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SHORT COMMUNICATIONS

Production of High-Affinity Monoclonal Antibodies for Azinphos-methyl from a Hapten Containing Only the Aromatic Moiety of the Pesticide

Keywords: *Azinphos; Guthion; hapten synthesis; monoclonal antibodies; ELISA; phosmet*

INTRODUCTION

Azinphos-methyl [*O,O*-dimethyl *S*-[(4-oxo-1,2,3-benzotriazin-3-yl)methyl] phosphorodithioate], also called Guthion or Gusathion, is a broad spectrum organophosphorous insecticide and acaricide. It is effective against biting and sucking insect pests and is mainly used on citrus, cotton, grapes, maize, some ornamental plants, top quality fruit, and vegetables (García and Aguilar, 1992). Azinphos-methyl is reportedly one of the most persistent of the many "nonsystemic" organic phosphate pesticides, and its toxicity range is similar to that of the parathions (McDougall, 1964). Schuytema et al. (1994) reported that over 520 000 kg of the active ingredient was used in the United States in 1991 on fruit crops and cotton in the major producing states. Azinphos-methyl has been listed in the 76/464/EEC Council Directive of dangerous substances discharged into the aquatic environment of the European Union (Barceló, 1993). All of these considerations make it desirable to monitor azinphos-methyl in food commodities as well as in environmental matrices.

Several methods have been reported for the determination of azinphos-methyl using a variety of techniques, such as spectrophotometry (Gunther et al., 1980), spectrofluorometry (García and Aguilar, 1992), mass spectrometry (Schulten and Sun, 1981), gas chromatography (Allmaier et al., 1986), and HPLC with UV (Carabias Martínez et al., 1992) or electrochemical detection (Carabias Martínez et al., 1993). Nevertheless, these methods are time-consuming, require sophisticated equipment, or otherwise lack the sensitivity and selectivity demanded for residue monitoring in agricultural and food products and in environmental matrices. Immunoassays are being demonstrated as suitable alternative tools for quick and sensitive, as well as cost-effective, analysis especially for screening large numbers of samples (Sherry, 1992; Niessner, 1993).

The growing interest in immunoassays leads to an increasing demand for antibodies that are able to detect the analytes in very low ranges (Giersch, 1993). For organophosphorous pesticides, sensitive immunoassays have already been reported (McAdam et al., 1992; Manclús et al., 1994). In the present paper, we describe the preparation of an azinphos hapten, consisting of the attachment of a spacer arm to one of the intermediate products of the synthesis of the pesticide. Using this hapten, which preserves the characteristic rings of the molecule, we were able to obtain, for the first time, four monoclonal antibodies (MAbs) that recognize azinphos-methyl, one of them in the low nanomolar range.

EXPERIMENTAL PROCEDURES

Materials and Instruments. Organic synthesis reagents and biological products were of the highest grade available from current commercial suppliers. Analytical standards of azinphos-methyl, azinphos-ethyl, phosmet, quinalphos, and chlorpyrifos, all of Pestanal grade, were purchased from Riedel-de-Haën AG (Seelze, Germany). Stock solutions of each pesticide were prepared in *N,N'*-dimethylformamide (DMF). ^{1}H NMR spectra were recorded with a Varian VXR-400S spectrometer (Varian, Sunnyvale, CA), operating at 400 MHz. Electron impact mass spectra (EIMS) were recorded on a 5988A Hewlett-Packard apparatus at 70 eV, and data are reported as *m/z* (relative intensity). A Hewlett-Packard 5890 gas chromatograph equipped with a 20 m × 0.20 mm (i.d.) HP-5 column was interfaced to this spectrometer for GC–MS analyses. Ultraviolet (UV) spectra were recorded on a UV-160A Shimadzu apparatus.

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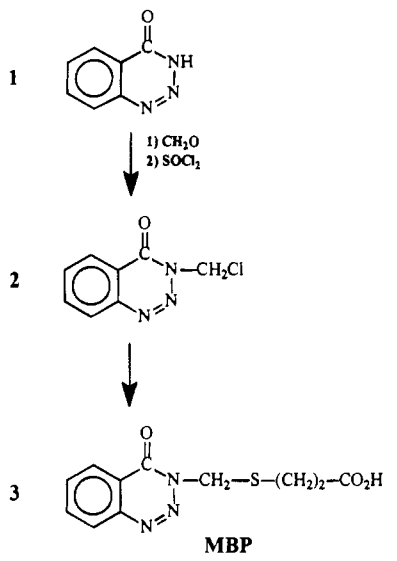


Figure 1. Synthesis pathway of MBP hapten.

Hapten Synthesis. The synthesis strategy is shown in Figure 1. The first step follows one of the pathways to industrially obtain azinphos-methyl, and the second one is a modification of the reaction published by Manclús et al. (1994), which links the spacer arm to the hapten.

3-(Chloromethyl)-1,2,3-benzotriazin-4-one [*N-(chloromethyl)benzazimide*, **2**]. This intermediate was synthesized from 1,2,3-benzotriazin-4(3*H*)-one (benzazimide, **1**, Sigma-Aldrich, Spain) according to established procedures (Sittig, 1977), and its structure was subsequently confirmed. ^{1}H NMR ($CDCl_3$) δ 8.65–7.90 (m, 4H, ArH_4), 6.30 (s, 1H, NCH_2Cl); GC–MS *m/z* 196 (M + 1, 2), 195 (M, 1), 160 (100), 133 (4), 132 (47), 105 (11), 104 (31), 90 (7), 77 (59), 76 (35), 75 (17), 74 (14), 63 (11), 51 (19), 50 (41), 49 (20).

3-[(4-Oxo-1,2,3-benzotriazin-3-yl)methylthio]propionic Acid (*MBP*, **3**). To a solution of 3-mercaptopropionic acid (50 mmol, 4.4 mL) in 20 mL of absolute ethanol was added 2 equiv of KOH (5.7 g) and heated until dissolved. Then, 25 mmol of **2** (4.9 g), previously dissolved in 50 mL of absolute ethanol, was slowly added under vigorous stirring. After 30 min, the solvent was removed at reduced pressure. To the residue was added 40 mL of water, followed by washing with hexane (2 × 50 mL). The aqueous layer was acidified to pH 4, extracted with ethyl acetate (3 × 50 mL), and dried over Na_2SO_4. The solution was concentrated to the minimum volume and left for 3 days at 4 °C for crystallization. The precipitate was dried, yielding 3.1 g (46%) of **3** as a yellowish solid. TLC analysis (dichloromethane/ethyl acetate/acetic acid 70:30:1) showed only one spot (R_f 0.54). ^{1}H NMR (acetone-d_6) δ 8.34–7.94 (m, 4H, ArH_4), 5.60 (s, 1H, NCH_2S), 3.04 (t, 2H, SCH_2C), 2.72 (t, 2H, CCH_2CO). A fraction of **3** was esterified with methanol and the product analyzed by GC–MS: *m/z* 280 (M, 1), 248 (1), 192 (1), 160 (74), 133 (5), 132 (43), 119 (1), 105 (19), 104 (38), 77 (100), 76 (28), 74 (8), 59 (15), 55 (28), 45 (27).

Preparation of Hapten–Protein Conjugates. MBP was coupled to bovine serum albumin (BSA) according to the mixed anhydride method described by Rajkowski et al. (1977) and to ovalbumin (OVA) according to the active ester method described by Langone and Van Vunakis (1975). BSA–MBP and OVA–MBP conjugates were purified by gel filtration on Sephadex G-25 (Pharmacia, Uppsala, Sweden).

Production of Monoclonal Antibodies. Six BALB/c female mice (8–10 weeks old) were intraperitoneally immunized with 200 μL of a 1:1 (v/v) mixture of complete Freund's adjuvant and PBS (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 100 μg of BSA–MBP conjugate. Three subsequent injections were given at 2 week intervals using incomplete Freund's adjuvant, and a last injection was made with no adjuvant. Mouse serum titers and analyte recognition properties were analyzed during the immunization schedule by indirect enzyme-linked immunosorbent assay (ELISA). Four days after the fifth injection, spleenocytes from mice were fused with P3-X63/Ag8.653 murine myeloma cells (ATCC, Rockville, MD) according to established protocols (Nowinski et al., 1979). Twelve days after fusion, hybridoma culture supernatants were screened by simultaneous indirect noncompetitive/competitive ELISA as described by Abad and Montoya (1994). Those hybridomas secreting MAbs that recognized azinphos-methyl in solution were cloned by limiting dilutions and expanded. MAbs were purified from late stationary phase culture supernatants by ammonium sulfate precipitation followed by anion exchange chromatography on DEAE-Sepharose (Sigma, St. Louis, MO). The purified MAbs were stored at 4 °C as ammonium sulfate precipitates [50% saturation, according to Montoya and Castell (1987)], and the immunoglobulin isotype was determined using a commercial kit from Bio-Rad (Richmond, CA).

Enzyme-Linked Immunosorbent Assays. Easy wash, modified flat-bottom polystyrene ELISA plates (Corning Inc., Corning, NY) were coated with the OVA–MBP conjugate in 50 mM carbonate–bicarbonate buffer (pH 9.6) by overnight incubation at 4 °C. Antibodies were diluted in PBS 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 four times with 0.05% Tween 20 in 0.15 M NaCl.

Noncompetitive ELISA. Plates coated with 1 μg/mL of OVA–MBP conjugate were incubated with serial dilutions of sera, culture supernatants, or purified MAbs for 1 h at 37 °C. Captured antibodies were measured by incubation for 0.5 h at 37 °C with 100 μL of a 1/2000 dilution of peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako, Denmark), followed by 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 with 2.5 M H_2SO_4 and the absorbance read at 490 nm.

Competitive ELISA. The procedure was a modification of the noncompetitive ELISA. After coating, a competition step was introduced by adding various concentrations of the analyte together with the appropriate concentration of antibody (serum, culture supernatant, or purified MAb) to reach a maximum absorbance near 1.0 at the zero dose of analyte. For each assay, the optimal concentrations of both coating conjugate and antibody were estimated by checkerboard titration. Competition curves were analyzed by mathematically fitting experimental points to a four-parameter logistic equation using a commercial software package (Sigmaplot, Jandel Scientific).

Determination of Cross-Reactivities. Different compounds were tested for cross-reactivity by performing competitive assays and determining the concentration producing 50% inhibition of antibody binding (I_{50}). Cross-reactivity was calculated as (I_{50} azinphos-methyl/I_{50} compound) × 100.

RESULTS AND DISCUSSION

In aromatic organophosphate pesticides, such as azinphos-methyl, two basic moieties can be differentiated: a phosphate group and an aromatic ring. The ring and its substituents constitute the major difference among the molecular structures of the many organophosphorous pesticides. The aromatic moiety of azinphos (methyl or ethyl) is composed by two rings, to which we intended to raise specific monoclonal antibodies. With this aim, we obtained a hapten (MBP) consisting of the two original rings of azinphos attached to a spacer arm. The synthesis pathway consisted of only two steps (Figure 1) and was very easy to perform with high yields, which made it an appropriate strategy for our purposes.

The UV absorbance spectrum of azinphos-methyl shows two absorbance peaks at 240 and 285 nm, with an important shoulder at 317 nm. This shoulder is also displayed by the MBP hapten, which allows the determination of hapten-to-protein molar ratios (MR) after

Table 1. Summary of the Monoclonal Antibody Selection Results

fusion no.	serum[a] I_{50} (μM)	no. of wells seeded	positive[b]	competitive[c]	no. of cloned hybridomas
1	>100	563	39	4	0
2	>100	305	6	0	0
3	91	288	19	4	2
4	40	576	50	8	1
5	>100	384	15	0	0
6	>100	480	16	4	1

[a] Sera collected just before fusion. Azinphos-methyl used as competitor. [b] Wells with antibodies that recognized the OVA–MBP conjugate by indirect noncompetitive ELISA. [c] Wells that showed near or more than 50% inhibition with 10 μM azinphos-methyl by indirect competitive ELISA.

conjugation. By assuming additive absorbance values, MR of 17 and 14 were estimated for BSA–MBP and OVA–MBP conjugates, respectively.

Six mice were immunized with BSA–MBP, and sera were periodically titrated by indirect noncompetitive ELISA using OVA–MBP as coating conjugate. Sera dilutions giving an absorbance in the range of 0.8–1.2 were subsequently used in indirect competitive ELISA with different concentrations of azinphos-methyl and MBP as competitors. The estimated I_{50} ranged from 11 μM to more than 100 μM for azinphos-methyl. For each mouse, the I_{50} for azinphos-methyl was essentially the same throughout the immunization schedule. Right before cell fusion, sera were also collected and assayed as before. Only two of these sera showed I_{50} below 100 μM for azinphos-methyl (Table 1).

After each fusion, culture supernatants were screened as described under Experimental Procedures, using OVA–MBP as coating conjugate and 10 μM azinphos-methyl (a concentration clearly below the I_{50} of sera) as competitor. For each culture supernatant, the signal obtained in noncompetitive conditions was compared to that of the competitive assay, and ratios near or below 50% inhibition were used as the criteria for selecting high-affinity antibody-secreting clones. Of a total of 2596 wells seeded, 145 were considered to be positive, but only 20 of them were competitive (Table 1). Finally, four hybridoma cell lines were cloned and stabilized. LIB/MBP-3.1 and LIB/MBP-3.4 were obtained from fusion 3, while LIB/MBP-4.1 and LIB/MBP-6.1 were from fusions 4 and 6, respectively. Supernatants of the selected hybridomas were titrated, and the I_{50} for azinphos-methyl was determined. LIB/MBP-4.1 and LIB/MBP-6.1 were not further characterized since their I_{50} values were too high (above 1 μM). On the other hand, LIB/MBP-3.1 and LIB/MBP-3.4, both of them being IgG_1 (κ) subclass, showed better affinity, so they were expanded and purified.

Characterization of LIB/MBP-3.1 and LIB/MBP-3.4 was conducted by homologous antigen-coated competitive ELISA. The concentration of OVA–MBP as coating conjugate and that of MAb were adjusted to obtain inhibition curves with a maximum absorbance near 1.0 in the absence of analyte, to ensure optimal competitive conditions. For LIB/MBP-3.1, 25 ng/mL of both coating conjugate and MAb was used in the competitive assay. In the case of LIB/MBP-3.4, 20 ng/mL of each reagent was used. Figure 2 shows the typical inhibition curves displayed by either MAb. LIB/MBP-3.1 had an I_{50} of 346 nM (110 μg/L) for azinphos-methyl. Compared with the I_{50} of the corresponding serum, an improvement of 2 orders of magnitude was achieved (curve C vs curve A). Even better results were obtained for LIB/MBP-3.4, which showed an I_{50} of 28 nM (9 μg/L) for azinphos-methyl, thus improving the serum I_{50} by 3 orders of magnitude (curve D vs curve A). Very similar I_{50} values were obtained when the MBP hapten was used as competitor (Table 2). On the basis of its low I_{50}, LIB/MBP-3.4 can be considered as a high-affinity MAb for azinphos-methyl, comparable to other antibodies reported for pesticides (McAdam et al., 1992; Manclús et al., 1994), including those involved in commercially available immunoassays (Niessner, 1993; Meulenberg et al., 1995).

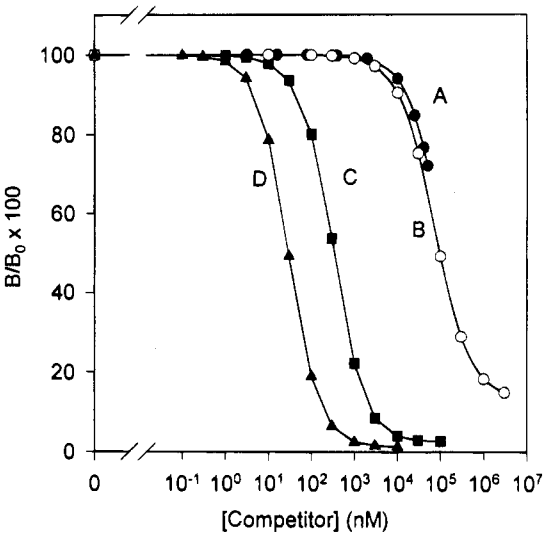
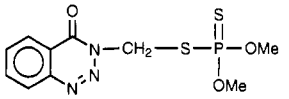
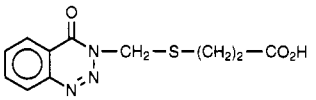
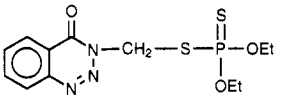
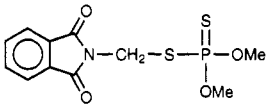
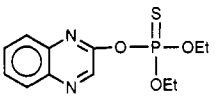
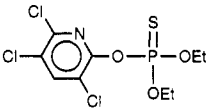
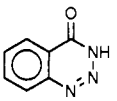
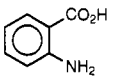


Figure 2. Normalized competitive ELISA standard curves of azinphos-methyl (solid symbols) and MBP hapten (open symbols), using fusion serum (●, ○), LIB/MBP-3.1 (■), and LIB/MBP-3.4 (▲). Experimental maximum absorbances were 0.9 for curves performed with the MAbs and 0.6 for those performed with fusion serum.

Both antibodies were further characterized by determining their cross-reactivity (CR) with a variety of pesticides related to azinphos-methyl. In Table 2 the I_{50} obtained for four pesticides and for the main decomposition products of azinphos-methyl are listed. Both LIB/MBP-3.1 and LIB/MBP-3.4 MAbs showed, as expected, an important CR with azinphos-ethyl (41.3% and 68.3%, respectively). LIB/MBP-3.1 also presented a significant CR with quinalphos (76.9%) and some CR with phosmet (11.9%). On the contrary, LIB/MBP-3.4 highly cross-reacted with phosmet (93.3%) but did not cross-react with quinalphos (0.4%). The decomposition products tested (benzazimide and anthranilic acid) presented no significant CR with either MAb. Besides the compounds listed in Table 2, diazinon, carbaryl, methidathion, phosalone, and dialifos were tested, all of them showing CR values below 1%. These results mainly suggest that both MAbs recognize the $-N-CH_2-S-$ group together with the unsubstituted aromatic ring. Moreover, LIB/MBP-3.4 seems to require the amide moiety at the heterocyclic ring for good interaction.

The sensitivity and specificity of pesticide immunoassays primarily depend on the ability to raise antibodies from haptens resembling the original analyte structure (Harrison et al., 1990; Mei et al., 1991). In this respect, an interesting approach was reported by Schlaeppi et al. (1992), who obtained excellent MAbs to triasulfuron from a hapten corresponding only to the chloroethoxy sulfonamide moiety of the pesticide. In the

Table 2. Cross-Reactivity of Structurally Related Compounds and Decomposition Products

compound	chemical structure	LIB/MBP-3.1 I_{50} (nM)	LIB/MBP-3.1 CR (%)	LIB/MBP-3.4 I_{50} (nM)	LIB/MBP-3.4 CR (%)
azinphos-methyl		346	100	28	100
MBP		428	80.8	25	112.0
azinphos-ethyl		837	41.3	41	68.3
phosmet		2900	11.9	30	93.3
quinalphos		450	76.9	6600	0.4
chlorpyrifos		$>10^5$	<0.3	$>10^4$	<0.2
benzazimide[a]		$>10^5$	<0.3	$>10^4$	<0.2
anthranilic acid[a]		$>10^5$	<0.3	$>10^6$	<0.2

[a] Main decomposition products of azinphos-methyl.

present paper, a similar strategy is addressed to easily obtain an azinphos hapten derived from only its aromatic moiety. MBP has been demonstrated to be a suitable hapten for protein conjugation, mice immunization, and MAb production. Our results confirm that the use of fragmentary haptens containing only the distinctive moiety of the analyte structure is potentially a very valuable approach to generate high-affinity antibodies for a particular pesticide. The application of this strategy could be of special interest for compounds belonging to the same structural family, such as the different organophosphorous pesticides.

The MAbs presented here constitute, to our knowledge, the first MAbs reported for azinphos-methyl. The features displayed by LIB/MBP-3.1, but furthermore, the results obtained with LIB/MBP-3.4, make them very promising immunoreagents for the future development of immunoassays for azinphos (methyl or ethyl). As known, the immunoassay sensitivity can be influenced by the ELISA format and the conjugates used (Schneider and Hammock, 1992). In this way, further evaluation of these MAbs is being done by using direct, enzyme-tracer ELISA, with both homologous and heterologous conjugates. Preliminary results suggest that the I_{50} could be lowered, although confirmation and subsequent proposal of azinphos immunoassays require additional work and will be the subject of future papers.

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Registry No. Supplied by the Author: Benzazimide (**1**), 90-16-4; azinphos-methyl, 86-50-0.

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