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Synthesis of haptens for immunoassay of organophosphorus pesticides and effect of heterology in hapten spacer arm length on immunoassay sensitivity

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Abstract

The synthetic method for haptens of organophosphorus (OP) pesticides with a spacer arm (amino carboxylic acid) attached at the pesticide thiophosphate group was simplified to a large extent. While the previous synthetic approach for this type of haptens requires seven steps, the present process involves only two steps. Using this process, five haptens of fenthion differing in spacer arm length (4–8 atoms) were synthesized and they were conjugated to bovine serum albumin and keyhole limpet hemocyanin to be used as immunogens. Rabbits were immunized with these hapten–protein conjugates for production of polyclonal antibodies against fenthion. The five haptens were conjugated to ovalbumin to be used as plate-coating antigens and twenty polyclonal antisera to the haptens were screened against each of the five coating antigens using noncompetitive and competitive indirect enzyme-linked immunosorbent assay (ELISA). The titer difference between the homologous and heterologous combinations was small, suggesting that heterology in spacer arm length is not important for the antigen recognition by antibodies. While the heterology in spacer length of the coating antigen had no significant effect on the sensitivity of ELISA, heterology in spacer structure of the coating antigen produced a remarkable improvement in the sensitivity of ELISA.
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Keywords: Organophosphorus pesticide; Hapten synthesis; Spacer arm; Fenthion; Enzyme-linked immunosorbent assay; ELISA

1. Introduction

Since the 1970s, organophosphorus (OP) pesticides have replaced the organochlorine insecticides previously used and are now widely used on agricultural crops (fruits, vegetables and cereals) to control a large variety of pests [1]. However, the OP pesticides generally have higher acute toxicity than chlorinated insecticides, due to the inhibition of the enzyme acetylcholinesterase, an essential component of the animal nervous system [1,2], which necessitates comprehensive monitoring programs for them. Analyses of OP pesticides have been based on chromatographic methods, mainly gas chromatography (GC) which are characterized by low limits of detection and high precision and sensitivity [3]. However, these methods have a few shortcomings: the need for time-consuming sample cleanup prior to detection, the need of sophisticated

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equipment available only in well-equipped laboratories, and inadequacy of on-site analysis. On the other hand, enzyme-linked immunosorbent assay (ELISA), which has emerged from the trials to overcome the weak points of GC and HPLC methods, is a simple, rapid, and cost-effective method for monitoring pesticide residues, and in particular, for high sample through-put and on-site screening analysis [4–6].

The application of ELISA requires the production of antibodies to the analyte. To obtain desirable antibodies to small molecules, such as pesticides, suitable haptens have to be synthesized and conjugated to proteins to elicit the immune response of the host animal. Haptens must have a spacer arm (linker) for their covalent attachment to carrier proteins. In the case of aromatic OP pesticides, the spacer arm can be attached at either one of the two basic moieties of the pesticides, the thiophosphate group and the aromatic ring. The haptens derived through the thiophosphate moiety using an amino carboxylic acid as a spacer arm have been used to obtain highly sensitive antibodies for paraoxon [7], fenitrothion [8], chlorpyrifos [9,10], chlorpyrifos-methyl [11,12], and pirimiphos-methyl [12]. The synthesis of these haptens [13,14], however, is not easy due to the large number of steps (i.e. seven) involved in their synthesis including protection and deprotection of both amino and carboxyl groups of the spacer arm reagent. We developed an easy process for synthesizing this type of hapten that requires only two steps with no protection and deprotection steps. This paper describes the result of the application of this new approach to the synthesis of haptens of the OP pesticide fenthion.

Five haptens of fenthion differing only in spacer arm length (Fig. 1) were synthesized using amino carboxylic acids as spacer arm reagents and were conjugated to bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) to be used as immunogens. Rabbits were immunized with these hapten–protein conjugates for production of polyclonal antibodies (PAbs) against fenthion. The five haptens were conjugated to ovalbumin (OVA) to be used as coating antigens and twenty polyclonal antisera were screened against each of the five coating antigens using non-competitive and competitive indirect ELISA.

It is generally accepted that the length of the hapten spacer arm is an important factor in the production of antibodies with high affinity to the analyte and that ELISA sensitivity improves with increasing spacer length [5,15,16]. A long spacer arm is thought to make possible the avoidance of the shielding effect of the carrier protein. However, a spacer arm that is too long may lead to hapten folding, thus reducing the exposure of the hapten molecule to the immune system. Thus, a medium size spacer arm with three or four to six atoms has often been quoted as an optimal range [5,16]. However, experimental results supportive of these hypotheses are limited. The objective of preparing five haptens with varying spacer arm length in this study was two-fold. The first objective was to obtain diverse antisera from a multitude of haptens for wider option of selecting a proper antiserum, and the other objective was to investigate the effect of spacer arm heterology between immunizing and coating antigen haptens on the affinity of antibodies to the haptens and sensitivity of ELISA. In order to examine the effect of spacer arm heterology on ELISA, twenty antisera against the five haptens were screened for each of the same five haptens and two additional haptens coated on the microtiter plate.

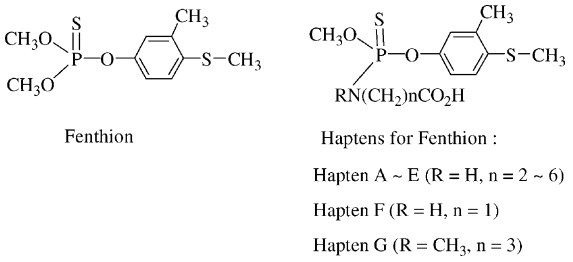

Fig. 1. Chemical structures of fenthion and the haptens for fenthion (Hapten A–E: immunizing haptens; Hapten A–G: plate-coating haptens).

2. Experimental

2.1. Reagents and materials

Fenthion was obtained from Dr. Ehrenstorfer (Augsburg, Germany). 4-(Methylthio)-*m*-cresol was obtained by hydrolysis of fenthion or purchased from Tokyo Kasei (Tokyo, Japan). β-Alanine, 4-aminobut-

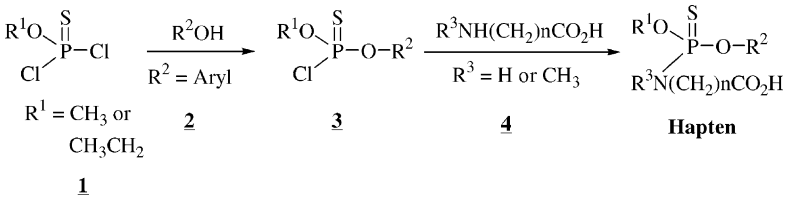

Fig. 2. The synthetic route developed in this laboratory for haptens of organophosphorus pesticides.

yric acid, 5-aminovaleric acid, 6-aminocaproic acid, 7-aminoheptanoic acid, 4-(methylamino)butyric acid, *N*-hydroxysuccinimide, 4-dimethylaminopyridine, *N*, *N*-dicyclohexylcarbodiimide, $CHCl_3$-*d*, and silica gel for column chromatography (60–230 mesh) were purchased from Aldrich (Milwaukee, USA). Analytical (silica gel F254) and preparative TLC plates (silica gel, 1 mm) were obtained from Merck (Darmstadt, Germany). Glycine, BSA ovalbumin OVA gelatin, alkaline phosphatase conjugated goat anti-rabbit IgG, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG, *p*-nitrophenyl phosphate, Freund's complete and incomplete adjuvants, phosphate-buffered saline (PBS, 10 mM, pH 7.4), Tween 20, and Sephadex G-25 were from Sigma (St. Louis, USA). KLH was from CalBiochem (La Jolla, USA) and tetramethylbenzidine (TMB) from Boehringer Mannheim (Mannheim, Germany). The dialysis membrane (MW cutoff 12 000–14 000) was a Spectra/Por product from Spectrum Laboratories (Rancho Dominguez, USA). Microtiter plates (Maxisorp, 442404 and 439454) were purchased from Nunc (Roskilde, Denmark).

2.2. Instruments

NMR spectra were obtained with a Bruker ARX spectrometer (300 MHz, Rheinstetten, Germany). Chemical shift values are given in parts per million (ppm) downfield from internal standard tetramethylsilane. Coupling constants are expressed in Hz and the abbreviations s, d, t, q, qn, sx, m, and ar represent singlet, doublet, triplet, quartet, quintet, sextet, multiplet, and aromatic, respectively. UV-Vis spectra were recorded on a Varian Cary 3 spectrophotometer. ELISA plates were washed with a Model 1575 ImmunoWash, and well absorbances were read with a Model 550 plate reader, both from Bio-Rad (Hercules, USA).

2.3. Hapten synthesis

The haptens used for immunization and antigen coating are presented in Fig. 1. The synthetic routes for this type of haptens are illustrated in Fig. 2. The following is the procedure for the synthesis of Hapten A. Hapten B–G were synthesized by the same procedure as that for Hapten A using the necessary amino carboxylic acids.

2.3.1. Methyl dichlorothiophosphate (**1**)

This starting material was obtained by a published procedure [17].

2.3.2. O-Methyl O-[3-methyl-4-(methylthio)phenyl] phosphorochloridothioate (**3**)

To a solution of **1** (7.64 g, 46.3 mmol) in acetonitrile (30 ml) were added finely-ground K_2CO_3 and a solution of 6.46 g (42.1 mmol) of **2** in acetonitrile. After stirring for 1 h at room temperature, the mixture was filtered through celite, and the solvent was removed under reduced pressure. Column chromatography (silica gel, benzene/hexane, 1:1, R_f: 0.68) of the residue gave 6.47 g (55%) of a colorless syrup. ^{1}H NMR ($CDCl_3$) δ: 7.16 (1H, d, $J = 8.6$, ar), 7.11 (1H, d, $J = 8.9$, ar), 7.07 (1H, s, ar), 4.01 (3H, d, $J = 16.3$, CH_3OP), 2.47 (3H, s, SCH_3), 2.35 (3H, s, $PhCH_3$); GC–MS molecular ion peak ($m/z = 282$) was the base peak.

2.3.3. O-Methyl O-[3-methyl-4-(methylthio)phenyl] N-(2-carboxyethyl)phosphoramidothioate (Hapten A)

To a stirred solution of 0.59 g (2.1 mmol) of **3** in methanol (3 ml) cooled in an ice–water bath was added dropwise a solution of KOH (321 mg, 5.72 mmol) and β-alanine (232 mg, 2.6 mmol) in methanol (1.7 ml). After stirring for 5 min, the reaction mixture was extracted with 1 N HCl–chloroform. The extract was dried over $MgSO_4$, and the solvent was evaporated.

Column chromatography (silica gel, chloroform/ethyl acetate/acetic acid, 65:35:1, R_f: 0.49) of the residue gave 441 mg (63%) of a white solid. ^{1}H NMR ($CDCl_3$) δ: 7.13 (1H, d, J = 8.8, ar), 7.05 (1H, d, J = 8.8, ar), 7.01 (1H, s, ar), 3.78 (3H, d, J = 14.4, CH_3OP), 3.37 (2H, sx, J = 6.6, NCH_2), 2.62 (3H, t, J = 5.9, CH_2CO), 2.44 (3H, s, SCH_3), 2.33 (3H, s, $PhCH_3$). ^{13}C NMR ($CDCl_3$) δ: 178.14 (COOH), 148.14, 138.88, 137.49, 126.15, 122.31, 118.83 (ar), 37.29, 35.67 (CH_2), 20.99, 20.76, 15.70 (CH_3).

2.3.4. O-Methyl O-[3-methyl-4-(methylthio)phenyl] N-(3-carboxypropyl)phosphoramidothioate (Hapten B)

This hapten was synthesized using **3** and 4-aminobutyric acid. Yield: 66%. TLC R_f: 0.50 (silica gel, chloroform/ethyl acetate/acetic acid, 65:35:1). ^{1}H NMR ($CDCl_3$) δ: 7.12 (1H, d, J = 8.7, ar), 7.06 (1H, d, J = 8.7, ar), 7.01 (1H, s, ar), 3.77 (3H, d, J = 14.0, CH_3OP), 3.32 (1H, qn, J = 7.3, NH), 3.18 (2H, sx, J = 7.1, NCH_2), 2.45 (2H, t, J = 7.2, CH_2CO), 2.43 (3H, s, SCH_3), 2.33 (3H, s, $PhCH_3$), 1.85 (2H, qn, J = 6.7, $CH_2C\underline{H}_2CH_2$). ^{13}C NMR ($CDCl_3$) δ: 179.16 (COOH), 148.21, 137.47, 133.82, 126.16, 122.32, 118.83 (ar), 41.15, 30.88, 26.14 (CH_2), 20.77, 20.05, 15.71 (CH_3).

2.3.5. O-Methyl O-[3-methyl-4-(methylthio)phenyl] N-(4-carboxybutyl)phosphoramidothioate (Hapten C)

The amino carboxylic acid used was 5-aminovaleric acid. Yield: 77%. TLC R_f: 0.51 (silica gel, chloroform/ethyl acetate/acetic acid, 65:35:1). ^{1}H NMR ($CDCl_3$) δ: 7.10 (1H, d, J = 8.7, ar), 7.04 (1H, d, J = 8.7, ar), 6.99 (1H, s, ar), 3.75 (3H, d, J = 14.0, CH_3OP), 3.15 (1H, qn, J = 7.0, NH), 3.05 (2H, sx, J = 6.9, NCH_2), 2.42 (3H, s, SCH_3), 2.36 (2H, t, J = 7.1, CH_2CO), 2.31 (3H, s, $PhCH_3$), 1.66 (2H, m, $NHCH_2C\underline{H}_2$), 1.55 (2H, m, $C\underline{H}_2CH_2CO$). ^{13}C NMR ($CDCl_3$) δ: 179.43 (COOH), 148.28, 137.48, 133.74, 126.20, 122.52, 118.83 (ar), 41.54, 33.38, 30.65, 21.52 (CH_2), 20.77, 20.07, 15.74 (CH_3).

2.3.6. O-Methyl O-[3-methyl-4-(methylthio)phenyl] N-(5-carboxypentyl)phosphoramidothioate (Hapten D)

The amino carboxylic acid used was 6-aminocaproic acid. Yield: 75%. TLC R_f: 0.52 (silica gel, chloroform/ethyl acetate/acetic acid, 65:35:1). ^{1}H NMR ($CDCl_3$) δ: 7.13 (1H, d, J = 8.7, ar), 7.06 (1H, d, J = 8.7, ar), 7.06 (1H, s, ar), 3.77 (3H, d, J = 14.0, CH_3OP), 3.23 (1H, qn, J = 7.1, NH), 3.07 (2H, sx, J = 6.8, NCH_2), 2.43 (3H, s, SCH_3), 2.33 (2H, t, J = 7.2, CH_2CO), 2.33 (3H, s, $PhCH_3$), 1.65 (2H, qn, J = 7.6, $NHCH_2C\underline{H}_2$), 1.53 (2H, m, $C\underline{H}_2CH_2CO$), 1.40 (2H, m, $CH_2C\underline{H}_2CH_2$). ^{13}C NMR ($CDCl_3$) δ: 179.87 (COOH), 148.29, 137.43, 133.70, 126.15, 122.25, 118.82 (ar), 41.73, 33.80, 30.98, 25.91, 24.09 (CH_2), 20.80, 20.06, 15.71 (CH_3).

2.3.7. O-Methyl O-[3-methyl-4-(methylthio)phenyl] N-(6-carboxyhexyl)phosphoramidothioate (Hapten E)

The amino carboxylic acid used was 7-aminoheptanoic acid. Yield: 75%. TLC R_f: 0.53 (silica gel, chloroform/ethyl acetate/acetic acid, 65:35:1). ^{1}H NMR ($CDCl_3$) δ: 7.13 (1H, d, J = 8.5, ar), 7.06 (1H, d, J = 10.0, ar), 7.02 (1H, s, ar), 3.77 (3H, d, J = 14.0, CH_3OP), 3.16 (1H, m, NH), 3.00 (2H, sx, J = 6.5, NCH_2), 2.44 (3H, s, SCH_3), 2.36 (3H, t, J = 7.4, CH_2CO), 2.33 (3H, s, $PhCH_3$), 1.63 (2H, m, $NHCH_2C\underline{H}_2$), 1.54 (2H, m, $C\underline{H}_2CH_2CO$), 1.36 (4H, m, $CH_2C\underline{H}_2C\underline{H}_2CH_2$). ^{13}C NMR ($CDCl_3$) δ: 179.54 (COOH), 148.34, 137.49, 133.74, 126.22, 122.35, 118.85 (ar), 41.92, 33.81, 31.09, 28.55, 26.19, 24.44 (CH_2), 20.11, 15.77 (CH_3).

2.3.8. O-Methyl O-[3-methyl-4-(methylthio)phenyl] N-(carboxymethyl) phosphoramidothioate (Hapten F)

The amino carboxylic acid used was glycine. Yield: 78%. TLC R_f: 0.48 (silica gel, chloroform/ethyl acetate/acetic acid, 65:35:1). ^{1}H NMR ($CDCl_3$) δ: 7.12 (1H, d, J = 8.5, ar), 7.05 (1H, d, J = 8.8, ar), 7.01 (1H, s, ar), 3.93 (2H, m, NCH_2), 3.79 (3H, d, J = 14.2, CH_3OP), 2.44 (3H, s, SCH_3), 2.32 (3H, s, $PhCH_3$).

2.3.9. O-Methyl O-[3-methyl-4-(methylthio)phenyl] N-(3-carboxypropyl) N-methyl phosphoramidothioate (Hapten G)

The amino carboxylic acid used was 4-(methylamino)butyric acid hydrochloride. Yield: 45%. TLC R_f: 0.48 (silica gel, chloroform/ethyl acetate/acetic acid, 65:35:1). ^{1}H NMR ($CDCl_3$) δ: 7.12 (1H, d, J = 8.7, ar), 7.00 (1H, d, J = 8.5, ar), 6.95 (1H, s, ar), 3.73 (3H, d, J = 13.6, CH_3OP), 2.84 (3H, d, J = 11.0, CH_3N), 3.18 (2H, sx, J = 7.1, NCH_2), 2.45 (2H, t, J = 7.2, CH_2CO), 2.43 (3H, s, SCH_3), 2.32 (3H, s, $PhCH_3$), 1.88 (2H, qn, J = 7.0, $CH_2C\underline{H}_2CH_2$).

2.4. Preparation of hapten–protein conjugates

The carboxylic acid haptens were covalently attached to BSA, KLH (immunogens) or OVA (coating antigens) by the active ester method [8,18]. The haptens were reacted with *N*-hydroxysuccinimide to obtain active esters and isolated esters were then reacted with proteins. The procedure for the synthesis of the active ester of Hapten A is described below. The procedures for the synthesis of other active esters were similar.

2.4.1. Ester of Hapten A

To a solution of *N*-hydroxysuccinimde (108 mg, 0.94 mmol) dissolved in dichloromethane (15 ml) were added Hapten A (286 mg, 0.85 mmol), 4-dimethylaminopyridine (10 mg, 0.085 mmol), and *N*,*N*-dicyclohexylcarbodiimide (194 mg, 0.94 mmol). The mixture was stirred for 3 h and then filtered to remove the dicyclohexylurea, and the solvent was evaporated. TLC of the resultant oil on silica gel (benzene/ethyl acetate/acetic acid, 65:35:1, R_f: 0.63) gave the ester as a syrup (294 mg, 80%). ^{1}H NMR ($CDCl_3$) δ: 7.13 (1H, d, $J = 8.4$, ar), 7.05 (1H, d, $J = 8.4$, ar), 7.01 (1H, s, ar), 3.78 (3H, d, $J = 14.1$, CH_3OP), 3.52 (2H, sx, $J = 6.7$, NCH_2), 3.36 (1H, qn, $J = 6.6$, NH), 2.87 (4H, s, succinyl), 2.62 (2H, t, $J = 5.9$, CH_2CO), 2.44 (3H, s, SCH_3), 2.33 (3H, s, $PhCH_3$). ^{13}C NMR ($CDCl_3$) δ: 169.25 (CO_2), 167.22 (C=O), 148.18, 137.47, 133.89, 126.15, 122.30, 118.81 (ar), 33.48, 25.50, 25.28 (CH_2), 20.60, 20.01, 15.67(CH_3).

2.4.2. Ester of Hapten B

Yield: 49%. TLC R_f: 0.65 (silica gel, benzene/ethyl acetate/acetic acid, 65:35:1). ^{1}H NMR ($CDCl_3$) δ: 7.13 (1H, d, $J = 8.6$, ar), 7.06 (1H, d, $J = 8.6$, ar), 7.01 (1H, s, ar), 3.79 (3H, d, $J = 14.0$, CH_3OP), 3.36 (1H, qn, $J = 6.9$, NH), 3.22 (2H, sx, $J = 6.6$, NCH_2), 2.85 (4H, s, succinyl), 2.72 (2H, t, $J = 7.5$, CH_2CO), 2.44 (3H, s, SCH_3), 2.33 (3H, s, $PhCH_3$), 1.98 (2H, qn, $J = 7.0$, $CH_2C\underline{H}_2CH_2$). ^{13}C NMR ($CDCl_3$) δ: 169.28 (CO_2), 168.16 (C=O), 148.18, 137.37, 133.74, 126.06, 122.25, 118.80 (ar), 40.69, 27.89, 25.89, 25.44 (CH_2), 20.63, 19.95, 15.60 (CH_3).

2.4.3. Ester of Hapten C

Yield: 58%. TLC R_f: 0.67 (silica gel, benzene/ethyl acetate/acetic acid, 65:35:1). ^{1}H NMR ($CDCl_3$) δ: 7.13 (1H, d, $J = 8.7$, ar), 7.06 (1H, d, $J = 8.7$, ar), 7.02 (1H, s, ar), 3.78 (3H, d, $J = 14.0$, CH_3OP), 3.32 (1H, qn, $J = 7.2$, NH), 3.12 (2H, sx, $J = 6.7$, NCH_2), 2.85 (4H, s, succinyl), 2.65 (2H, t, $J = 7.1$, CH_2CO), 2.44 (3H, s, SCH_3), 2.33 (3H, s, $PhCH_3$), 1.82 (2H, m, $NHCH_2C\underline{H}_2$), 1.65 (2H, m, $C\underline{H}_2CH_2CO$). ^{13}C NMR ($CDCl_3$) δ: 169.16 (CO_2), 168.26 (C=O), 148.30, 137.44, 133.71, 126.17, 122.25, 118.82 (ar), 41.34, 30.42, 30.25, 25.49, 21.54 (CH_2), 20.97, 20.04, 15.71 (CH_3).

2.4.4. Ester of Hapten D

Yield: 55%. TLC R_f: 0.69 (silica gel, benzene/ethyl acetate/acetic acid, 65:35:1). ^{1}H NMR ($CDCl_3$) δ: 7.13 (1H, d, $J = 8.5$, ar), 7.06 (1H, d, $J = 8.5$, ar), 7.02 (1H, s, ar), 3.78 (3H, d, $J = 14.0$, CH_3OP), 3.24 (1H, qn, $J = 7.3$, NH), 3.09 (2H, sx, $J = 6.3$, NCH_2), 2.84 (4H, s, succinyl), 2.62 (2H, t, $J = 7.2$, CH_2CO), 2.44 (3H, s, SCH_3), 2.33 (3H, s, $PhCH_3$), 1.78 (2H, qn, $J = 7.4$, $NHCH_2C\underline{H}_2$), 1.57 (2H, m, $C\underline{H}_2CH_2CO$), 1.49 (2H, m, $CH_2C\underline{H}_2CH_2$). ^{13}C NMR ($CDCl_3$) δ: 169.25 (CO_2), 168.42 (C=O), 148.34, 137.48, 133.69, 126.21, 122.30, 118.80 (ar), 41.65, 33.64, 30.74, 25.54, 25.31, 24.09 (CH_2), 20.65, 20.10, 15.76 (CH_3).

2.4.5. Ester of Hapten E

Yield: 81%. TLC R_f: 0.71 (silica gel, benzene/ethyl acetate/acetic acid, 65:35:1). ^{1}H NMR ($CDCl_3$) δ: 7.13 (1H, d, $J = 8.5$, ar), 7.07 (1H, d, $J = 8.5$, ar), 7.02 (1H, s, ar), 3.77 (3H, d, $J = 14.0$, CH_3OP), 3.19 (1H, qn, $J = 7.5$, NH), 3.07 (2H, sx, $J = 6.8$, NCH_2), 2.84 (4H, s, succinyl), 2.62 (2H, t, $J = 7.3$, CH_2CO), 2.44 (3H, s, SCH_3), 2.33 (3H, s, $PhCH_3$), 1.73 (2H, qn, $J = 7.4$, $NHCH_2C\underline{H}_2$), 1.60 (2H, m, $C\underline{H}_2CH_2CO$), 1.40 (4H, m, $CH_2C\underline{H}_2C\underline{H}_2CH_2$).

2.4.6. Ester of Hapten F

Yield: 50%. TLC R_f: 0.62 (silica gel, benzene/ethyl acetate/acetic acid, 65:35:1). ^{1}H NMR ($CDCl_3$) δ: 7.13 (1H, d, $J = 8.4$, ar), 7.06 (1H, d, $J = 8.4$, ar), 7.02 (1H, s, ar), 3.87 (1H, qn, $J = 6.5$, NH), 3.80 (3H, d, $J = 14.1$, CH_3OP), 2.84 (4H, s, succinyl), 2.43 (3H, s, SCH_3), 2.33 (3H, s, $PhCH_3$).

2.4.7. Ester of Hapten G

Yield: 34%. TLC R_f: 0.58 (silica gel, benzene/ethyl acetate/acetic acid, 65:35:1). ^{1}H NMR ($CDCl_3$) δ: 7.12 (1H, d, $J = 8.6$, ar), 7.00 (1H, d, $J = 9.0$, ar), 6.97

(1H, s, ar), 3.74 (3H, d, $J = 13.5$, CH_3OP), 3.36 (2H, qn, $J = 7.2$, NCH_2), 2.85 (3H, d, $J = 10.5$, CH_3N), 2.84 (4H, s, succinyl), 2.65 (2H, t, $J = 7.0$, CH_2CO), 2.43 (3H, s, SCH_3), 2.32 (3H, s, $PhCH_3$), 1.99 (2H, qn, $J = 7.3$, $CH_2C\underline{H}_2CH_2$).

The procedure for the coupling of haptens to proteins was as follows. To a vigorously stirred solution of 50 mg of either BSA, KLH or OVA in 0.2 M borate buffer (5 ml, pH 8.7) was added DMF (1 ml). A solution of the ester (0.045 mmol for BSA, 0.023 mmol for KLH, 0.067 mmol for OVA) of hapten in DMF (0.25 ml) was added dropwise to the stirred protein solution over a 20 min period, and stirring was continued for 1 h at room temperature and then at 4 °C overnight.

Conjugates were separated from uncoupled haptens by gel filtration (Sephadex G-25, 2 g), using PBS as eluant. The fractions showing high absorbance at 280 nm were combined and dialyzed against water at 4 °C overnight. The final solution was lyophilized and stored at −20 °C.

The protein concentration of these conjugates was determined according to the Lowry method [19]. The degree of hapten substitution was estimated by measuring the loss of free amino groups on the protein using the trinitrobenzenesulfonic acid (TNBS) assay [20]. The density of fenthion to KLH was not obtainable due to poor solubility of hapten–KLH conjugates in the buffer (0.2 M borate, pH 9.5).

2.5. Immunization of rabbits

Female New Zealand white rabbits weighing 2–2.5 kg were used for obtaining PAbs to the five immunogens (two rabbits per immunogen). The immunogen (500 μg) dissolved in PBS (0.5 ml) was emulsified with Freund's complete adjuvant (1:1 volume ratio) and injected intradermally at multiple sites (20–25 sites) on the back. After 2 weeks, each animal was boosted with an additional 500 μg of the immunogen emulsified with Freund's incomplete adjuvant and bled 7–10 days later. After this, boosting and bleeding was continued on a monthly basis. Serum was isolated by centrifugation and sodium azide was added as a preservative at a final concentration of 0.02%. Antiserum was aliquotted and stored at −70 °C.

2.6. Titration of antisera

The titers of the antisera from different bleeds and different animals were determined by measuring the binding of serial dilutions of the antisera to the coating antigen (Hapten C-OVA) using noncompetitive ELISA. Flat-bottom polystyrene microtiter plates were coated with the coating antigen (10 μg/ml, 100 μl per well) in 50 mM carbonate–bicarbonate buffer (pH 9.6) by overnight incubation at 4 °C. The following day, the coated plates were washed five times with PBSTA (PBS containing 0.05% Tween 20 and 0.02% NaN_3, pH 7.4) and were blocked by incubation with 1% gelatin in PBS (200 μl per well) for 1 h at 37 °C. After another washing step 100 μl per well of antiserum diluted with PBSTA (1/16 000–1/1 024 000) was added to the plate and incubated for 1 h at 37 °C. After another washing step 100 μl per well of goat anti-rabbit IgG conjugated with alkaline phosphatase diluted 1:2000 with PBSTA was added to the plate and incubated for 1 h at 37 °C. Then the plates were washed again, and 100 μl per well of *p*-nitrophenyl phosphate (1 mg/ml) dissolved in 10% diethanolamine buffer (pH 9.8) was added to the plate. After incubation at 37 °C for 30 min, the reaction was stopped by adding 50 μl of 3 N NaOH and absorbance was read at 405 nm.

2.7. Determination of the effect of hapten spacer length on the affinity of antisera to coating antigens

The affinity of each of the twenty antisera to each of the five coating antigens (hapten–OVA conjugates) was determined by noncompetitive indirect ELISA as follows. All the incubations were performed at room temperature, except for the incubation with coating antigens. Microtiter plates were incubated with hapten–OVA conjugate (20, 100, and 500 ng per well) in 50 mM carbonate–bicarbonate buffer (pH 9.6) by overnight incubation at 4 °C. The following day the coated plates were washed four times with PBS (containing 0.05% Tween 20, pH 7.4) and were blocked by incubation with 0.2% OVA in PBS (200 μl per well) for 1 h. After another washing step 100 μl per well of antiserum diluted with PBST (1/10 000, 1/40 000, and 1/160 000) was added to the plates and incubated for 1 h. After another

washing step 100 μl per well of HRP-conjugated goat anti-rabbit IgG diluted 1:3000 with PBST was added to the plates and incubated for 1 h. Then the plates were washed again, and 100 μl per well of TMB solution (400 μl of 0.6% TMB–DMSO and 100 μl of 1% H_2O_2 diluted with 25 ml of citrate–acetate buffer, pH 5.5) was added to the plates and incubated for 10 min. The reaction was stopped by addition of 50 μl of 2 M H_2SO_4, and absorbance was read at 450 nm.

Effect of spacer length heterology on binding of antibodies to the coating antigen was also evaluated by comparing the affinity of antibodies to Hapten F having the shortest spacer group with those to Hapten A and B. Effect of spacer structure heterology on binding of antibodies to the coating antigen was evaluated by comparing the affinity of antibodies to Hapten G having different spacer structure with those to Hapten A, B and F. For these experiments, the antibodies against Hapten A were used.

2.8. Determination of the effect of spacer length heterology on the sensitivity of ELISA

The effect of spacer length heterology between immunogen and coating antigen on the sensitivity of ELISA was evaluated by competitive indirect ELISA for all the possible combinations of antiserum and coating antigen. The assay procedure was as follows. All the incubations were performed at room temperature except for the incubation with coating antigens. Microtiter plates were coated with hapten–OVA conjugate (200 ng/ml, 100 μl per well) in 50 mM carbonate–bicarbonate buffer (pH 9.6) by overnight incubation at 4 °C. The plates were washed four times with PBST and were blocked by incubation with 200 μl per well of 1% OVA in PBS for 1 h. After another washing step 50 μl per well of serial dilutions of the analyte in 10% methanol–PBS was added followed by 50 μl per well of antiserum diluted 1:10 000 with PBST. After incubation for 30 min antibody binding was assessed as described above using HRP-conjugated goat anti-rabbit IgG diluted 1:5000 with PBST. Competition curves were obtained by plotting absorbance against the logarithm of analyte concentration. Sigmoidal curves were fitted to a four-parameter logistic equation [21] from which IC_{50} values (concentration at which binding of the antibody to the coating antigen is inhibited by 50%) are determined.

Effect of spacer length heterology on the sensitivity of ELISA was also evaluated by comparing the results of the ELISA using Hapten F as the coating antigen with those using Hapten A and B. Effect of spacer structure heterology on ELISA sensitivity was investigated by comparing the results of the ELISA using Hapten G as a coating antigen with those using Hapten A, B and F. For these experiments, the antibodies against Hapten A were used.

3. Results and discussion

3.1. Hapten synthesis

The initial and critical step in the development of effective immunoassays for low molecular weight chemicals, such as pesticides, lies in the selection of appropriate haptens which elicit the production of antibodies demonstrating maximum specificity and sensitivity for the target molecule. In designing haptens for antibody production, it is preferable to retain functional groups that are unique to the target molecule and attach a spacer at other site where members of a class have identical structural features. In case of aromatic OP pesticides it is desirable to place a spacer at the thiophosphate group that is common to OP pesticides, maximizing exposure of aromatic structure, the unique determinant of each pesticide. Heldman et al. [7] was the first to synthesize a hapten for OP pesticide with a spacer arm at the thiophosphate group. However, a generic method was developed later by McAdam et al. [13]. They successfully applied this synthetic method to the synthesis of haptens of fenitrothion [8], chlorpyrifos [9,10], chlorpyrifos-methyl [11,12], and pirimiphos-methyl [12]. The strategy they adopted requires the preparation of a carboxyl-pretected spacer arm reagent from an amino carboxylic acid and involves a synthetic route including seven steps. In an effort to simplify the synthetic process for this type of haptens we developed a simpler process which requires only two steps without the need of preparing protected amino carboxylic acid (Fig. 2). It involves reaction of *O*-methyl (ethyl) dichlorothiophosphate (**1**) with a phenol (**2**, not sodium salt) in the presence of K_2CO_3 followed by reaction of the substitution

product (**3**) with an amino carboxylic acid (**4**, not protected) in the presence of KOH. Secondary amino acids could also be attached for the spacer arm. As described in experimental section, this process was successfully applied to the synthesis of seven haptens for the OP insecticide fenthion. The reactions proceeded facilely with relatively high yield (55% and 45–78% in the first and second reaction, respectively). The reaction time was relatively short (1 h and a few minutes for the first and second reaction, respectively). This method was successfully applied in this laboratory for the synthesis of haptens of several other OP pesticides such as chlorpyrifos, chlorpyrifos-methyl, diazinon, fenitrothion, parathion-methyl, isofenphos, etc. and was applied for a patent. All the carboxylic acid haptens could be converted to the succinimide esters, which are active esters for coupling haptens to carrier proteins. The conjugation of the active ester of each hapten to proteins was verified by measuring the modification of free amino group on the protein by TNBS reaction, and the result is presented in Table 1. The hapten–protein ratios were fairly uniform.

3.2. Effect of spacer arm length of hapten on the affinity of antisera to coating antigens

In general, immunizing haptens should be designed as a close mimic of the target molecule, preserving as much as possible the original steric and electronic characteristics of the analyte. However, even when all or most of the molecule's functional groups are unaltered, they still could be masked by the protein tertiary structure [5,16]. A small molecule covalently linked to a carrier protein may suffer a considerable amount of masking in the region of the hapten near the site of linkage. Antisera raised would suffer a lack of specificity for those determinant groups that have been masked. Therefore, it is necessary to ensure that the hapten structure is set apart from the carrier surface. An obvious means to avoid this problem would be to extend the hapten out in space via a spacer arm. However, too lengthy a spacer arm may cause folding of hapten molecule bringing the molecule close to the protein surface which would result in the masking of the hapten determinant groups. Thus, some researchers believe that the optimum spacer arm is medium-sized, i.e. 3 or 4–6 atoms [5,16]. However, there has been limited experimental data supportive of these notions.

In order to gain clues to the question of the extent of the effect of the spacer arm length of the immunizing hapten on the affinity and specificity of the antibodies as well as the question as to the optimum spacer length for immunoassay, this study used the five haptens of fenthion (Hapten A–E, Fig. 1) differing only in the length of the spacer arm attached to the thiophosphate group of the pesticide. The twenty antisera against these haptens (five haptens coupled to BSA and KLH, two rabbits per immunizing hapten) were screened after each boost against the coating antigen Hapten C-OVA. All of the antisera against hapten–BSA conjugates showed the highest titer at the fourth boost and those against hapten–KLH conjugates at the third boost. This study used BSA and KLH as carriers for immunization because they are the most often mentioned in publications. As can be seen in the tables presented below, there was no remarkable difference between the two proteins regarding the quality of the antisera raised against the immunogens prepared from each of them.

In order to examine the effect of the spacer arm length of both immunizing and coating haptens on the affinity of the antibodies to the coating antigens, the twenty antisera showing the highest titer were screened against each of the five coating haptens coupled to OVA. It would be difficult to draw solid conclusions from the results of this type of experiments, since the immune responses may present a large

Table 1
Hapten density in protein-hapten conjugates[a]

Conjugate	Covalent modification (%)	Fenthion/protein mole ratio
Immunogen		
Hapten A-BSA	63	38.4
Hapten B-BSA	65	39.7
Hapten C-BSA	80	48.8
Hapten D-BSA	77	47.0
Hapten E-BSA	77	47.0
Coating antigen		
Hapten A-OVA	73	14.6
Hapten B-OVA	82	16.4
Hapten C-OVA	71	14.2
Hapten D-OVA	71	14.2
Hapten E-OVA	75	15.0

[a] The hapten/protein ratio was determined by measuring the loss of free amino groups on the protein using the trinitrobenzenesulfonic acid (TNBS) assay.

Table 2
Titer values of the antisera against Hapten A–E for each of the same five haptens coated on the microtiter plate[a]

Immunogen	Antiserm (1:40 000)	Coating antigens				
		Hapten A-OVA	Hapten B-OVA	Hapten C-OVA	Hapten D-OVA	Hapten E-OVA
Hapten A-BSA	593	1.07	1.02	0.95	0.75	0.62
	581	1.19	1.24	1.16	1.20	1.29
Hapten B-BSA	228	1.12	1.17	1.03	0.72	0.68
	445	0.74	0.83	0.82	0.76	0.80
Hapten C-BSA	976	0.75	0.78	0.78	0.54	0.45
	834	0.85	0.97	1.08	1.01	0.98
Hapten D-BSA	657	0.92	0.94	0.72	0.64	0.60
	334	0.32	0.39	0.40	0.40	0.35
Hapten E-BSA	799	0.81	0.78	0.70	0.58	0.58
	325	0.65	0.79	0.84	0.84	0.80
Hapten A-KLH	663	0.57	0.56	0.53	0.46	0.38
	478	0.59	0.54	0.51	0.47	0.38
Hapten B-KLH	946	0.67	0.73	0.64	0.55	0.52
	237	1.13	1.14	1.03	0.96	0.81
Hapten C-KLH	748	0.74	0.79	0.71	0.58	0.53
	314	0.31	0.30	0.31	0.29	0.23
Hapten D-KLH	582	0.72	0.71	0.59	0.61	0.50
	821	0.86	0.89	0.80	0.84	0.76
Hapten E-KLH	795	0.70	0.70	0.60	0.58	0.55
	122	0.68	0.81	0.77	0.75	0.70

[a] Absorbencies were measured in a checkerboard pattern with several coating antigen concentrations and several antibody dilutions. For simplicity only data from a coating antigen concentration of 500 ng per well and an antibody dilution of 1:40 000 are shown. Absorbances were measured after a 15 min incubation with TMB at room temperature. Titer values are the means of triplicates. The relative standard deviations (CV) were all below 10%.

interindividual variation among animals and the nature of the target compounds and carrier proteins may affect the final behavior of the antibodies. However, the results of the experiment showed that there are certain trends in the behavior of the antibodies.

The result of the screening of the twenty antibodies with the highest titer against each of the five coating antigens is presented in Table 2. A notable feature in Table 2 is that, for each coating antigen, the titer difference among the antisera is small. Some exceptionally low titer values (334 and 314) appear to have resulted from divergence in the host animal rather than the difference in spacer arm length because the other sera against the same haptens (657 and 748) showed much higher titers. The relationship between the spacer arm length of the immunizing hapten and the affinity of the antisera to the plate-coating antigens is unclear. The length of a spacer arm varies depending on which atom is considered the starting point of the spacer arm. For example, Hapten A with the shortest spacer arm has four atoms (N, C, C, C excluding the carboxyl OH group which is eliminated in the hapten–protein conjugation) in the spacer arm. However, since $NHCH_2$ group may be considered as a substitute of the methoxy group (O, C, H) of the pesticide, it may be considered as having only one atom in the spacer arm. The antisera from this hapten show titers that are not particularly low, thereby masking the hapten determinant groups by using a short spacer arm appear to be not real but imaginary. In agreement with our view are the several reports that describe development of an acceptable assay

using immunizing haptens with zero spacer arm length [22,23].

Another feature to note in Table 2 is that titer difference between the homologous and heterologous ones is small, suggesting that heterology in spacer arm length is not important for the antigen recognition by antibodies. The examination of Table 2 also reveals that the affinity of each antiserum continues to decrease with increasing spacer arm length of plate-coating hapten. Of the twenty antisera, 14 show the lowest titer against the hapten with longest spacer arm (Hapten E). Theoretically this may result from masking the functional groups of coating hapten due to folding of the hapten molecule. This fact may be utilized in adjusting the affinity of a coating antigen to antibody to achieve higher assay sensitivity.

In order to see further how much a short spacer arm causes the masking of important determinant groups by carrier proteins for antibody binding, titers of antisera 593 and 663 against a coating antigen with a zero spacer length (Hapten F, Fig. 1) were measured and compared with those against some other coating antigens (Table 3). The titer difference was small, suggesting no significant masking effect by the carrier protein (OVA).

The sensitivity of immunoassays can be improved by reducing spacer recognition of antibodies and thereby elevating analyte recognition. Thus, estimating the affinity of antibodies for the spacer group would be helpful for improving assay sensitivity. For the estimation, the titers of the antisera 593 and 663 against coating antigen with a spacer of different structure [Hapten G, the spacer arm material was 4-(*N*-methylamino)butyric acid] were measured (Table 3). This new coating antigen lowered the titer values considerably, thereby, suggesting appreciable affinity of antibodies for spacer arms with a secondary, instead of a tertiary amino group.

3.3. Effect of spacer arm length on the sensitivity of ELISA

The sensitivity of immunoassay increases with the corresponding increase in affinity of antibodies to the analyte. If the spacer arm length of immunizing hapten has some influence on the affinity of antibodies to the analyte, it may affect the sensitivity of immunoassay. In order to examine the effect of spacer arm length of immunizing hapten on the sensitivity of immunoassay, we screened each of the antisera for analyte recognition by competitive indirect ELISA using each of the five coating antigens. IC_{50} values of each assay are given in Table 4. For some assays exact IC_{50} values could not be determined due to incomplete sigmoidal curve that was obtained (no plateau at either high or low analyte concentration). The table shows no notable trend suggesting irrelevance of spacer arm length to the sensitivity of immunoassay. There have been several studies that used two immunizing haptens differing only in spacer arm length. In some studies, haptens with a medium sized (3–6 atoms) spacer arm provided more sensitive assay compared to those with shorter spacer arm [24–26]. In other studies, opposite result was obtained [10,27,28]. The results of these studies and ours suggest that the spacer arm length of the immunizing hapten is not important for assay sensitivity.

Heterology in indirect (antigen-coated) immunoassay means that the immunizing hapten is different from the plate-coating hapten. Heterology is commonly used to eliminate problems associated with the strong affinity of the antibodies to the spacer arm that leads to no or poor inhibition by the target compound. Heterology usually results in weaker recognition of plate-coating antigens compared to recognition of the target compound. Thus, lower analyte concentrations can compete with these reagents, which result in better assay sensitivity. With indirect competitive ELISA,

Table 3
Titer values of the two antisera against Hapten A for four haptens coated on the microtiter plate[a]

Immunogen	Antiserm (1:40 000)	Coating antigens			
		Hapten F-OVA	Hapten A-OVA	Hapten B-OVA	Hapten G-OVA
Hapten A-BSA	593	0.93	1.09	0.97	0.65
Hapten A-KLH	663	0.39	0.48	0.35	0.20

[a] Absorbances were measured in 500 ng per well of coating antigen concentrations and 1:40 000 of antibody dilutions. Titer values are the means of triplicates. The relative standard deviations (CV) were all below 10%.

Table 4
IC_{50} values of the antibodies against Hapten A–E using each of the same five haptens coated on the microtiter plate[a]

Immunogen	Antiserum (1:10 000)	Coating antigens				
		Hapten A-OVA	Hapten B-OVA	Hapten C-OVA	Hapten D-OVA	Hapten E-OVA
Hapten A-BSA	593	2	1	2	0.6	0.1
	581	3	3	4	3	1
Hapten B-BSA	228	4	4	3	11	72
	445	5	12	3	4	1
Hapten C-BSA	976	2	3	3	39	2
	834	2	–	6	5	3
Hapten D-BSA	657	4	3	10	11	7
	334	7	–	6	9	3
Hapten E-BSA	799	1	4	3	5	–
	325	1	5	–	4	1
Hapten A-KLH	663	6	3	4	5	2
	478	6	9	1	7	0.9
Hapten B-KLH	946	9	5	3	3	2
	237	5	7	6	5	7
Hapten C-KLH	748	4	3	3	4	3
	314	0.6	3	4	4	6
Hapten D-KLH	582	10	6	7	10	19
	821	3	3	6	5	3
Hapten E-KLH	795	0.4	2	2	8	–
	122	2	3	7	7	20

[a] Data shown are concentrations in μg/ml of fenthion providing 50% inhibition of antibody binding. For some assays indicated by "–" exact IC_{50} values could not be obtained, due to incomplete sigmoidal curve obtained (no plateau at high and/or low analyte concentration). Data are the means of duplicates. The relative standard deviations (CV) were typically between 0 and 30% and maximally 60%.

site heterology (different site of spacer arm attachment) is commonly used to avoid spacer recognition of the antisera. Linker heterology may be implemented using spacer groups with different lengths and structures. There has been little data useful for estimating the degree of the effect of spacer length heterology on assay sensitivity. If spacer length heterology is indeed important for better assay sensitivity, IC_{50} values for the most heterologous combinations in Table 4 (Hapten A versus Hapten E) would be lower than those for other combinations of the same antisera. Of eight such combinations (antiserum 593 and 581 versus Hapten E-OVA, antiserum 799 and 325 versus Hapten A-OVA, etc.), seven showed the lowest IC_{50} (antiserum 593 versus Hapten E-OVA is the only exception). However, since these IC_{50} values are not much lower than other combinations, it may be concluded that spacer length heterology is not very important in improving assay sensitivity. On the other hand if spacer length heterology is important for better assay sensitivity, IC_{50} values for the most homologous combinations would be higher than those in other combinations. Of the twenty such combinations (antiserum 593 and 581 versus Hapten A-OVA, antiserum 228 and 445 versus Hapten B-OVA, etc.), only seven show the highest IC_{50}. Again, spacer length heterology seems to be not very important in improving assay sensitivity.

In order to see if a very short spacer of coating antigen may help improve the assay sensitivity by diminishing the affinity of antibodies for coating antigen, IC_{50} values for the combinations involving the coating antigen with the shortest spacer (Hapten F-OVA) were measured and compared with some other combinations (Table 5). Only a small improvement was observed. On the other hand, heterology in spacer group structure of coating antigen (Hapten G-OVA)

Table 5
IC_{50} values of the antibodies against Hapten A using four haptens coated on the microtiter plate[a]

Immunogen	Antiserum (1:10 000)	Coating antigens			
		Hapten F-OVA	Hapten A-OVA	Hapten B-OVA	Hapten G-OVA
Hapten A-BSA	593	0.4	1	2	0.3
Hapten A-KLH	663	1	2	7	0.07

[a] Data shown are concentrations in μg/ml of fenthion providing 50% inhibition of antibody binding. Data are the means of duplicates.

brought a remarkable improvement in assay sensitivity (Table 5). Therefore, to improve assay sensitivity, priority would have to be given to the heterology in structure of coating antigen spacer rather than the length of the spacer.

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