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Production of polyclonal antibodies towards the immunodetection of insecticide phosalone

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Summary. Hapten synthesis for the production of specific insecticide phosalone polyclonal antibodies was carried out starting from an intermediate of the phosalone synthesis, 6-chloro-2-benzoxazolone **1**. Two haptens containing different spacers have been prepared: N-5-carboxypentyl-6-chloro-2-benzoxazolone **7** and N-(2-oxo-3-aza-5-carboxypentyl)-6-chloro-**2**-benzoxazolone **12**. Each of these two haptens conjugated to bovine serum albumine (BSA) was used to immunize four rabbits. Immunoassays of phosalone were performed with ELISA using solid-phase bound hapten thyroglobulin conjugate and horseradish peroxidase labelled goat antirabbit IgG. The more sensitive response was observed when the antiserum obtained from the rabbit immunized with the hapten-BSA conjugate containing the N-2-oxo-3-aza-5-carboxypentyl spacer was in competition with the same hapten coupled to thyroglobulin. An identical response was obtained under the same conditions when using benzoxazolone instead of phosalone as competitor, showing a good recognition of the specific aromatic part of the organophosphate insecticide phosalone. Reduction of coating conjugate concentration (from 2 to 0.05 μg/well) and also the use of heterologous coating protein instead of homologous did improve the sensitivity, resulting in a concentration of phosalone required to inhibit binding by 50% of 2 mg/l and a detection limit of 0.02 mg/l.

Keywords: Amino acids – Polyclonal antibodies – Phosalone – Immunodetection – Hapten synthesis

Introduction

Phosalone (S-6-chloro-2,3-dihydro-2-oxobenzoxazol-3-ylmethyl phosphorodithioate) (Fig. 1) is an organophosphorus insecticide still widely used for

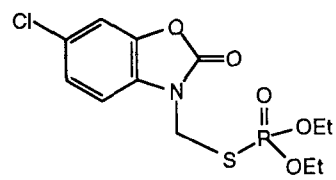

Fig. 1. Chemical structure of phosalone

the control of chewing and sucking insects and spider mites on stone fruits, citrus fruits and ornementals. It is a non-systemic insecticide and acaricide and a cholinesterase inhibitor. The maximum allowed residue limit is 2 ppm in stone fruit and peaches, 1 ppm in citrus fruits and strawberries and 0.1 ppm in roots vegetables and olives (Index Phytosanitaire, 1995). Phosalone residues analyses are carried out by chromatographic techniques, mainly gas chromatography (Worthing and Hance, 1991). However, chromatographic methods are time consuming and require sophisticated equipment only available in well-equipped laboratories. As enzyme-linked immunosorbent assay (ELISA) offers an alternative to conventional methods with high specificity, sensitivity, simplicity and suitability for the analysis of a large number of samples in a short period of time (Vanderlaan et al., 1988; Van Vukanis, 1990; Kaufman and Clower, 1991; Van Emon and Lopez-Avila, 1992), we have focused on a feasability study for the development of an ELISA test aimed at controlling phosalone residues in fruits.

The critical component of an immunoassay is the production of antibodies presenting maximum specificity and sensitivity for the target molecule. In immunology, most of pesticides are haptens: they can be recognized by antibodies but their low molecular weight does not allow themselves to induce an immune response. They have to be covalently linked to carriers such as proteins to form an immunogenic conjugate. Between hapten and protein, a spacer is needed to facilitate recognition of the target structure by antibodies. It is the reason why synthetized haptens (mimicking the structure of the target compound and having a handle that is 3 to 6 atoms long and that contains a functional group for conjugation with protein) are generally used (Jung et al., 1989).

In order to optimize specificity of immune response, several haptens and several carrier proteins have to be tested. The spacer can be either grafted directly on the target analyte or on a structural analog, otherwise a total synthesis of the hapten is required.

In the present study, we describe two routes of hapten synthesis, which consist of attaching a spacer on a synthetic intermediate, 6-chloro-2-benzoxazolone which has an amide group allowing a direct alkylation. This intermediate corresponds to phosalone without its thiophosphate group which is common to other organophosphate pesticides and therefore undesirable for specific antibody production. From protein conjugates of these haptens, polyclonal antibodies have been produced and used for preliminary tests to detect phosalone by ELISA.

Materials and methods

*Experimental procedure for the preparation of haptens **7** and **12***

1. Hexanoic acid spacer

*t-Butyl 6-bromohexanoate **4***

To a solution of 12.5 g (64 mmol) of 6-bromohexanoic acid in 20 ml of ethyl ether, conc. sulfuric acid (2 ml) was added dropwise at −15°C. After cooling to −80°C, isobutene was introduced in excess (100 ml, 1.3 mol) by bubbling. The mixture was then stirred at room temperature for 48 h. After neutralization with a saturated solution of $NaHCO_3$, the organic phase was dried with $MgSO_4$ and evaporated to give 14.9 g (92%) **4** as white crystals. *^1H NMR* ($CDCl_3$, TMS) δ 1.39 (s, 9H, —C($\mathbf{CH_3}$)$_3$), 1.44 (m, 2H, —$\mathbf{CH_2}$—), 1.57 (m, 2H, —$\mathbf{CH_2}$—CH_2—COOtBu), 1.82 (q, 2H, —$\mathbf{CH_2}$—CH_2Br, J = 7.2 Hz, 6.8 Hz), 2.18 (t, 2H, —$\mathbf{CH_2}$—COOtBu, J = 7.2 Hz), 3.36 (t, 2H, —$\mathbf{CH_2}$Br, J = 6.8 Hz).

*t-Butyl 6-iodohexanoate **5***

To a solution of 14.9 g (59.2 mmol) of t-butyl 6-bromohexanoate **4** in 30 ml of dimethylformamide (DMF), KI (59 g, 355.2 mmol) in 170 ml of DMF was added. The mixture was stirred at 50°C for 21 h. After filtration of the precipitated KBr and evaporation of DMF, the residue was dissolved in 100 ml CH_2Cl_2, washed with 1 M Na_2SO_3 (2 × 60 ml) and H_2O (60 ml). The organic phase was dried with $MgSO_4$ and evaporated to give 13.2 g (75%) of **5**. *^1H NMR* ($CDCl_3$, TMS) δ 1.37 (s, 9H, —C($\mathbf{CH_3}$)$_3$), 1.38 (m, 2H, —$\mathbf{CH_2}$—), 1.53 (q, 2H, —$\mathbf{CH_2}$—CH_2—COOtBu, J = 8 Hz, 7.1 Hz), 1.77 (q, 2H, —$\mathbf{CH_2}$—CH_2—I, J = 7.3 Hz, 7 Hz), 2.15 (t, 2H, —$\mathbf{CH_2}$—COOtBu, J = 7.3 Hz), 3.12 (t, 2H, —$\mathbf{CH_2}$—I, J = 7 Hz).

*t-Butyl ester of N-5-carboxypentyl-6-chloro-2-benzoxazolone **6***

To a suspension of 0.525 g (17.5 mmol) of NaH (initially at 80% in mineral oil and then washed with hexane (2 × 10 ml)) in 10 ml of tetrahydrofuran (THF), 6-chloro-2-benzoxazolone (2.7 g, 15.9 mmol, oven dried overnight at 80°C) in 20 ml of THF was added at −10°C. After the mixture was stirred at room temperature for 45 min, 9.5 g (31.8 mmol) of the iodide **5** in 10 ml of THF and 19.2 g (0.15 mol) of DMPU (1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone) dried overnight on molecular sieves were added at −10°C. The mixture was stirred at room temperature for 22 h. After evaporation of THF, the residue was dissolved in 30 ml of H_2O and extracted with petroleum ether (3 × 30 ml). The organic phase was washed with water (3 × 40 ml), dried over $MgSO_4$ and evaporated. Residual iodide was separated by column chromatography on silica gel (eluted with up to 20% AcOEt in CH_2Cl_2). After evaporation of the solvent, compound **6** 3.8 g (yield: 50%) was obtained. *^1H NMR* ($CDCl_3$, TMS) δ 1.42 (s, 9H, —C($\mathbf{CH_3}$)$_3$), 1.42 (m, 2H, —$\mathbf{CH_2}$—), 1.64 (m, 2H, —$\mathbf{CH_2}$—CH_2—COOtBu), 1.78 (m, 2H, —$\mathbf{CH_2}$—CH_2—NR_1R_2), 2.22 (t, 2H, —$\mathbf{CH_2}$—COOtBu, J = 7.3 Hz), 3.81 (t, 2H, —$\mathbf{CH_2}$—NR_1R_2, J = 7.2 Hz)., 6.89 (d, 1H$_{ar}$, J = 8.3 Hz), 7.17 (dd, 1H$_{ar}$, J = 1.9 Hz, 8.3 Hz), 7.25 (d, 1H$_{ar}$, J = 1.85 Hz); *MS* (GT, Fab$^+$) m/z: 340 (M+H), 284 (M+H-56(tBu)).

*N-5-Carboxypentyl-6-chloro-2-benzoxazolone **7***

To 1 g (2.94 mmol) of the ester **6** in 10 ml of CH_2Cl_2, 14.9 g (131 mmol) of TFA in 10 ml of CH_2Cl_2 and 1 ml of water were added at −10°C. The mixture was stirred at 0°C for 20 min then at room temperature for 1 h. After evaporation to dryness (excess of TFA was coevaporated with methanol), **7** in quantitative yield (0.83 g) was obtained. *^1H NMR* (DMSO, TMS) δ 1.30 (m, 2H, —$\mathbf{CH_2}$—), 1.50 (q, 2H, —$\mathbf{CH_2}$—CH_2—COOH, J = 7.45 Hz, 7.2 Hz), 1.67 (q, 2H, —$\mathbf{CH_2}$—CH_2—NR_1R_2, J = 7.45 Hz, 7.15 Hz), 2.15 (t, 2H, —$\mathbf{CH_2}$—COOH, J = 7.4 Hz), 3.79 (t, 2H, —$\mathbf{CH_2}$—NR_1R_2, J = 7 Hz), 7.28 (dd, 1H$_{ar}$, J = 1.8 Hz,

8.4 Hz), 7.17 (d, $1H_{ar}$, J = 8.4 Hz), 7.55 (d, $1H_{ar}$, J = 1.8 Hz); *MS* (GT, Fab^-) m/z 282 (M-H).

2. N-(2-Oxo-3-aza-5-carboxypentyl) spacer

N-(t-Butoxycarbonylmethyl)-6-chloro-2-benzoxazolone ***9***

To a suspension of 0.64 g (26.7 mmol) of NaH in 30 ml of THF, 6-chloro-2-benzoxazolone (4.3 g, 25.3 mmol, oven dried overnight at 80°C) in 40 ml of THF was added at −10°C. After the mixture was stirred at room temperature for 30 min, t-butyl bromoacetate (10 g, 51.3 mmol) in 10 ml of THF was added at −10°C. The mixture was stirred at room temperature for 2 h. After neutralization with a solution of 1 N HCl, filtration of the precipitated NaBr and evaporation of THF, the resulting solid was washed with hexane (3 × 10 ml) to eliminate the excess of ester. After filtration, compound **9** was obtained (6.2 g, yield = 85%). *Rf* (hexane/dichloromethane, 1:1) 0.65; *^1H NMR* ($CDCl_3$, TMS) δ 1.47 (s, 9H, $C(CH_3)_3$), 4.45 (s, 2H, —N—CH_2—COOR), 6.81 (d, $1H_{ar}$, J = 8.35 Hz), 7.16 (dd, $1H_{ar}$, J = 8.35 Hz, 1.9 Hz), 7.26 (d, $1H_{ar}$, J = 1.9 Hz); *MS* (GT, Fab +) m/z 284 (M + H).

N-Carboxymethyl-6-chloro-2-benzoxazolone ***10***

To a solution of 4 g (14.1 mmol) of the ester **9** in 40 ml of CH_2Cl_2, 59.2 g (5.19 mmol) of TFA in 60 ml of CH_2Cl_2 and 2 ml of water were added at −10°C. The mixture was stirred at 0°C for 20 min then at room temperature for 2.5 h. After evaporation of the solvent, compound **10** in 96% yield (2.98 g) was obtained. *^1H NMR* (CD_3OD, TMS) δ 4.96 (s, 2H, —N—CH_2—COOH), 7.09 (d, $1H_{ar}$, J = 8.4 Hz), 7.16 (dd, $1H_{ar}$, J = 8.35 Hz, 1.9 Hz), 7.20 (d, $1H_{ar}$, J = 1.9 Hz); *MS* (GT, Fab +) m/z 228 (M + H).

Hydroxysuccinimide ester of N-carboxymethyl-6-chloro-2-benzoxazolone ***11***

To a solution of 0.91 g (4 mmol) of the acid **10** in 20 ml of AcOEt, 10.51 g (4.4 mmol) of N-hydroxysuccinimide (HOSu) in 20 ml of DMF and 0.82 g (4 mmol) of dicyclohexyl-carbodiimide (DCC) were added at −10°C. The reaction mixture was stirred at 0°C for 2 h and at room temperature for 6 h. After filtration of the precipitated DCU (dicyclohexyl urea) and dry evaporation of the filtrate, the residue was dissolved in 20 ml of acetone and left overnight at −20°C. After filtration and evaporation of the solvent, the compound **11** is obtained in 43% yield (0.56 g).

N-(2-Oxo-3-aza-5-carboxypentyl)-6-chloro-2-benzoxazolone ***12***

To a solution of 0.56 g (1.7 mmol) of the activated ester **11** in 5 ml of pyridine, 0.3 g (0.17 mmol) of β alanine and 0.021 g (0.17 mmol) of dimethylaminopyridine (DMAP) were added. The mixture was stirred overnight at room temperature. After evaporation to dryness (pyridine being coevaporated with methanol), 0.18 g (35%) of compound **12** was obtained. *^1H NMR* ($DMSOD_6$, TMS) δ 2.44 (t, 2H, 6.5 Hz, —NH—CH_2—CH_2—COOH), 3.6 (m, 2H, —NH—CH_2—CH_2—COOH), 4.69 (s, 2H, —N—CH_2—CONH—), 7.2–7.4 (m, $1H_{ar}$), 7.6–7.7 (m, $2H_{ar}$).

Conjugation to carrier proteins

The haptens were covalently attached to bovine serum albumine (BSA) and thyroglobulin (TG) after activation of the acid function via the HOSu ester method by a procedure similar to that described by Bauminger and Wilchek (Bauminger and Wilchek, 1980).

Activation of haptens via HOSu ester

Compound ***8***. To a solution of 0.81 g (2.86 mmol) of N-5-carboxypentyl-6-chloro-2-benzoxazolone **7** in 10 ml of DMF, 0.46 g (4 mmol) of HOSu and 0.59 g (2.86 mmol) of DCC were added at −10°C. After stirring 2 h at 0°C and 24 h at room temperature, the precipitated DCU was removed by filtration. After concentration of the filtrate, the residue was taken up in 20 ml of acetone and left overnight at −20°C. Evaporation of the filtrate yielded 1.16 g (100%) of the activated compound **8**. *^{1}H NMR* ($CDCl_3$, TMS) δ 1.48 (m, 2H, —CH_2—), 1.65 (m, 2H, —CH_2—CH_2—COOR), 1.80 (m, 2H, —CH_2—CH_2—NR_1R_2), 2.31 (t, 2H, —CH_2—COOR, J = 7.23 Hz), 2.62 (t, 4H, —CH_2—CONR, J = 7.18 Hz), 3.82 (m, 2H, —CH_2—NR_1R_2), 6.92 (m, $1H_{ar}$,), 7.17 (dd, $1H_{ar}$, J = 1.9 Hz, 8.3 Hz), 7.23 (d, $1H_{ar}$, J = 1.8 Hz); *MS* (GT, Fab −) m/z 282 (M-H-OSu).

Compound ***13***. To a solution of 0.18 g (0.6 mmol) of compound **12** in 5 ml of DMF, 0.123 g (1.07 mmol) of HOSu and 0.13 g (0.63 mmol) of DCC, were added at −10°C. The mixture was stirred for 2 h at 0°C and 1 h at room temperature. After elimination of the residual DCU as described above, 0.23 g (35%) of compound **13** was obtained. *^{1}H NMR* ($CDCl_3$, TMS) δ 2.82 (t, 2H, 6.5 Hz, —NH—CH_2—CH_2—COOH), 2.88 (s, 4H, —CO CH_2—CH_2—CO—), 3.66 (t, 2H, 6.5 Hz, —NH—CH_2—CH_2—COOH), 4.95 (s, 2H, —N—CH_2—CONH—), 6.96 (d, $1H_{ar}$, J = 9 Hz), 7.24 (dd, $1H_{ar}$, J = 2 Hz), 7.28 (d, $1H_{ar}$, J = 2 Hz).

Coupling to the carrier protein

To either bovine serum albumin (BSA) or thyroglobulin (TG) at a concentration of 1.67 mg/ml in phosphate buffer (Na_2HPO_4 0.2 M, pH 9.1), **8** (200 mg/ml) or **13** (100 mg/ml) dissolved in DMF was added. Molar excess of hapten was respectively 14, 38, 15 and 18 for conjugation of **8**-BSA, **8**-TG, **13**-BSA and **13**-TG. The solutions were stirred at room temperature for 24 h. After concentration to 2 ml and centrifugation to separate the excess of precipitated uncoupled hapten from soluble hapten-protein conjugate. The soluble fraction was then purified through a Sephadex G25 column using distilled water as eluent. The elution of modified proteins was controlled with a differential refractometer and a qualitative Bradford test. Fractions containing modified protein were then lyophilized.

Determination of hapten density of conjugates

The yield of coupling was determined by assuming the principal coupling was with free amines on the proteins. The number of amines coupled was determined by the difference in free amines of coupled protein and untreated proteins, as determined using trinitrobenzene sulfonic acid TNBS (9). After an evaluation of the modified protein concentration (in comparing absorbance at 280 nm of the modified protein solution with a native protein solution), absorbance of these same solutions treated with TNBS was measured at 335 nm. By assuming the molar absorption coefficients are the same for native or modified proteins to a given wavelength, the following molar ratio of coupling were obtained: 0.68, 0.47, 0.95 and 0.28 for hapten-protein conjugate **8**-BSA, **8**-TG, **13**-BSA and **13**-TG, respectively.

Polyclonal antibody production

Male white New Zealand rabbits, 2 groups of 2 animals each, were used to obtain antibodies against the hapten **8**-BSA and hapten **13**-BSA conjugates. The lyophilized conjugates were dissolved in phosphate-buffered saline (PBS, 1.4 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136 mM NaCl, 2.7 mM KCl, pH 7.4) at a concentration of 1.2 μmol hapten equivalent/ml (2.25 mg of the hapten **8**-BSA conjugate/ml and 1.77 mg of the hapten **13**-BSA conjugate/ml). Rabbits were injected intradermally at 5 sites with 300 μl of an emulsion of 150 μl of conjugate and 150 μl of Freund's complete adjuvant. For the booster injections, solutions of conjugate were emulsified with an equal volume of Freund's incomplete adjuvant.

These booster injections were performed 2, 4 and 7 weeks later with respectively 200, 250 and 350 μl of conjugate. Blood was collected from the marginal ear vein 2 weeks after each boost.

Animals were exsanguinated under anesthesia 8 weeks after the initial immunization. Sera were isolated by centrifugation, and sodium azide was added at a final concentration of 0.02%. Serum was then aliquoted and stored at −20°C and used without further treatment.

Enzyme-linked immunosorbent assay (ELISA)

Polystyrene ELISA plates were coated in carbonate buffer pH 9.6, overnight at 4°C. Plates were then washed three times in PBS buffer containing 0.05% Tween 20 (PBST) and unbound active sites were blocked with 0.5% gelatine and 3% gelatine hydrolysate in PBS (150 μl) for 1 h at 20°C. Antibodies were diluted in 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 hapten-thyroglobulin conjugate (2 μg/ml) were incubated with serial dilutions of sera (in the range $1:10^2 - 1:10^6$) at 37°C for 2 h. The ratio of captured antibodies was measured by incubation at room temperature for 1 h with goat anti-rabbit peroxidase labeled immunoglobulins (diluted at 1:5000), followed by the addition of the substrate solution (0.2 mg/ml *o*-phenylene diamine, 0.014% H_2O_2 in citrate buffer, pH 5). After 15 min into darkness, the reaction was stopped by adding 1 M H_2SO_4 and the absorbance was read at 492 nm. Serum titer was defined as the serum dilution corresponding to inflexion point of the sigmoide curve given by OD = f (dilution).

Competitive indirect ELISA. The protocol was the same as for the indirect ELISA except that after coating with hapten **13**-TG conjugate (TG-2), a competition step was established by adding the appropriate concentration of serum to different concentrations of competitor (phosalone or benzoxazolone). Competitor in PBS containing 0.03% NaN_3 was previously mixed with equal volume of serum and preincubated overnight at 4°C. Competition curves were obtained by plotting %OD vs the logarithm of competitor concentration and were fitted to the following two-parameter logistic equation: $\%OD = 100/[1 + (x/C)^B]$, where B is the curvature parameter and C is the concentration giving 50% inhibition (Rodbard, 1981).

Results and discussion

Hapten synthesis

The initial heterocycle of the synthetic intermediate, 6-chloro-2-benzoxazolone **1**, has been modified by two routes ending in two spacers with a similar length but a different structure. One of them had a carbon chain structure, the other had a carbamoylated chain structure. First alkylation assays of the secondary amine of benzoxazolone performed according to Sam works (Sam et al., 1971), and consisting in adding an alkyl halide under basic conditions did not provide satisfying results. During the alkylation with 6-bromohexanoic acid, in the presence of KOH, polyalkylated compounds were formed as a result of the esterification of the alkylating chain by the alkyl halide ($R\text{-}[(CH_2)_5\text{-}COO]_{n=0,1,2,3}\text{-}(CH_2)_5\text{-}COOH$). The ester saponification under basic aqueous conditions (NaOH) lead to ring opening of the benzoxazolone. The alkylation of the ethyl ester of 6-bromohexanoic acid in the presence of KOH allowed the preparation of the aimed alkylated compound but the ester saponification led again to the opening of the lactone. These results prompted us to use less nucleophilic base as well as esters which

could be deprotected under acidic conditions. The two routes of hapten synthesis are illustrated in Fig. 2.

The synthesis of N-5-carboxypentyl spacer (Fig. 2A), required in the first step the protection of the acid function of the alkylating agent (6-bromohexanoic acid, **3**) by a t-butyl group. Reactivity of the resulting ester **4** was improved by substituting bromine atom by iodine. The alkylation was carried out with a non nucleophilic base (NaH). This key step of the synthesis was improved by the addition of dimethyl propylene urea (DMPU) as cosolvent. After addition of the iodide **5** to 6-chloro-2-benzoxazolone, the alkylated compound **6** was deprotected in acid medium to give **7**.

The synthesis of N-(2-oxo-3-aza-5-carboxypentyl) spacer (Fig. 2B) was carried out with t-butyl bromoacetate as the alkylating agent. Because of the high reactivity of this compound, addition of DMPU was unnecessary. The following steps of the synthesis was a matter of classical peptide synthesis: the acid function was deprotected by trifluoroacetic acid (TFA), activated as hydroxysuccinimide ester **11** and coupled with unprotected β-alanine to give **12**.

Antiserum production

The titers of each antiserum produced by the two rabbits immunized with hapten **8**-BSA (BSA 1) were determined using a coating with hapten **8**-TG (TG 1). The antisera of the two other rabbits immunized with hapten **13**-BSA (BSA 2) were analyzed using a coating with hapten **13**-TG (TG 2). The results of the titration experiments are illustrated in Fig. 3. Antibodies raised again BSA 1, containing an aliphatic spacer, showed higher titer in one case out of 2 (the antiserum B1 can be diluted 8,000 times to produce an absorbance around of 1). The very low titer observed with the antisera A1 could be a result of the physiologic state of the rabbit. When the spacer contained an amide group, a decrease in the recognition occured (the antisera have to be diluted 2,500 times to produce an absorbance around of 1).

Evaluation of the specificity of the antibodies

Preliminary immunoassays of phosalone using the highest titer antiserum B1 at a dilution 1/5000 and a coating with 0.2 μg of TG1 revealed that a phosalone concentration of 0.05 mg/l could not be detected. We assumed that this antiserum was not the most specific of the target analyte. Each of the four antisera at a dilution giving an absorbance of about 1 in the titration experiments were then tested using a coating with 0.2 μg of TG1 or TG2 and a phosalone concentration of 0.5 mg/l. Before the assays, 2 ml of standard solution of phosalone (1 mg/l) was preincubated overnight at room temperature with 2 ml of each of the diluted antisera (resp. 1/5000, 1/500, 1/2000, 1/2000 for antisera A1, B1, B2) in the presence of NaN_3 0.03%; 100 μl of this preincubated solution was used for ELISA. The same assays were carried out using the competitor benzoxazolone instead of phosalone in order to compare the affinity of antibodies for these two compounds. The results of these experiments are illustrated in Table 1. The greatest affinity for phosalone

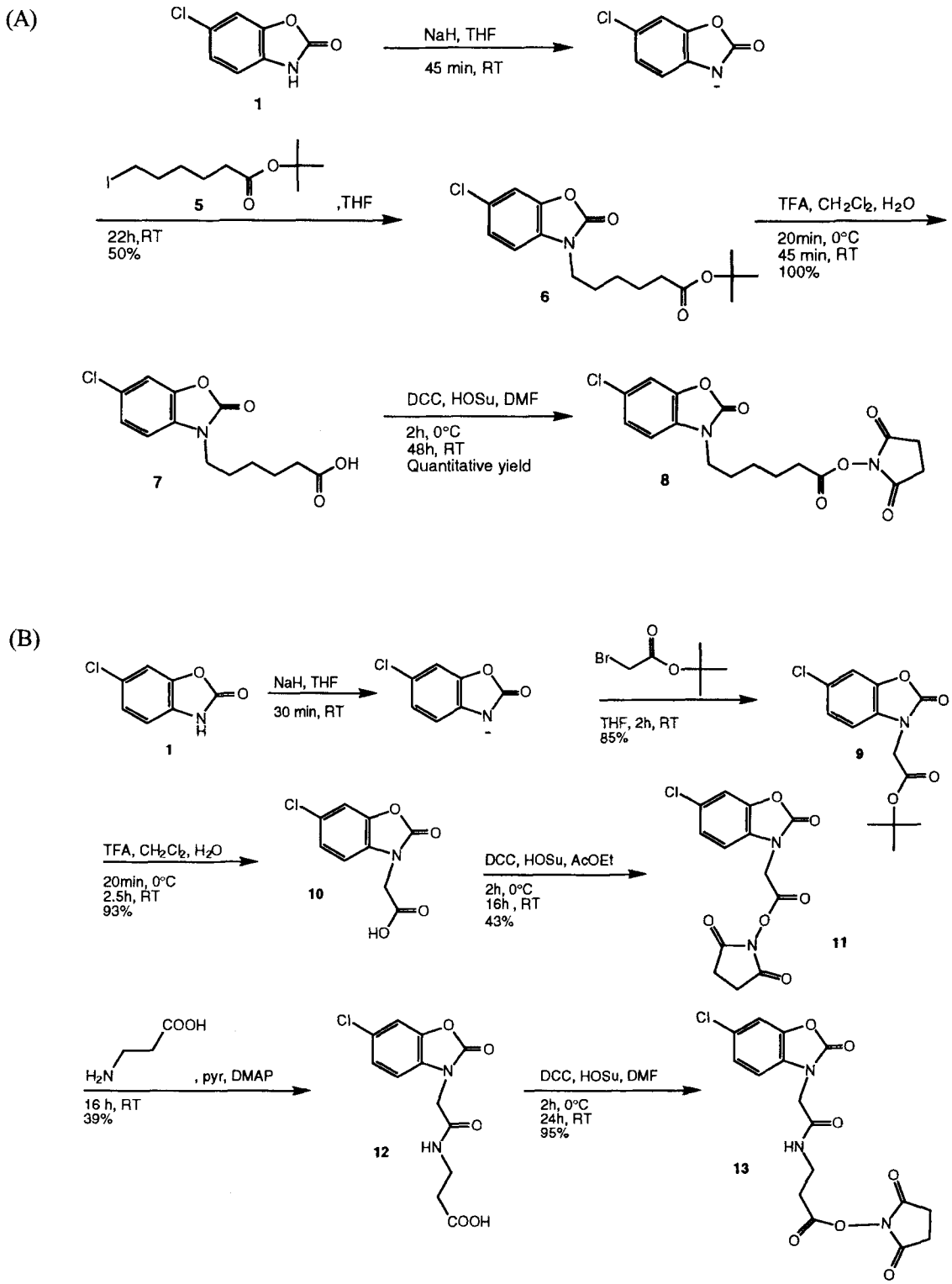

Fig. 2. Synthetic scheme for the production of two haptens. (**A**) N-5-carboxypentyl spacer; (**B**) N-(2-oxo-3-aza-5-carboxypentyl) spacer

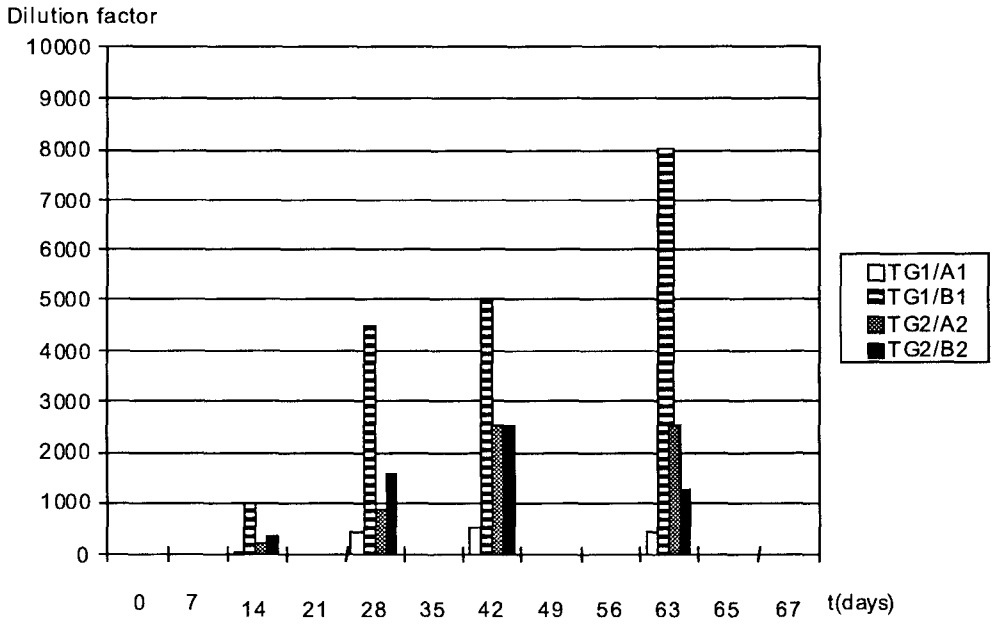

Fig. 3. Immune response in four rabbits: rabbits A1 and B1 were immunized with hapten(8)-BSA conjugate (BSA1) and titrated with hapten(8)-TG conjugate (*TG1*); rabbits A2 and B2 were immunized with hapten(13)-BSA conjugate (BSA2) and titrated with hapten(13)-TG conjugate (*TG2*)

Table 1. Influence of antiserum and coating conjugate on the immunoassay of phosalone and benzoxazolone

	Percentage inhibition of antibody binding			
Antiserum	B1	A1	A2	B2
Dilution	1/10000	1/1000	1/4000	1/4000
Coating		TG1		TG2
*Competitor				
phos	14	12	5	31
Coating		TG2		TG1
*Competitor				
phos	8	3	0	25
bzx	16	8	2	28

* Competitor: phos. (Phosalone 0.5 mg/l), bzx. (benzoxazolone 0.5 mg/l).

appeared with antiserum B2 for which the titer was not the highest. A slightly better response was obtained when coating conjugate contained the same hapten that the one of immunization conjugate. These results lead us to use the antiserum B2 and the coating conjugate TG2 for the following phosalone analysis. The affinity of antiserum for benzoxazolone was slightly higher than

Table 2. Affinity of the diluted antisera for non conjugated coated proteins TG, KLH and BSA at 0.2μg/well

	Optical density			
Serum	B1	A1	A2	B2
Dilution	1/5000	1/500	1/2000	1/2000
Coating				
TG	0	0.02	0.03	0.02
KLH	0.14	0.05	0.16	0.06
BSA	NT	NT	NT	0.06

NT not tested.

the one for phosalone with antiserum B1, and was the same with the three others antisera, although we could expect a greater affinity in all cases. This showed a good recognition of the specific part of the insectide phosalone.

Because of the low sensitivity of the assays described above, the degree of recognition of non conjugated proteins was evaluated. Each of the three proteins BSA, TG and KLH were used as coating protein at 0.2μg/well and ELISA was performed without competitor. The very low absorbances observed in all cases (Table 2) illustrated the poor affinity of antisera for this non conjugated coated proteins, showing either that the binding to the conjugate occured only to the hapten or that antibodies could recognize protein only under a given conformation corresponding to that the one observed in the conjugated form.

These assumptions were evaluated by complementary experiments using antiserum B2 (dilution 1/5000), a coating with a conjugate (TG2 or BSA2) at 0.2μg/well and a competition with conjugated or not conjugated protein as competitor (resp. TG2, BSA2, TG and BSA). The results were compared to a negative control (no competitor) and to an assay where phosalone at 0.05 mg/l was the competitor (Table 3). The concentrations of conjugate competitors were chosen to correspond to a concentration of hapten equivalent to 0.05 mg/l; the concentrations of free proteins were the same as those of conjugates. When the same conjugate was used as competitor and as coating, the equilibrium of binding was totally deplaced to the soluble form; this was illustrated by an inhibition of 100% while the inhibition by phosalone or free proteins was low (resp. 25% and 15% with a TG2 coating, and only 5% and 8% with a BSA2 coating). Moreover, TG2 is better recognized than BSA2. These results showed that the greatest affinity of antibodies was raised to a given conformation of the conjugate TG2 that was not the one used for immunization BSA2.

Optimization of reagents

A checkerboard experiment was set up in which various dilutions of the coating conjugate were titrated against various dilutions of antiserum B2. The

Table 3. Affinity of the diluted antisera B2 for conjugated or free proteins (resp. TG2, BSA2 at a hapten equivalent concentration of 0.5 mg/l and TG, BSA at the same concentrations than the conjugates) compare to the one for phosalone at 0.5 mg/l using a coating with TG2 or BSA2 at 0.2 μg/well

Percentage inhibition of antibody binding		
Coating	TG2	BSA2
Competitor		
BSA2	39	100
TG2	100	67
BSA	NT	7
TG	15	NT
Phos.	28	5

NT not tested.

results illustrated in Fig. 4 showed that a decreasing of the ELISA sensitivity was observed when antibody concentration was doubled (dilution of 1/1000 instead of 1/2000) but the sensitivity was improved by a decrease of the coating conjugate from 0.1 to 0.05 μg/well, provided an immunoassay capable of detecting phosalone with an I_{50} of 2 mg/l and a detection limit around 0.02 mg/l (Fig. 4).

Conclusion

This paper describes a first approach to obtain specific antibodies against phosalone. Polyclonal antibodies produced from immunization of rabbits by the hapten benzoxazolone coupled to BSA through a 6 atom long handle used for the phosalone competitive ELISA test in a format where the hapten-thyroglobulin conjugate were on solid phase provided a low sensitive immunoassay in comparison with major pesticide immunoassays described in the literature where the I_{50} value is generally from 100 to 1,000 times lower (Newsome et al., 1993; McAdam et al., 1992; Marco et al., 1993).

However, as compared to the maximal admissible concentration of phosalone which is 1 mg/kg in fruits and vegetables, this immunoassay could be applied as a qualitive monitoring test of phosalone in such matrices after an adequate preparation of samples that imply an extraction and a concentration of the target analyte.

Moreover we could expect an improvement of the immunoassay using either another format as a solid phase antibodies or other labels as chemiluminescent compounds. The most specific antibodies could also be partially purified and then enriched after passing the pool of the polyclonal antibodies through an immunoaffinity column. The production of more specific antibodies depends on various parameters concerning either the conju-

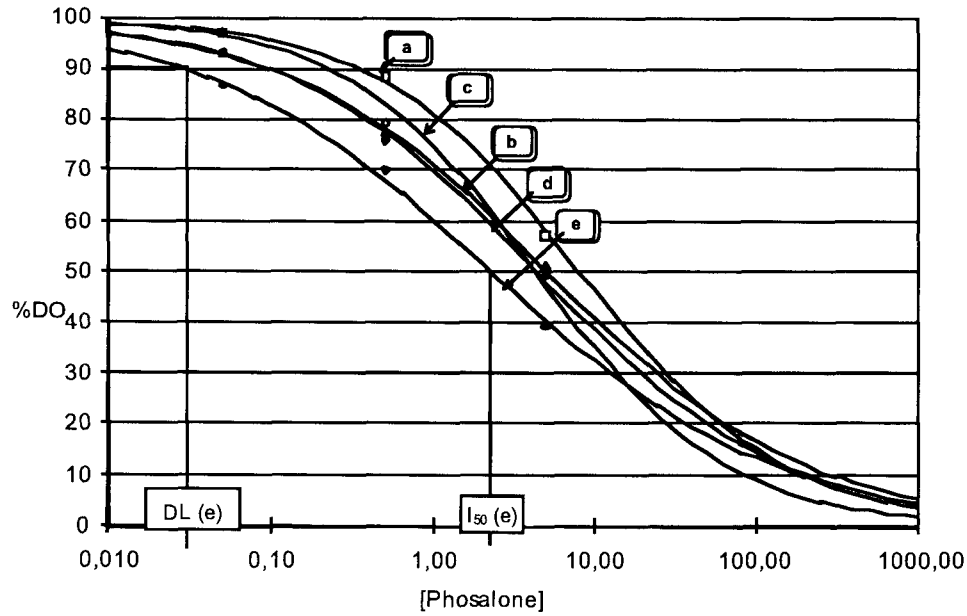

Fig. 4. Standard curves of phosalone in PBS-T buffer using different concentrations of the antiserum B2 (dilution 1/1000 in (*a*), 1/2000 in (*b*), (*c*), (*d*)), of the coating conjugate (0.2μg/l in (*a*), (*b*), 0.1μg/l in (*c*), (*d*), 0.05μg/l in (*c*), (*d*)), and of the labelled anti-IgG (dilution 1/5000 in (*a*), (*b*), (*c*), and 1/2500 in (*d*), (*e*)). *DL* Detection limit, I_{50} 50% inhibition of antibody binding

gate of immunization (the hapten structure, the structure and the location of the spacer, the selection of the protein, the selection of coupling chemistry) or the immunization schedule.

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