conjugates was then estimated from the spectral data of the hapten, the protein, and the corresponding conjugate. By assuming that the molar absorptivity of haptens was the same for the free and conjugated forms, apparent molar ratios in the range 25−35 and 8−15, for BSA and OVA conjugates, respectively, were estimated.

Enzyme Conjugates. HRP was used to prepare the enzyme tracers. Following the same procedure as before, haptens were activated and then conjugated to HRP (5 mg/mL) using a 20 molar excess of activated hapten. Enzyme tracers were purified by gel filtration and stored at 4 °C in a 1:1 mixture of saturated ammonium sulfate and PBS containing 0.1% BSA, without loss of activity for at least 2 months. HRP conjugate concentrations and molar ratios were estimated spectrophotometrically. With the same assumptions as before, the estimated molar ratios were around 2 for all tracers.

Production of Monoclonal Antibodies to Chlorpyrifos. *Immunization.* BALB/c female mice (8−10 weeks old) were immunized with BSA−**AR1**, −**AR2**, −**PO**, −**PN1**, and −**PN2** conjugates. First dose consisted of 100 μg of conjugate intraperitoneally injected as an emulsion of PBS and complete Freund's adjuvant. Two and 4 weeks after the initial dose, mice received booster injections with the same amount of immunogen emulsified in incomplete Freund's adjuvant. One week after the last injection, mice were tail-bled and sera tested for antihapten antibody titer by indirect ELISA and for analyte recognition properties by competitive indirect ELISA. After a resting period of at least 3 weeks from the last injection in adjuvant, mice selected to be spleen donors for hybridoma production received a final soluble intraperitoneal injection of 100 μg of conjugate in PBS, 4 days prior to cell fusion.

Cell Fusion. P3-X63/Ag 8.653 murine myeloma cells (ATCC, Rockville, MD) were cultured in high-glucose Dulbecco's Modified Eagle's medium supplemented with 2 mM L-glutamine, 1 mM nonessential amino acids, 25 μg/mL gentamicin, and 15% fetal bovine serum (referred to as s-DMEM). Cell fusion procedures were carried out essentially as described by Nowinski *et al.* (1979). Mouse spleen lymphocytes were fused with myeloma cells at a 5:1 ratio using PEG 1500 as the fusing agent. The fused cells were distributed in 96-well culture plates at an approximate density of 4×10^5 cells/100 μL of s-DMEM per well; 24 h after plating, 100 μL of HAT selection medium (s-DMEM supplemented with 100 μM hypoxanthine, 0.4 μM aminopterine, and 16 μM thymidine) was added to each well. Half of the medium of the wells was replaced by fresh HAT medium on days 4 and 7 postfusion. Cells were grown in HAT medium for 2 weeks and then HAT was substituted by HT medium (HAT medium without aminopterine).

Hybridoma Selection and Cloning. Eight to 10 days after cell fusion, culture supernatants were screened for the presence of antibodies that recognized chlorpyrifos. The screening consisted of the simultaneous performance of noncompetitive and competitive indirect ELISAs, to test the ability of antibodies to bind the OVA conjugate of the immunizing hapten and to recognize chlorpyrifos, respectively. Optimum conditions were pursued for the screenings. Thus, the coating conjugate concentrations were those selected when the analyte recognition by mouse sera was evaluated, and culture supernatants were appropriately diluted to obtain ELISA absorbance below 2.0. Selected hybridomas were cloned by limiting dilution on a feeder layer of BALB/c thymocytes (*ca.* 10^6 cells/well) and peritoneal macrophages (*ca.* 5000 cells/well). Stable antibody-producing clones were expanded and cryopreserved in liquid nitrogen.

Purification of Monoclonal Antibodies. Antibodies were purified on a small scale directly from late stationary phase culture supernatants by affinity chromatography on protein A−agarose (Pharmacia, Sweden) according to the method of Schuler and Reinacher (1991). For scaling-up, ascitic fluid was obtained from Pristane-primed mice. Antibodies were purified from clarified ascites by saline precipitation followed by anion-exchange chromatography on DEAE-Sepharose (Sigma).

Enzyme-Linked Immunosorbent Assays. Flat-bottom polystyrene ELISA plates (Costar High Binding No. 3590, Cambridge, MA) were coated overnight with conjugate or antibody solutions in 50 mM carbonate buffer (pH 9.6). Standards were prepared in PBS by serial dilutions from a stock solution in DMF, using borosilicate glass tubes instead of the usual plastic ones, since we found that the latter material adsorbs chlorpyrifos, as also reported by Hill *et al.* (1994). Although chlorpyrifos adsorption may also occur on polystyrene ELISA wells during the competition step of the assay, this phenomenon would equally affect standards and samples. Therefore, the immunochemical detection and quantification of chlorpyrifos would be accurately carried out. A volume of 100 μL per well was used throughout all assay steps, and all incubations were carried out at room temperature. After each incubation, plates were washed four times with washing solution (0.15 M NaCl containing 0.05% Tween 20). Two basic formats were used depending on the assay component immobilized into the ELISA plates. In the conjugate-coated format, an indirect ELISA was used for the estimate of mouse serum antibody titers and for the screening of culture supernatants, and a competitive indirect ELISA was used for the study of antibody sensitivity and specificity to chlorpyrifos. In the antibody-coated format, the specific antibody was coated directly or by using a capture auxiliary antibody, and competitive ELISAs were followed to evaluate the assay properties using different enzyme tracers.

For competition assays, the concentrations of antibodies, hapten conjugates, or enzyme tracers were optimized by checkerboard titration. Usually, several combinations of the immunoreagents, under subsaturating conditions giving absorbances around 1.0, were evaluated to select those providing the highest sensitivity. Competitive curves were obtained by plotting absorbance against the logarithm of analyte concentration. Sigmoidal curves were fitted to a four-parameter

logistic equation (Raab, 1983), using Sigmaplot software package (Jandel Scientific, Germany).

Conjugate-Coated Format. Plates were coated with the selected concentrations of OVA−hapten conjugates. Then, serum, culture supernatant, or antibody dilutions in PBS containing 0.05% Tween 20 (PBST) were added and incubated for 1 h. Next, plates were incubated for 1 h with peroxidase-labeled rabbit anti-mouse immunoglobulins (Dako, Denmark) diluted 1/2000 in PBST. Finally, peroxidase activity bound to the wells was determined by adding the substrate solution (2 mg/mL OPD and 0.012% H_2O_2 in 25 mM citrate, 62 mM sodium phosphate, pH 5.35). After 10 min, the reaction was stopped with 2.5 M sulfuric acid, and the absorbance at 490 nm was read and recorded. For competitive assays, the procedure was the same except that after coating a competition step was introduced by adding 50 μL of the competitor (chlorpyrifos or related compounds) followed by 50 μL of the appropriate concentration of antibody (serum, culture supernatant, or purified MAb).

Antibody-Coated Format. In this format, plates were coated with antibodies at the selected concentrations. Next, the competition was established for 1 h between chlorpyrifos standards and the selected dilutions of enzyme tracers (hapten−HRP conjugates). Peroxidase activity was measured as above.

Indirect Antibody-Coated Format. The difference with the previous format was that plates were first coated with goat anti-mouse immunoglobulins (Dako, Denmark) at 2 μg/mL in carbonate buffer, followed by an incubation for 2 h with the specific antibodies at appropriate concentrations in PBST.

RESULTS AND DISCUSSION

Synthesis of Immunizing Haptens. Two approaches were followed to prepare haptens resembling as much as possible the chlorpyrifos chemical structure (Figure 1): (I) introduction of a spacer arm as an aromatic ring substituent and (II) attachment of a spacer through the thiophosphate group. The first type of haptens were synthesized directly from chlorpyrifos by substitution of activated chlorine in the 6-position of the aromatic ring with alkyl ω-mercaptoacids of different length. Thus, haptens bearing spacers with two (**AR1**) and five (**AR2**) methylene groups (Figure 1) were readily prepared with acceptable yields (24% and 17%, respectively). In the second type of haptens, the pyridyl ring moiety was retained unchanged, and one of the *O*-ethyl groups was replaced by a spacer arm. This strategy was based on the reaction of ethyl dichlorothiophosphate with TCP and suitable spacers in the adequate order. Spacer attachment was accomplished as ester or amide bond depending on the use of alkyl ω-hydroxy or ω-amino acids, respectively. Introduction of these bifunctional spacer arms required the use of appropriate protection/deprotection reactions. For sodium 4-hydroxybutanoate, direct protection of the carboxylic group as a benzyl ester was successfully achieved using benzyl bromide and phase-transfer catalysis. Attachment of the carboxy-protected alcohol to the thiophosphate moiety, followed by treatment with HBr to remove the benzyl protective group, gave the thiophosphate hapten **PO**. For amino acids, two protection/deprotection reactions were required for spacer introduction, according to the procedure described by McAdam and Skerritt (1993). Following this strategy, the thiophosphoramide haptens **PN1** and **PN2**, differing in their spacer length, were synthesized (Figure 1).

Mouse Immune Response to Conjugates of Chlorpyrifos Haptens. To test the suitability of the synthesized immunizing haptens to elicit an appropriate antibody response to chlorpyrifos, several mice were immunized with each of the BSA−hapten conjugates.

Table 1. Properties of the Sera of Mice Immunized with Chlorpyrifos Haptens

immunizing hapten[a]	serum titer[b]	chlorpyrifos I_{50}[c] (μM) OVA−**AR1**	OVA−**AR2**	OVA−**PO**	OVA−**PN1**	OVA−**PN2**
AR1	3×10^5	**2.5**	3.1	nd[d]	nd	nd
AR2	3×10^6	2.5	**ni**[e]	nd	nd	nd
PO	3×10^4	nd	nd	**0.9**	1.2	0.8
PN1	3×10^5	nd	nd	0.4	**1.5**	1.8
PN2	1×10^6	nd	nd	2.1	2.1	**3.2**

[a] Representative sera obtained 1 week after the third booster injection of the respective BSA−hapten conjugate. [b] Serum dilution that gave 3 times the background absorbance in the ELISA using the homologous hapten. [c] Data obtained from competitive ELISAs performed with optimum concentrations of OVA−hapten conjugates and serum dilutions giving absorbances around 1.0. Boldface characters indicate homologous assays. [d] Not determined, since the coating OVA−hapten conjugates were not recognized by the sera. [e] Indicates no inhibition up to 50 μM chlorpyrifos.

After the third injection, mouse sera were collected and subsequently characterized for the presence of antibodies recognizing the conjugated immunizing haptens (serum titer) and for their ability to bind chlorpyrifos, represented by their I_{50} value (concentration giving 50% inhibition of the maximum response). Results of the characterization are summarized in Table 1. Serum titers (serum dilution giving 3 times the background absorbance) were estimated by indirect ELISA using the homologous OVA−hapten conjugates (coating conjugate concentration = 1 μg/mL). All mouse sera showed high levels of polyclonal antibodies recognizing each respective homologous hapten conjugate, with titers ranging from 1/30000 to 1/3000000. Next, the ability to recognize chlorpyrifos was evaluated by competitive indirect ELISA using homologous and heterologous haptens. Except the sera obtained from the BSA−**AR2** conjugate, the rest of the sera provided competitive inhibition curves in homologous assays giving comparable recognition of chlorpyrifos (I_{50} ranging from 0.9 to 3.2 μM) and showing shallow slopes as well as no complete inhibition of antibody binding (high background, absorbance 0.15−0.35). In heterologous assays, sera recognized the conjugated haptens of the same type but not those of the other type. Under optimized conditions, for all sera except those raised from BSA−**AR1**, at least one heterologous hapten was found to provide higher recognition of chlorpyrifos (lower I_{50}) than the homologous one (Table 1). Particularly, OVA−**AR1** provided competitive curves with the sera derived from BSA−**AR2**, for which no inhibition was achieved with the homologous hapten.

As evidenced by the polyclonal mouse response, antibodies recognizing chlorpyrifos with I_{50} in the micromolar order were obtained from haptens derivatized through either site of the analyte structure. Similar approaches to hapten design were used to raise antibodies to other organothiophosphate insecticides (McAdam *et al.*, 1992). In this study, better affinity antibodies were obtained derivatizing through the thiophosphate moiety than through the aromatic ring. The lesser suitability of the latter type of haptens, also evidenced from the works of Vallejo *et al.* (1982) and Brimfield *et al.* (1985), might be a consequence of important modifications of the ring electronic distribution produced by either direct or spacer-mediated conjugation of the haptens to proteins. In addition, most spacers attached through the aromatic ring contained functional groups that were found to be important antigenic determinants. This led to an undesirable antibody response character-

Table 2. Summary of the Results of Cell Fusions and Hybridoma Selection

immunizing hapten	fusion no.	no. of wells: seeded	positive[a] (hapten)	competitive[b] (analyte)	no. of cloned hybridomas[c]
AR1	1	384	18	5	4
	2	384	48	3	1
	3	288	10	3	–[d]
AR2	4	384	190	0	0
PO	5	576	270	3	3
	6	384	18	8	1
	7	384	22	8	–
PN1	8	480	470	6	2
	9	384	170	9	–
PN2	10	480	200	4	2
	11	384	25	10	–

[a] Wells with antibodies that recognized the OVA–hapten conjugates (homologous assays) by indirect ELISA (absorbance >1.0). [b] Wells with antibodies that recognized free chlorpyrifos (inhibition >50% by 1 μM chlorpyrifos). Competitive ELISAs were carried out with the OVA–hapten concentrations previously selected for evaluating mouse sera, and culture supernatants giving absorbances out of range were diluted until absorbance <2.0. [c] Hybridomas secreting antibodies with the lowest I_{50} for chlorpyrifos were stabilized and cloned. [d] Indicates that none of the antibodies improved the results of the previous fusion carried out from the same hapten.

ized by a strong recognition of haptens but a poor recognition of the analyte (Vallejo *et al*., 1982). Herein, the introduction of a simple spacer arm in the chlorpyrifos pyridyl ring by substitution of a chlorine with a thioether linkage may not involve major modification of analyte chemical structure, as suggested by Goodrow *et al*. (1990) for a similar case of triazine hapten design. Therefore, the aromatic approach seemed to be as suitable as the thiophosphate approach to elicit antibodies recognizing chlorpyrifos.

Production of Hybridomas Secreting MAbs to Chlorpyrifos. At least one fusion was performed from each immunizing hapten. Results of the hybridoma selection are summarized in Table 2. As shown, very different yields of positive wells (wells containing antibodies recognizing the homologous coating conjugate) were obtained, even in fusions performed from the same immunogen. Competitive wells (wells with hybridomas secreting antibodies showing I_{50} values lower than the established cutoff concentration of 1 μM chlorpyrifos) were found in fusions derived from four of the five immunogens used. Consistent with the polyclonal immune response, competitive wells were not found in the homologous screening of the fusion performed from the **AR2** immunogen. From culture supernatants of competitive wells obtained in the first series of fusions, antibody binding inhibition curves were performed to roughly estimate the ability to recognize chlorpyrifos (I_{50}). Those wells with hybridomas secreting antibodies showing the lowest I_{50} values were cloned by limiting dilutions. Afterward, new fusions were undertaken provided that competitive wells had been found in the previous ones. The strategy was continued until antibodies of higher affinity for chlorpyrifos were not found. Only the second fusions from **AR1** and **PO** immunogens gave higher affinity antibodies, while the third fusions of these immunogens did not improve the antibody affinities obtained in the previous ones. Selected MAbs were small-scale-purified from culture supernatant and subsequently characterized.

Characterization of the MAbs. MAbs selected from each immunizing hapten were characterized for affinity and specificity to chlorpyrifos using homologous and/or heterologous ELISA in the conjugate-coated format.

Table 3. Affinity to Chlorpyrifos of MAbs Raised from Type I Haptens Using Homologous and Heterologous Haptens for the Coating Conjugates[a]

coating hapten	I_{50}[b] (nM): LIB–**AR1.1** MAb	LIB–**AR1.4** MAb
AR1	**219**	**197**
AR2	74	102
AR3	85	952
AR4	153	282
AR5	35	33

[a] Conjugates of type II haptens were not recognized. For each coating hapten/MAb combination, optimum conditions were selected. [b] Chlorpyrifos concentration reducing the ELISA maximum response to 50%. Data represent the mean of four experiments. Boldface characters indicate homologous assays.

Affinity in Homologous Assays. The five MAbs derived from hapten **AR1** displayed comparable I_{50} for chlorpyrifos, ranging from 197 to 300 nM. With respect to MAbs derived from haptens of type II, a wider range of values was found. Thus, the I_{50} values of MAbs raised from hapten **PO** ranged from 19 to 440 nM, those from hapten **PN1** were 85 and 184 nM, and those from hapten **PN2** were 205 and 606 nM. Therefore, the highest affinity antibodies in homologous assays were obtained from hapten **PO**, which may be related with the fact that this hapten is the most structurally similar to chlorpyrifos. Nevertheless, antibodies derived from each immunizing hapten showing the lowest I_{50} values, namely LIB–**AR1.1** (I_{50} of 219 nM for chlorpyrifos), LIB–**AR1.4** (197 nM), LIB–**PO** (19 nM), LIB–**PN1** (85 nM), and LIB–**PN2** (205 nM), were further characterized.

Affinity in Heterologous Assays. Heterologous assays are well-known procedures to improve the sensitivity of immunoassays, since the structure of the assay hapten modulates the equilibrium conditions of the competition (Harrison *et al*., 1991). As recently reviewed by Marco *et al*. (1995), suitable heterology can be accomplished at three different levels: by hapten, site, and spacer modifications. These possibilities of heterology were contemplated for both types of haptens used in this study. For type I haptens, several chemical modifications of hapten **AR1** were evaluated. Haptens **AR2,** presenting a longer spacer arm, **AR3**, being a phosphate instead of a thiophosphate, **AR4**, with methyl instead of ethyl esters, and **AR5**, with a different spacer attached through an adjacent position, were synthesized and conjugated to OVA. For type II haptens, apart from **PO**, **PN1**, and **PN2** used as immunogens, hapten **PNC**, with a phenyl instead of the pyridyl ring of hapten **PN1**, and haptens **TR1** and **TR2**, lacking the thiophosphate moiety and differing from each other in the *O*-alkyl spacer length, were used for heterology evaluation. The five selected MAbs were subsequently tested against the OVA conjugates of all haptens, in noncompetitive and competitive ELISAs, to check their ability to recognize the haptens and chlorpyrifos, respectively.

MAbs derived from type I haptens (LIB–**AR1.1** and LIB–**AR1.4**) recognized all heterologous conjugates of type I haptens but none of those of type II haptens. Under optimized conditions, competitive curves for chlorpyrifos were obtained from all recognized conjugates, displaying different I_{50} values, as shown in Table 3. For both MAbs, hapten **AR5** with spacer and

Table 4. Affinity to Chlorpyrifos of MAbs Raised from Type II Haptens Using Homologous and Heterologous Haptens for the Coating Conjugates[a]

	I_{50}[b] (nM)		
coating hapten	LIB−**PO** MAb MAb	LIB−**PN1** MAb	LIB−**PN2** MAb
PO	**19**	55	52
PN1	15	**85**	90
PN2	40	191	**205**
PNC	12	125	98
TR1	9	100	405
TR2	16	41	203

[a] Conjugates of type I haptens were not recognized. For each coating hapten/MAb combination, optimum conditions were selected. [b] Chlorpyrifos concentration reducing the ELISA maximum response to 50%. Data represent the mean of four experiments. Boldface characters indicate homologous assays.

attachment site modifications was the best heterologous hapten, providing a 6-fold improvement of assay sensitivity.

All OVA−type II hapten conjugates, but none of the type I, were recognized by the MAbs LIB−**PO**, LIB−**PN1**, and LIB−**PN2**, derived from type II haptens. As shown in Table 4, MAb affinities for the analyte were improved using heterology, but the best heterologous hapten differed for each antibody. Thus, the affinity of LIB−**PO** and LIB−**PN1** MAbs for chlorpyrifos increased 2-fold using haptens without the thiophosphate group to prepare coating conjugates, *i.e.* hapten **TR1** (I_{50} of 9 nM) and hapten **TR2** (41 nM), respectively. For LIB−**PN2** MAb, the affinity for chlorpyrifos was 4-fold better using OVA−**PO** as coating conjugate.

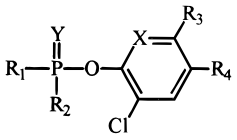
Our results confirmed the suitability of the hapten heterology approach to improve the sensitivity of pesticide immunoassays. However, the heterology should not involve the presentation of sites of the analyte opposite to the part exposed in the immunogen. Otherwise, haptens could be unrecognized by the antibodies, as exemplified by the absence of reactivity of MAbs derived from one type of chlorpyrifos haptens against conjugates of the other type of aptens. In our work, suitable heterologous haptens resulted to be those prepared by maintaining unchanged the hapten moiety distal to the attachment site to the carrier protein, while modifying the attachment site or the moiety closer to this site.

Specificity. The specificity of the selected MAbs was investigated by conjugate-coated ELISA using the conjugated hapten giving the highest affinity for chlorpyrifos, *i.e.* hapten **AR5** for LIB−**AR1.1** and LIB−**AR1.4**, hapten **TR2** for LIB−**PN1**, hapten **PO** for LIB−**PN2**, and haptens **PNC** and **TR1**, which gave similar affinity, for LIB−**PO** MAb. Under optimum conditions for each combination, specificity was evaluated by obtaining competitive curves with several related and nonrelated organophosphorus insecticides and metabolites as competitors. Relative cross-reactivity (CR) data for each compound and for each MAb/conjugated hapten combination are summarized in Table 5. The specificity pattern differed significantly among MAbs derived from different type of haptens, from haptens of the same type, and even from the same hapten, especially for the most structurally similar compounds (chlorpyrifos-methyl and chlorpyrifos-oxon). On the contrary, the specificity of LIB−**PO** MAb using different coating haptens (**PNC** or **TR1**) did not change significantly, suggesting that what determines specificity is actually the MAb, whatever the assay hapten used. A general feature observed was that the change from pyridyl- to phenyl-type compounds involved large reduction of their recognition, although at different extent for each MAb. Cross-reactivity of the major chlorpyrifos metabolite, TCP, was negligible for most of the antibodies. Chlorpyrifos-oxon, another chlorpyrifos metabolite, showed a wide range of cross-reactivity, from the poor recognition by LIB−**AR1.1** MAb (3.6% CR, I_{50} = 972 nM) to the high affinity displayed by LIB−**AR1.4** (264% CR, I_{50} = 12 nM), although interestingly both MAbs originated from the same immunizing hapten. Taking advantage of the different specificity, an adequate combination of MAbs might be used to differentially determine chlorpyrifos and chlorpyrifos-oxon in samples where both compounds could be encountered. Except LIB−**PN1** MAb, all MAbs showed higher affinity to chlorpyrifos-methyl than to chlorpyrifos. A similar behavior has been reported for a rabbit polyclonal immunoassay raised from an OVA−**PN1** immunogen (Edward *et al*., 1993). In this respect, LIB−**AR1.1** MAb provided a highly sensitive ELISA for chlorpyrifos-methyl (670% CR, I_{50} = 5 nM). On the basis of their CR to related organophosphorus insecticides, LIB−**AR1.4** and LIB−**PO** MAbs can be considered as the most specific to chlorpyrifos. On the contrary, LIB−**PN1** MAb cross-reacted to a greater or lesser extent with all of the organothiophosphates tested, and among them is remarkable the high recognition of azinphos-methyl (39% CR, I_{50} = 105 nM).

Evaluation of the Antibody-Coated Format. From the characterization study, LIB−**PO** proved to be the best MAb with regard to chlorpyrifos affinity and specificity and was then selected to evaluate the antibody-coated (enzyme tracer) ELISA format. With this aim, haptens of type II were conjugated to HRP and used as enzyme tracers. The subsequent bidimensional titration of the MAb versus each tracer allowed the selection of appropriate concentrations for competitive immunoassays. **PO**, **PN1**, **PN2**, and **PNC** were the tracers best recognized by LIB−**PO** MAb, while **TR2** was poorly recognized and **TR1** was nearly unrecognized. To perform competitive assays, heterologous tracers required higher concentrations of immunoreagents than the homologous one, especially for the hapten lacking the thiophosphate group (10 μg/mL of MAb, 5 μg/mL of HRP−**TR2**). Since the **TR1** hapten provided the highest affinity for chlorpyrifos using LIB−**PO** MAb in the conjugate-coated format, additional effort was devoted to find out whether the lack of reactivity was related with a low hapten to HRP molar ratio. Thus, a new HRP−**TR1** tracer with a molar ratio of 4 was prepared, under the strongest conjugation conditions afforded by the mixed anhydride method (Tijssen, 1985). This tracer with higher hapten loading was also unrecognized. Therefore, it is possible that changes in the three-dimensional structure of the HRP−hapten conjugate, *i.e.* changes in the electrostatic environment and/or accessibility to the hapten (steric hindrance) or antibody conformational changes due to immobilization may impair the MAb recognition of the HRP−**TR1** tracer. As shown in Table 6, competitive ELISAs obtained by direct coating of this MAb in combination with the recognized tracers provided I_{50} values for chlorpyrifos similar to those obtained with the conjugate-coated format.

The enzyme tracer format with indirect coating of the MAb was also evaluated. In this respect, the use of a capture anti-mouse antibody reduced considerably the MAb concentration necessary to give adequate signals.

Table 5. Specificity of Chlorpyrifos MAbs



	structure						CR[a], %					
							LIB-AR1.1	LIB-AR1.4	LIB-PO		LIB-PN1	LIB-PN2
compound	R_1	R_2	X	R_3	R_4	Y			OVA-PNC	OVA-TR1		
chlorpyrifos	EtO	EtO	N	Cl	Cl	S	100	100	100	100	100	100
chlorpyrifos-oxon	EtO	EtO	N	Cl	Cl	O	3.6	264	2.6	3	19	72
chlorpyrifos-methyl	MeO	MeO	N	Cl	Cl	S	670	183	121	118	55	182
fenchlorphos	MeO	MeO	C	Cl	Cl	S	35	3.8	5.9	6.4	19	17
bromophos	EtO	EtO	C	Cl	Br	S	7	1.8	3.6	3.6	25	27
trichloronate	EtO	Et	C	Cl	Cl	S	17	1.1	4.9	5.6	3.8	7.4
dichlofenthion	EtO	EtO	C	H	Cl	S	5	0.67	0.37	0.33	6.5	0.7
parathion	other organothiophosphorus insecticides						0.64	0.16	< 0.01	< 0.01	2.3	< 0.01
diazinon							7.7	< 0.01	0.03	0.03	3.3	< 0.01
azinphos-methyl							0.03	< 0.01	< 0.01	< 0.01	39	0.23
3,5,6-trichloro-2-pyridinol	(TCP) metabolite						< 0.01	1.5	0.03	0.01	< 0.01	0.03

[a] Percentage of cross-reactivity = (I_{50} of chlorpyrifos/I_{50} of other compound) × 100. Competitive ELISAs in the conjugate-coated format were performed in the following conditions: OVA−**AR5** (0.5 μg/mL)/LIB−**AR1.1** MAb (0.05 μg/mL); OVA−**AR5** (0.5 μg/mL)/LIB−**AR1.4** MAb (0.5 μg/mL); OVA−**PNC** (0.5 μg/mL)/LIB−**PO** MAb (0.20 μg/mL); OVA−**TR1** (1 μg/mL)/LIB−**PO** MAb (0.3 μg/mL); OVA−**TR2** (2 μg/mL)/LIB−**PN1** (0.04 μg/mL); and OVA−**PO** (0.25 μg/mL)/LIB−**PN2** (0.3 μg/mL).

Table 6. Affinity to Chlorpyrifos Obtained for LIB−PO MAb Using Different Assay Haptens and Formats

	I_{50}[a] (nM)		
	conjugate-coated format	antibody-coated format	
hapten		direct coating	indirect coating
PO	19	19	15
PN1	15	17	13
PN2	40	23	14
PNC	12	**11**	**8**
TR1	**9**	nd[b]	**7**
TR2	16	20	15

[a] Chlorpyrifos concentration reducing the ELISA maximum response to 50%. Data represent the mean of four experiments. Boldface characters indicate the heterologous hapten giving the highest sensitivity for each ELISA format. [b] Not determined due to low color development.

Competitive assays with this format resulted in only a slight improvement of assay sensitivities, as compared to those obtained with the direct coating format. **PNC** and **TR1** tracers provided the highest affinity for chlorpyrifos, although the color development achieved with the last one was so low that it, in practice, handicapped its use as enzyme tracer. Hence, the **PNC** tracer was the best hapten to be used as enzyme tracer for LIB−**PO** MAb, for both direct and indirect MAb coating, as indicated by the lowest I_{50} for chlorpyrifos (11 and 8 nM, respectively).

Competitive curves obtained with LIB−**PO** MAb, in the three configurations evaluated and with the assay hapten giving the lowest I_{50} for chlorpyrifos in each format, are presented in Figure 7. As shown, the immunoassays developed displayed similar standard curve characteristics. In these circumstances, the choice among them for a particular application should be based on the assay improvements achievable by a subsequent optimization procedure and on the assay performance on the matrix wherein the determination of chlorpyrifos is intended to be carried out.

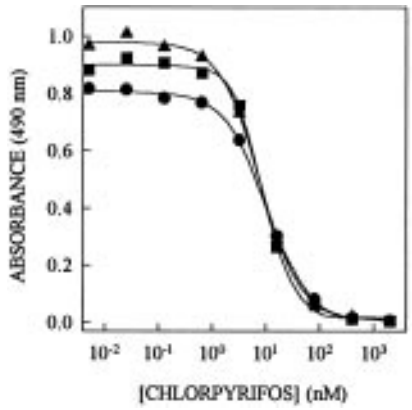


Figure 7. Chlorpyrifos competitive curves obtained for LIB−**PO** MAb in the three formats tested, using the assay hapten showing the lowest I_{50} in each case: (■) conjugate-coated format [OVA−**TR1** (1.0 μg/mL)/LIB−**PO** (0.30 μg/mL)]; (●) antibody-coated format [LIB−**PO** (10.0 μg/mL)/HRP−**PNC** (0.50 μg/mL)]; (▲) indirect antibody-coated format [goat anti-mouse (2 μg/mL)/LIB−**PO** (1 μg/mL)/ HRP−**PNC** (0.50 μg/mL)].

CONCLUSIONS

Following general guidelines about hapten design, several haptens were synthesized and examined for their ability to produce suitable antibodies to chlorpyrifos. Two series of haptens, characterized by the presentation of different parts of the chlorpyrifos molecular structure, were prepared. Immunological responses to protein−hapten conjugates were satisfactory and finally rendered sensitive, specific MAbs to chlorpyrifos from both types of haptens. Antibody affinities to chlorpyrifos were improved using different types of heterology. Apart from the recognition of chlorpyrifos with variable affinity, MAbs cross-reacted to a greater or lesser extent with the closely related compounds chlorpyrifos-methyl (another insecticide) and chlorpyrifos-oxon (a chlorpyrifos metabolite with higher toxic-

ity), which could be used to detect them sensitively if required.

Among the MAbs obtained one was selected for further studies on the basis of its highest affinity to chlorpyrifos. This MAb derived from the hapten that better resembles the chlorpyrifos molecular structure, since the spacer arm is an elongation of one of the ethoxy groups. After the best enzyme-labeled hapten was selected, the enzyme tracer format in two different configurations did not result in remarkable improvements of sensitivity to chlorpyrifos, although other repercussions on immunoreagent consumption and assay performance should be taken into account to select the appropriate format for a particular application.

Throughout this study, the convenience of the synthesis of a collection of haptens for a given analyte has been verified, to obtain MAbs with striking differences in sensitivity and specificity, which may expand the applicability of these immunological tools. In an accompanying paper, the influence of relevant physicochemical factors on the ELISAs to chlorpyrifos is reported, to optimize the assay and to adapt it to environmental water determinations.

ABBREVIATIONS USED

BSA, bovine serum albumin; CR, cross-reactivity; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; ELISA, enzyme-linked immunosorbent assay; EtAc, ethyl acetate; HRP, horseradish peroxidase; Hx, hexane; MAb, monoclonal antibody; I_{50}, concentration giving 50% inhibition of maximum response; NHS, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance; OPD, *o*-phenylenediamine; OVA, ovalbumin; PBS, 10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4; PBST, PBS containing 0.05% Tween 20; PEG, poly(ethylene glycol); TCP, 3,5,6-trichloro-2-pyridinol; THF, tetrahydrofuran; TLC, thin-layer chromatography.

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