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SHORT COMMUNICATIONS

Development of a Chlorpyrifos Immunoassay Using Antibodies Obtained from a Simple Hapten Design

INTRODUCTION

Chlorpyrifos [*O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] is a broad spectrum organophosphorus insecticide, widely used for the control of various crop pests in soil and on foliage, household insects, and animal parasites (Worthing and Hance, 1991). Its utilization is undoubtedly beneficial, but there are toxicological and environmental risks associated with the remaining residues after its application. Increasing chlorpyrifos and other organophosphate pesticide occurrence, along with recent concerns over long-term/low-level exposure to agrochemicals, has led to more comprehensive environmental and food monitoring programs (Vanderlaan et al., 1990; Yess et al., 1993).

Chlorpyrifos analyses are carried out by chromatographic techniques, mainly gas chromatography (Iwata et al., 1983; Sanz et al., 1991). Chromatographic methods are laborious and time-consuming and require sophisticated equipment only available in well-equipped centralized laboratories. Immunoassay technology is being demonstrated as a rapid and cost-effective alternative to traditional methods when a high sample throughput or on-site screening analyses is required (Vanderlaan et al., 1990; Sherry, 1992).

For the production of antibodies to small molecules such as chlorpyrifos, suitable haptens have to be synthesized and conjugated to carrier proteins, to render the analyte structure immunogenic. In aromatic organophosphate pesticides, two basic moieties can be differentiated for conjugation purposes: a phosphate group and an aromatic ring. An elaborated generic strategy for the synthesis of organothiophosphate haptens, with spacer arm attachment through the thiophosphate moiety, has been recently published (McAdam et al., 1992). Using this hapten, an immunoassay for the analysis of chlorpyrifos-methyl in cereal matrices, with appropriate sensitivity and specificity, was developed (Skerritt et al., 1992). On the other hand, organophosphate hapten immunogens prepared by modification through the aromatic ring rendered antibodies with relatively poor recognition properties of the target analyte (Vallejo et al., 1982; Brimfield et al., 1985; McAdam et al., 1992).

In the present study, we describe the one-step synthesis of a chlorpyrifos hapten, which consists of the attachment of a spacer arm through the aromatic ring with minor modification of the chemical properties of the original structure. From protein conjugates of this hapten, several monoclonal antibodies (MAb) that recognize chlorpyrifos were obtained. We also report the use of these MAb in the development of a sensitive and specific competitive enzyme immunoassay for the analysis of chlorpyrifos.

EXPERIMENTAL PROCEDURES

Materials and Instruments. Organic synthesis reagents, standards, and biological products were of the highest grade available from current commercial suppliers. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded with a Varian VXR-400S spectrometer (Varian, Sunnyvale, CA), operating at 400 and 100 MHz, respectively. Absorbances obtained in enzyme-linked immunosorbent assays (ELISA) were read and recorded with a Dynatech MR-700 microplate reader (Sussex, U.K.).

Hapten Synthesis. The structures of haptens **1** and **2** are shown in Figure 1. Synthetic intermediates were analyzed by TLC and confirmed by ^1H NMR.

O,O-Diethyl O-[3,5-Dichloro-6-[(2-carboxyethyl)thio]-2-pyridyl] Phosphorothioate (**1**). To a solution of 3-mercaptopropanoic acid (1.06 g, 10 mmol) in 50 mL of absolute ethanol, 2 equiv of KOH (1.42 g) was added and heated until dissolved. Then, chlorpyrifos (technical grade, 3.51 g, 10 mmol) dissolved in 50 mL of absolute ethanol was added. After 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 (2 × 50 mL). The aqueous layer was acidified to pH 4 and extracted with dichloromethane (3 × 50 mL). The extract was dried over Na_2SO_4 and concentrated, and the residue was subjected to column chromatography [hexane/tetrahydrofuran (THF)/acetic acid 75:25:1]. Fractions showing only one spot on TLC (R_f 0.41, same solvent mixture) were pooled and concentrated to provide 1.07 g of **1** (26%), which

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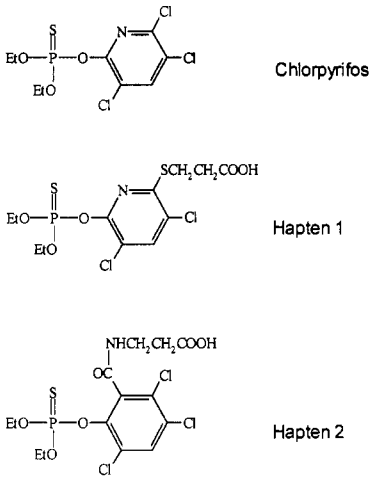


Figure 1. Structures of chlorpyrifos and the haptens used for preparing conjugates.

solidified on standing: mp 124–125 °C; ^{1}H NMR ($CDCl_3$) δ 7.76 (S, 1H, ArH), 4.37 (q+q, 4H, 2 CH_2O), 3.45 (t, 2H, SCH_2), 2.97(t, 2H, CH_2COO), 1.42 (t, 6H, 2 CH_3); ^{13}C NMR ($CDCl_3$) δ 177.1 (COOH), 153.8 (C6, Ar), 151.6 (C2, Ar), 139.2 (C4, Ar), 125.2 (C5, Ar), 116.2 (C3, Ar), 65.4 (2 OCH_2), 34.2 (SCH_2), 25.4 (CH_2COOH), 15.9 (2 CH_3).

O,O-Diethyl O-[3,4,6-Trichloro-2-[[(2-carboxyethyl)amino]carbonyl]phenyl] Phosphorothioate (2). The protected amino acid *tert*-butyl 3-aminopropanoate was synthesized as described by McAdam et al. (1992). To a stirred solution of 3,5,6-trichlorosalicylic acid (486 mg, 2 mmol), *tert*-butyl 3-aminopropanoate (580 mg, 4 mmol), and (dimethylamino)pyridine (20 mg) in 3 mL of THF was added dicyclohexylcarbodiimide (DCC; 453 mg, 2.2 mmol) in 1 mL of THF. After 5 h at room temperature, the mixture was filtered and the solvent evaporated under reduced pressure. The yellowish oil was subjected to column chromatography (hexane/ethyl acetate/acetic acid 70:30:1) to provide 246 mg (0.667 mmol) of *tert*-butyl 3-[(2-hydroxy-3,5,6-trichlorophenyl)methanamido]propanoate (33%, R_f 0.63) as a colorless solid. To a solution of this product in THF/water (1:1, 5 mL) was added 1 M NaOH (667 μL). The solvent was removed under reduced pressure and the residue was heated at 80 °C until complete dryness to give the corresponding sodium salt. To a heterogeneous mixture of this salt in 8 mL of acetonitrile was added diethyl chlorothiophosphate (150 mg, 0.8 mmol). The mixture was refluxed for 1 h and then filtered, and the solvent was evaporated. Without further purification, the residue was treated with 50% trifluoroacetic acid in 2 mL of dichloromethane for 1 h to deprotect the carboxylic group. After evaporation of the solvent, the residue was chromatographed (hexane/ethyl acetate/acetic acid 50:50:1) to yield 157 mg of 2 (51%) (R_f 0.54 in TLC, same solvent mixture): mp 161–162 °C; ^{1}H NMR ($CDCl_3$) δ 7.57 (S, 1H, ArH), 6.71 (S, 1H, NH), 4.27 (q+q, 4H, 2 CH_2O), 3.69 (d, 2H, NCH_2), 2.75 (t, 2H, CH_2COO), 1.38 (t, 6H, 2 CH_3).

Preparation of Hapten–Protein Conjugates. Haptens 1 and 2 were coupled to proteins (bovine serum albumin, BSA; ovalbumin, OVA) by the active ester method described by Langone and Van Vunakis (1975). Conjugates (BSA–1, OVA–1, and OVA–2) were purified by gel filtration chromatography on Sephadex G-25 (Pharmacia, Uppsala, Sweden).

Production of Monoclonal Antibodies. Five BALB/c female mice (8–10 weeks old) were intraperitoneally immunized with 2 week-interval injections of the immunogen (BSA–1, 100 μg per mouse) emulsified with Freund's adjuvants. Mouse serum titers and analyte recognition properties were analyzed during the immunization schedule by indirect ELISA. Splenocytes from mice showing the highest serum titers were fused with P3-X63/Ag8.653 murine myeloma cells (ATCC, Rockville, MA) according to established protocols (Nowinski et al., 1979). Hybridoma culture supernatants were screened by indirect ELISA for antibodies binding to OVA–1. Positive hybridomas were further analyzed by competitive indirect ELISA, and those selected were cloned by limiting dilutions. MAb were purified from late stationary phase culture supernatants by ammonium sulfate precipitation followed by anion-exchange chromatography on DEAE-Sepharose (Sigma, St. Louis, MO).

Enzyme-Linked Immunosorbent Assay. Flat bottom polystyrene ELISA plates (Nunc, Roskilde, Denmark) were coated in carbonate buffer overnight at 4 °C. Antibodies were diluted in phosphate-buffered saline containing 0.05% Tween 20 (PBST). A volume of 100 μL per well was used throughout all assay steps. After each step, plates were washed with PBST.

Indirect ELISA. Plates coated with the OVA–1 conjugate (1 μg/mL) were incubated with culture supernatants or serial dilutions of sera at room temperature for 1 h. Captured antibodies were measured by incubation at room temperature for 1 h with goat anti-mouse peroxidase-labeled immunoglobulins, followed by the addition of the substrate solution (2 mg/mL *o*-phenylenediamine, 0.012% H_2O_2 in phosphate–citrate buffer, pH 5.4). After 10 min, the enzymatic reaction was stopped by adding H_2SO_4 and the absorbance was read at 490 nm.

Competitive Indirect ELISA. The protocol was the same as for the indirect ELISA except that after coating with OVA–1 or OVA–2 (homologous or heterologous assay, respectively), a competition step was established by adding the appropriate concentration of antibody (serum, culture supernatant, or purified MAb) to different concentrations of competitor (chlorpyrifos or related compounds). For each assay, the optimal concentrations of both coating conjugate and antibody were estimated by checkerboard titration. Competition curves were obtained by plotting absorbance vs the logarithm of competitor concentration and were fitted to four-parameter logistic equations (Raab, 1983).

RESULTS AND DISCUSSION

The chlorpyrifos structure (Figure 1) contains at least two sites for spacer attachment. One option is the introduction of the spacer as a thiophosphate ester. This approach was successfully applied by McAdam et al. (1992) for the development of a chlorpyrifos-methyl immunoassay, but a multistep synthesis procedure to prepare the thiophosphoramide haptens was followed. The second possibility, which is studied here, is spacer coupling through the pyridyl ring by chlorine substitution using a suitable nucleophilic compound. In this sense, 3-mercaptopropanoic acid was previously used in a similar case for synthesizing *s*-triazine herbicide haptens (Goodrow et al., 1990).

Hapten 1 (Figure 1) was directly prepared from chlorpyrifos by reaction with 3-mercaptopropanoic acid. Its structure was confirmed by ^{1}H and ^{13}C NMR. Special analytical attention was directed to finding out which chlorine (ring position 3, 5, or 6) was preferentially substituted. Position 6 is activated for nucleophilic substitution by the nitrogen in ortho. In fact, attempts to carry out the same reaction with the chlorpyrifos analog fenchlorphos-ethyl, containing carbon instead of nitrogen as the unique structural modification, failed to provide detectable quantities of any substituted compound. This result confirmed the strong activation exerted by the nitrogen on the adjacent chlorine. Furthermore, by comparing the ^{13}C NMR chemical shifts of chlorpyrifos and hapten 1, a clear displacement of the signal assigned to C6 (McAdam and Skerritt, 1993) from 144.2 to 153.8 ppm was observed. Therefore, spacer attachment was very likely accomplished in the chlorine in position 6.

Using the active ester method, suitable hapten–protein conjugates were obtained. A clear spectrum modification of protein–hapten 1 conjugates at 315 nm after coupling allowed reliable estimates of the hapten to protein molar ratio (23 and 8 for BSA–1 and OVA–1, respectively).

Five mice were immunized with BSA–1. After three injections, serum titers (serum dilution that gave 3 times background absorbance) in the range 1:100000–1:300000 were found by indirect ELISA, using the homologous OVA–1 as coating conjugate. Serum titers did not

Table 1. Summary of the Monoclonal Antibody Selection Results

fusion no.[a]	no. of wells seeded	no. of wells positive[b]	no. of wells competitive[c]	no. of cloned hybridomas
1	384	18	8	4
2	384	48	3	1

[a] Mice used for fusion 1 or 2 received three or five biweekly intraperitoneal injections of BSA–1, respectively. [b] Wells with antibodies that recognized the OVA–1 coating conjugate by indirect ELISA (absorbance >0.5). [c] Wells with antibodies that recognized free-chlorpyrifos (inhibition >50% by 3 or 1 μM chlorpyrifos, for fusion 1 or 2, respectively). Competitive ELISAs were carried out with 0.3 μg/mL of OVA–1 as coating and the appropriate supernatant dilutions giving absorbances in the range 0.8–1.5.

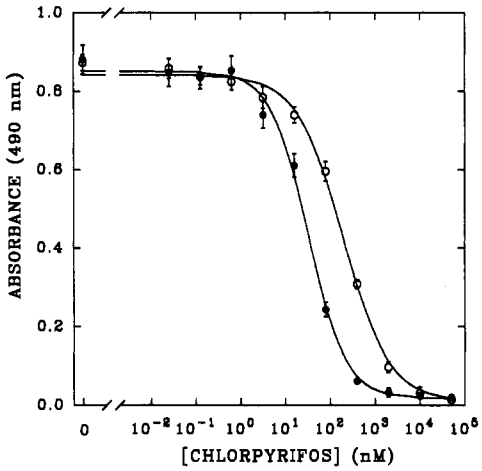


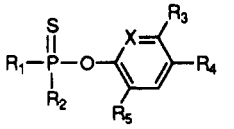
Figure 2. ELISA standard curves of chlorpyrifos using LIB-C4 MAb: (○) homologous assay (0.2 μg/mL of OVA–1 as coating, 0.05 μg/mL of MAb); (●) heterologous assay (1 μg/mL of OVA–2 as coating, 0.2 μg/mL of MAb). Each point represents the mean ± SD for eight replicates.

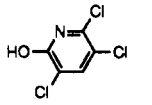
significantly improve after five injections. Sera were subsequently tested for their ability to bind chlorpyrifos by indirect competitive ELISA. The concentration producing 50% inhibition of antibody binding (IC_{50}) was 2.5 μM chlorpyrifos, confirming the usefulness of the designed hapten to elicit antibodies that recognize the free analyte.

Two fusions were carried out using splenocytes from the mice showing the highest serum titers. The results of the hybridoma culture supernatant screening are presented in Table 1. Of a total of 768 wells seeded, 66 were considered positive by indirect ELISA. Of the 66, 11 were selected by a restrictive indirect competitive ELISA (inhibition >50% with 3 μM chlorpyrifos). Finally, five hybridoma cell lines were cloned and stabilized. MAb were purified and characterized for chlorpyrifos recognition properties. One of them, LIB-C4, showed the highest affinity for chlorpyrifos (IC_{50} = 197 nM, 69 ng/mL) in a homologous indirect competitive ELISA (Figure 2). This result, obtained from a simple hapten derivatized through the aromatic ring, is comparable to those previously reported for chlorpyrifos-methyl haptens derivatized through the thiophosphate group (Skerritt et al., 1992).

Heterologous assays are well-known procedures to improve hapten immunoassay sensitivity (Harrison et al., 1991). Attachment position and spacer modification have been proved to be adequate types of heterology (Wie and Hammock, 1984; Szurdoki et al., 1992). The heterologous hapten 2 (Figure 1), with both site and spacer heterologies, was synthesized as described under Experimental Procedures and coupled to OVA with an approximate molar ratio of 12. The OVA–2 conjugate was subsequently used as coating antigen in a heterologous indirect competitive

Table 2. Cross-Reactivity of Related Compounds in the Heterologous Competitive Indirect ELISA for Chlorpyrifos



compound	structure R_1	R_2	X	R_3	R_4	R_5	IC_{50}, nM	CR,[a] %
chlorpyrifos	EtO	EtO	N	Cl	Cl	Cl	33	100
chlorpyrifos-methyl	MeO	MeO	N	Cl	Cl	Cl	18	183
fenchlorphos	MeO	MeO	C	Cl	Cl	Cl	835	3.8
bromophos	EtO	EtO	C	Cl	Br	Cl	1760	1.8
trichloronate	EtO	Et	C	Cl	Cl	Cl	2880	1.1
dichlofenthion	EtO	EtO	C	H	Cl	Cl	4950	0.67
parathion	EtO	EtO	C	H	NO_2	H	20700	0.16
3,5,6-trichloro-2-pyridinol (metabolite)							2160	1.5

[a] Percentage of cross-reactivity = (IC_{50} of chlorpyrifos/IC_{50} of other compound) × 100.

ELISA. The typical standard curve of chlorpyrifos using LIB-C4 in the heterologous assay is shown in Figure 2. With this ELISA format, sensitivity was improved 6 times (IC_{50} = 33 nM, 11.6 ng/mL for chlorpyrifos).

The heterologous assay was further characterized regarding its limit of detection (LOD) and specificity. The assay LOD, calculated as the chlorpyrifos concentration corresponding to the absorbance of the zero dose minus three standard deviations, was 3.7 nM (1.3 ng/mL). Specificity was evaluated by performing competitive assays with several related organophosphorus insecticides as competitors. IC_{50} and relative cross-reactivity (CR) values for each compound are given in Table 2. Chlorpyrifos-methyl showed an even lower IC_{50} (18 nM, 183% CR), suggesting that the methyl groups are better accommodated in the antibody binding site. Another important feature was that the change from pyridyl to phenyl-type compounds markedly influences the interaction, since other halogenated organothiophosphates bearing this change cross-reacted less than 2% (bromophos, 1.8%; trichloronate, 1.1%; dichlofenthion, 0.67%), except fenchlorphos (3.8%) which is in consonance with being a methyl analog. CR of other tested organophosphates were <0.2% (parathion, chlorfenvinphos). The major metabolite of chlorpyrifos (3,5,6-trichloro-2-pyridinol) was weakly recognized (1.5% CR).

From previous studies of organophosphate hapten design (McAdam et al., 1992; Vallejo et al., 1982; Brimfield et al., 1985), it might be inferred that better immunoassay characteristics could be achieved by introducing the spacer through the thiophosphate ester moiety than through the aromatic ring. In spite of these results, we undertook the synthesis of a chlorpyrifos hapten by attaching the spacer arm through the pyridyl ring, taking advantage of the presumably easy nucleophilic substitution of the chlorine in position 6 and assuming that this strategy would produce minor modifications of analyte structure and properties. The synthesis consisted of only one step using chlorpyrifos as starting material. With this appropriate hapten design, MAb were obtained with which we have demonstrated that a sensitive, specific immunoassay for chlorpiryfos can be developed. The assay could be used to analyze chlorpyrifos and chlorpyrifos-methyl if a standard curve was obtained for each pesticide. Although the quantification would not be reliable for samples containing both pesticides, the occurrence of such samples is unlikely since the simultaneous application of both pesticides to the same

crop makes no sense, provided that a good agricultural practice is observed. The intended use of the immunoassay is as a screening method of a high number of foodstuff samples suspected to have been treated with chlorpyrifos or chlorpyrifos-methyl. In this sense, studies on the assay performance in several matrices and validation with a reference analytical method are in progress.

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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

[†] Laboratorio Integrado de Bioingeniería.
[‡] Instituto de Tecnología Química.