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Synthesis of haptens for immunoassay of Chlorpyrifos-ethyl as organophosphorus pesticides

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ABSTRACT

In this article, we tackle the synthesis of hapten of Chlorpyrifos-ethyl (O,O-Diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate) as an organophosphorus insecticide. The global aim is to develop an immunoassay tool to evaluate this pesticide's residue level in agricultural products. Indeed, the presented method involves the hapten synthesis with a spacer arm which the structure was identified by nuclear magnetic resonance and verified using infrared spectrometry. Then, the obtained hapten has been used for the generation of polyclonal antibodies in rabbit using the hapten-protein (bovine serum albumin) conjugate carrier through N-hydroxysuccinimide active esters to make immunogens. Later, this antibody will be used to performing enzyme-linked immunosorbent assay (ELISA) test for immunoassay Chlorpyrifos-ethyl monitoring.

1. Introduction

Currently, the development of agriculture is accompanied by the high use of pesticides worldwide. Thus, this use has shown its benefits including increased production yields. However, these products have a negative impact on the environment, the quality of agricultural products and the health of populations .

Most used pesticides belong to organophosphorus class which has been used in several applications in industrial, agricultural and medicinal chemistry fields, owing to their biological and physical characteristics besides their use as synthetic intermediates [1-5]. However, they are among the very dangerous neurotoxins known to have direct effects on the central nervous system of animals. Indeed, they inhibit the acetyl cholinesterase enzyme which is an essential component of the animal nervous system necessitating comprehensive monitoring programs [6,7].

The Chlorpyrifos-ethyl (O,O-Diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate)is an organophosphorus product that is widely used in agriculture as insecticide. Its chemical structure is in the form of white crystals and very slightly soluble in water (1.05 mg/L at 20 °C). Its developed form is shown in figure 1.

As most organophosphorus, the analysis of Chlorpyrifos-ethyl is mainly based on chromatographic methods, which are characterized by low limits of detection and high precision and sensitivity [8-9]. However, these methods have many limits, like the need for time-consuming sample clean-up prior to detection, the need of sophisticated equipment available only in well-equipped laboratories and inadequacy of on-site analysis. Therefore, there is a growing need of more rapid and economical methods for determining pesticide residues.

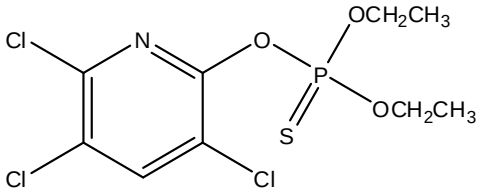

Figure 1: Chemical structure of Chlorpyrifos-ethyl [10]

During last decades, immunoassays have emerged as alternative and promising methods. These techniques that have been used extensively in clinical laboratories began recently to gain acceptance as a fast, simple to perform, sensitive and cost-effective tool for detecting trace amounts of chemicals such as pesticides [11-13].
The development of an immunoassay involves the production of antibodies to the analyte. Since pesticides are small molecules, the synthesis of their derivatives, namely haptens, is performed. The haptens are coupled to carrier proteins to induce antibody production [2,14].In this work, we describe the application of the method used by Bako et al. [15], to synthesize hapten for Chlorpyrifos-ethyl, and how to create a hapten-protein conjugate by coupling hapten to carrier proteins in order to get the immunogen that will be used for the generation of polyclonal antibodies in rabbit.

2. Material and methods

2.1. Reagents used
All chemicals (analytical grade) were from Fluka, Sigma-Aldrich, Scharlau, or Reidel-de Haein.

2.2.Hapten synthesis
In immunoassays, designing specific haptens is an important step. It aims to obtain a molecule similar to the structure of the studied product. This molecule should present a suitable functional groups (-COOH; -NH_2, -OH, -SH, etc.) to link the molecule to a carrier protein with [16-17].
Obtaining hapten involves a multi-step synthesis from an inexpensive commercial product. In our case, the O-ethyl phosphorodichloridothioate is selected. Thus, its chemical structure is similar to the common part of organophosphorus. The difficulty of this synthesis consists in the presence of a phosphorus atom, which is sensitive to water. To overcome this difficulty, the synthesis method is based on obtaining an intermediate "key" with a spacer arm that contains a certain functional group. The spacer arm must consist of a single hydrocarbon chain not too long or too short. Also, it must not have functional groups recognizable by antibodies. Therefore, the role of the spacer arm is to outdistance the hapten to the protein in order to ensure a better presentation thereof[15].The best compromise is obtained by a spacer arm consisting of a simple carbon chain which ideal length would be between two and five carbon atoms[18]. In our study, we reach to use a spacer arm that contains an acid. This will create a C-N bond via selective reaction with primary amines on the carrier protein (Figure 2). In addition, Alkylation is an important step in the synthesis of hapten. It provides an intermediate "key" close to the desired target molecule in its protected form. The role therefore of the spacer is to outdistance the hapten to the protein in order to ensure a better presentation thereof [15].
The synthesis of the hapten can be made in several ways. In our study, we performed this task on two steps as follow.As first step, we placed in an ice bath, a mixture (0.1 mol) of 3,5,6-trichloro-2-pyridinol, 15ml of triethylamine and 0.2 mole of O-ethyledichlorophosphate. Is added therein, dropwise, 50 mL of NaOH (2.3 mol/l), for 30 minutes under stirring. The resulting mixture was washed with a 5% NaOH solution (3x25 ml) and then withdistilled water (3x50 ml). The organic phase is dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue obtained was extracted with petroleum ether (3x50 ml) and then concentrated.
In the second step, is mixed in an ice bath and under stirring, 10mmol of 4-aminobutyric acid and 10mmol of NaOH. Then, O-ethyl O- (3,4,6-trichloropyridinyl) dichlorophosphate (5mmol, diluted in 5 ml of 4,4-dimethyl-1,3-dioxane) is added dropwise. Thereafter, a solution of 10 ml of NaOH (1 mole / l) is also added dropwise. After 1h of stiring, the residue is washed with a mixture of petroleum ether / ethyl acetate (9: 1, 3x10ml). The aqueous phase is acidified (pH = 3) with HCl (2 mol/l) and then extracted with ethyl acetate (3x20 mL). The organic phase is evaporated and the obtained solid is crystallized using a mixture of ethyl acetate / hexane (1: 9). These two steps can be summarized by the chemical reaction shown in figure 3 bellow.

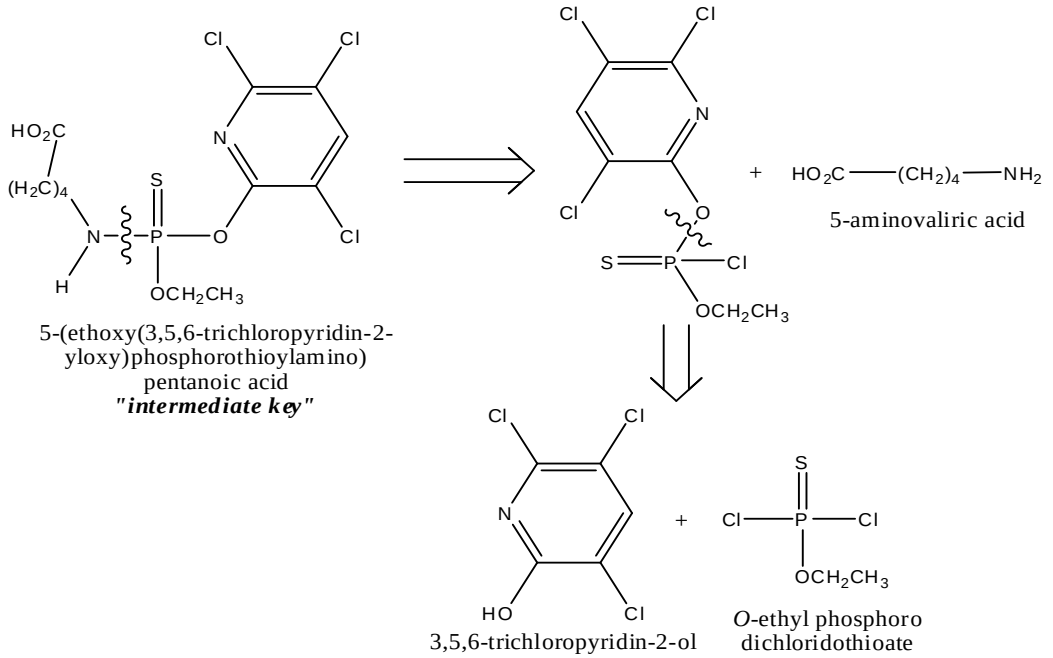

Figure 2: Retro synthesis of Chlorpyrifos-ethyl.

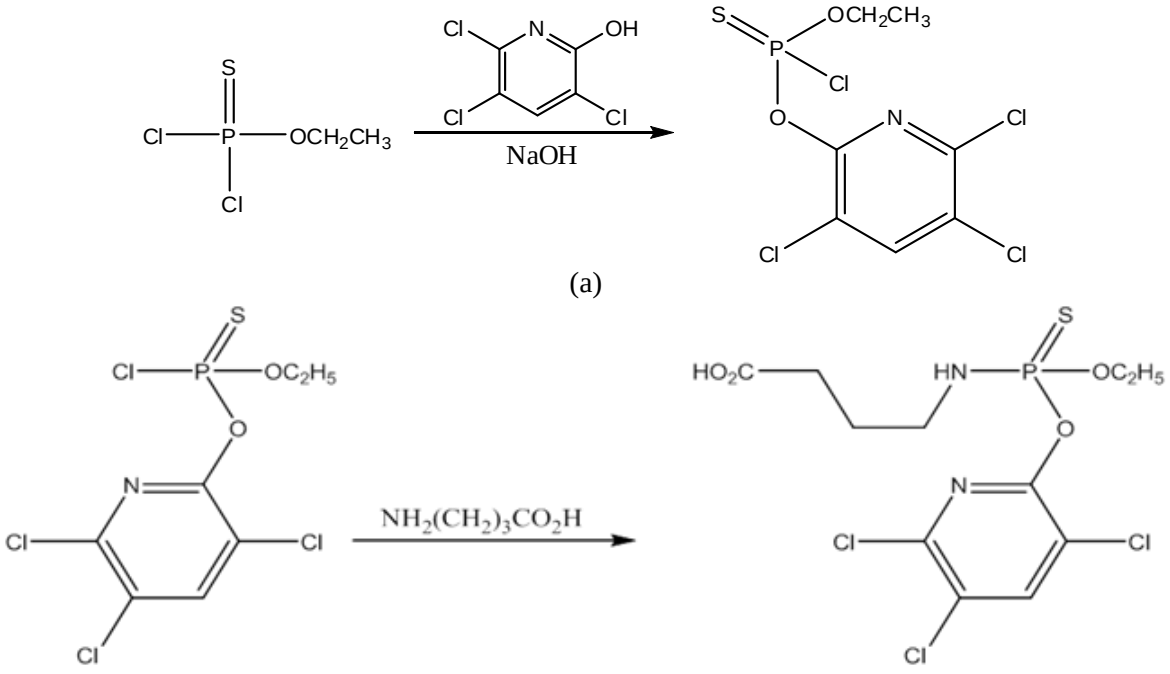

Figure 3: The chemical reactions of the two steps used in Chlorpyrifos-ethyl hapten synthesis.
(a) 1st Step. (b) 2nd step.

2.3. Characterization of hapten

Product thus obtained in the form of white powder was further characterized by Nuclear magnetic resonance (NMR) using Bruker DPX-300 spectrometer (300 MHz), gas chromatography coupled to mass-spectroscopy (GC-MS) and infrared spectroscopy (ATR Miracle Diamant).

2.4. Preparation of Chlorpyrifos–protein conjugates

In order to reach an immunoassay of Chlorpyrifos-ethyl, it is necessary to prepare the hapten–protein conjugates. Indeed, as most scientific researches dealing with immunoassay technique quote, the pesticides are in general unable to prompt a suitable immune response due to their low molecular weight [19, 20].
The hapten-protein conjugates can be obtained using covalent binding of the hapten to a carrier protein, through a coupling spacer, to synthesize the immunogen [19]. In this study, the carboxylic acid haptens were covalently attached to bovine serum albumin (BSA) or human serum albumin (HSA) (coating antigen) by the active ester method described by Aurora N. et al. [21]. The haptens were reacted with N-hydroxysuccinimide (NHS) and DCC to obtain active esters and reacted with proteins.This experiment protocol consists in dissolving 100mmol of DCC, 100mmolof hapten and 200mmol of NHS in 1ml of dimethylformamide (DMF) under agitation for 1h at room temperature. The solution is then filtered through 0.2µm nylon filters to remove the precipitate of dicyclohexylurea. The intermediate Chlorpyrifos-ethyl-dihydrosuccinimide ester (25mmol) is added to the mixture of 0.25mmol of BSA (0.0375g) in 2ml of 0.13M $NaHCO_3$ and 100ul of DMF. The solution is left to stand overnight at 4 °C. The resulting solution is dialyzed against eight changes of water 48 at 4 °C. To confirm that the coupling was performed we use electrophoresis.

2.5. Anti-Chlorpyrifos Antibodies production

A New Zealand white rabbit (1.5kg) [22] was injected with 1mg of the obtained Chlorpyrifos-BSA conjugate with the traditional procedure in aqueous solution with incomplete Freund's adjuvant. After 21 days, a second dose of 800 mg was injected. After 1 week, a third dose of 500 mg was then injected. One week later, 50 ml of rabbit blood was collected and the serum was separated by letting it coagulate overnight at 4°C and centrifuging it. The obtained serum, containing nonspecific anti-Chlorpyrifos polyclonal antibodies, was sampled and stored at -20 °C [23].

2.6. Dot blotting

Immunoblot assays (Dot Blot) is a simple and quick assay that may be employed to determine if the antibodies and detection system are effective. Dot blotting of HA-Chlorpyrifos conjugate was carried out after addition of 1µg of this solution directly onto the surface of the nitrocellulose membrane. The membrane was letting to dry at ambient temperature for 30 min, then incubated for overnight in blocking solution containing 5% (W/V) non-fat dry milk in 50mM Tris-HCl (pH 7.5), 150mM NaCl (TBS) with 0.01% (W/V) NaN3, followed by incubation with the anti-Chlorpyrifos antiserum (1:1000 dilution) as the first antibody for 1h. The membrane was then washed four times (15 min each) in TBS plus 0.05% (v/v) Tween 20 (TBSt) and incubated for 45 min with a goat anti-rabbit immunoglobulin G antibody-peroxidase conjugate (1:1000; Boehringer Mannheim). After four 15 min rounds of washing with TBSt, the nitrocellulose filter was developed under a mixture of TBS, 2mM H2O2, and 10mM 4-chloro-3-naphtol in methanol .

3. Results and Discussion

3.1. Hapten synthesis

NMR spectra were further confirmed by Proton-Nuclear Magnetic Resonance (^{1}H NMR)and carbon Nuclear Magnetic Resonance (^{13}C NMR) using $CDCl_3$as the solvent. Coupling constants are expressed in Hz and the abbreviations, t, q, m, and ar represent singular, triplet, quartet, multiplet, and aromatic, respectively.^{1}H NMR($CDCl_3$ 300MHz) δ 7,76 (1H, s, OH), 2,38 (2H, t, J=3,6 CH_2), 1,66 (4H, m, CH_2-CH_2), 2,98 (2H, t, J=5,4Hz, CH_2-NH), 3,70 (1H, s, NH), 4,61(2H, q, J=5,9Hz, OCH_2), 1,42(3H, t, J=3,45Hz, CH_3),7,26 (1H, s, ar); ^{13}C NMR ($CDCl_3$ 75MHz) δ: 178,95(COOH), 33,39 (CH_2), 30,63 (CH_2-CH_2), 41,26 (C-N), 63,06 (C-O), 15,51 (CH3), 178,86 (C=N), 141,02 (C-Cl,), 64,32 (C=C), 141,32 (Cl-C-N). GS-MS molecular ion peak (m/z=408) was the base peak.The structure was verified using infrared spectroscopy which confirms that the spacer arm has been integrated to our molecule and thus the target hapten structure has been reached. This is clearly visible on the recorded spectra at the wavelength's range [2982-2856 cm^{-1}]. The complete spectra of our hapten, is as follows: 2982, 2937(CH_3-CH_2), 2856(CH_2), 1428, 1407(CH_2), 953(P=S), 815(C-Cl, ar), 653(C-Cl).

3.2. Dot blot analysis

In order to achieve the dot blot analysis, a polyclonal antibody raised against the Chlorpyrifos-BSA (spaced than 4 carbons) was used. As shown in figure 4, this antibody clearly recognized a polypeptide corresponding to the

BSA (Blot 2) and Chlorpyrifos coupled to the HSA (spaced than 5 carbons) (Blot 3). A polypeptide corresponding to the HSA was not recognized by this antibody (Blot 1).

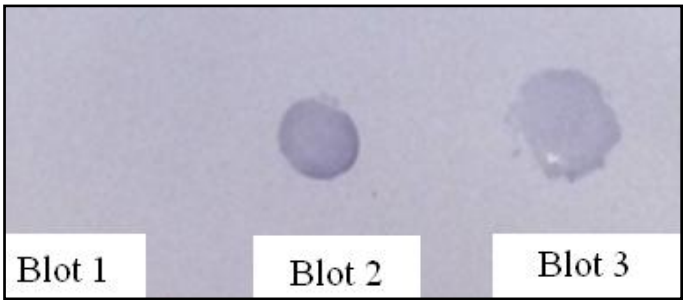

Figure 4: Dot blot analysis of anti-Chlorpyrifos.

The result shows that the antiserum contains both the anti-BSA and anti-Chlorpyrifos antibodies. Indeed, the change of the protein and the length of the spacer arm will permit us, through the results of the dot blot, to ensure that, among the antibodies produced, there are some anti-Chlorpyrifos and can be used in ELISA tests.

Conclusions

Immunoassay is a novel and promising analytical technique that we aim to use for the evaluation of Chlorpyrifos-ethyl (O,O-Diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate) pesticide's residue level in agricultural products. For this reason, we synthesize its hapten using a spacer arm of 5 carbon chain with an acid function. The 1H-NMR, 13C-NMR, GS-MS and Infrared spectroscopy results permit us to confirm that the chemical structure is the one reached. The obtained hapten was coupled to two proteins, BSA (immunogen) and HAS (coating protein). Chlorpyrifos-BSA conjugate was injected to rabbits whereas Chlorpyrifos-HSA was used in dot blot analysis. The dot blot results were positive against the Chlorpyrifos-BSA. This indicates that the antibodies correspond to our pesticide. In forthcoming works, we aim to complete our tests by performing enzyme-linked immunosorbent assay (ELISA) tests in order to achieve the development of Chlorpyrifos immunoassay tool. Also, we will compare it to other classical assays (HPLC, GC / MS ...) to ensure its reliability. Moreover, all assays must undergo validation tests. They are only validated if the results are concordant.

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