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Short Communication

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Synthesis of Hapten for Indirect Competitive Immunoassay for Measuring 3,5,6-trichloro-2-pyridinol

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Abstract

BACKGROUND: In this study, we have attempted to identify a urinary biomarker to assess chlorpyrifos exposure in farmers. The major metabolite and the excretion pathway of chlorpyrifos is 3,5,6-trichloro-2-pyridinol (TCP) in urine. Herein, we describe an adequate synthetic method for TCP hapten for measuring urinary TCP of farmers.
METHODS AND RESULTS: First, TCP was prepared by spacer attachment through hydrolysis of thiophosphate ester from chlorpyrifos. After reaction with benzyl bromide, the TCP was transformed into 2,3,5-trichloro-6-benzyloxypyridine. Next, the chlorine in the 2nd position of the pyridyl ring was substituted into 3-mercaptopropanoic acid spacer arm. Finally, the phenyl group attached to the 6th position in pyridyl ring was removed for producing the targeted product, 3-(3,5-Dichloro-6-hydroxy-2-pyridyl) thiopropanoic acid.
CONCLUSION: Henceforth, this TCP hapten would be used in developing immunoassay studies for the detection and quantitation of urinary TCP of farmers.

Key words: Chlorpyrifos, Hapten, TCP

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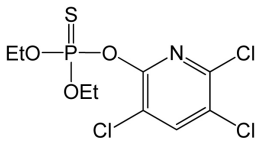
Chlorpyrifos is an organophosphorus insecticide that has been widely used in farms and in home gardens. Exposure to chlorpyrifos by breathing, ingestion or skin may affect the central nervous system as well as the cardiovascular and respiratory functions, since it acts as a cholinesterase-inhibiting compound (ATSDR, 1997; US EPA 2000).

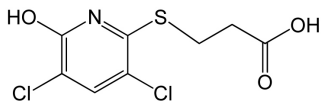
Once chlorpyrifos enters the body, the liver converts it to 3,5,6-trichloro-2-pyridinol (TCP). TCP is primarily eliminated from the body in the urine and can be detected in the urine using readily available laboratory equipments such as 96-microwell plate immunoassay fomat (Chuang et al., 2004). TCP does not inhibit cholinesterase and it is not mutagenic. Therefore, it could be considered as a valuable biomarker to estimate the total pesticide uptake via ingestioninhalation and dermal exposure (Mauriz et al., 2007).

Recently, immunoassay techniques such as enzyme-linked immunosorbent assay (ELISA) have been developed to quantify exposure to chlorpyrifos detection in biological samples (Zhang et al., 2013). Those immunoassays were demonstrated as simple, rapid, and cost-effective alternatives compared to the traditional methods (Kim et al., 2011). A competitive immunoassay requires a well-defined low-molecular-weight chemical grouping that is not immunogenic on

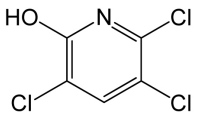
ANALYTES **HAPTEN**



Chlorpyrifos

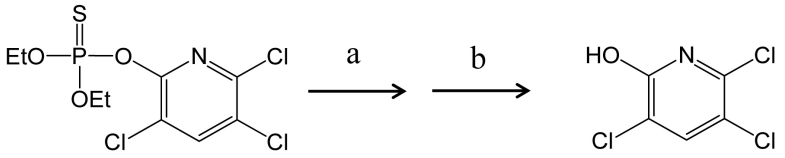


3-(3,5-Dichloro-6-hydroxy-2-pyridyl)thiopropanoic acid



3,5,6-trichloro-2-pyridinol (TCP)

Fig. 1. Structures of analytes and hapten synthesized for this study.



1 **2**

Scheme 1. (a) Addition of anhydrous EtOH and KOH, then refluxed for 1hr, (b) 2N HCl.

its own, but will react with preformed antibodies elicited by injecting the hapten linked to an immunogen (Gorton, 2005).

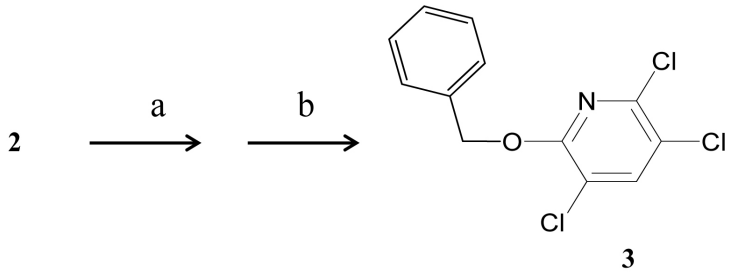
TCP does not have any suitable reactive group to be conjugated with carrier proteins (Lee et al., 2003). Therefore, hapten design is a key step in the development of immunoassays for small molecules like TCP (Manclús and Montoya, 1995). We conducted studies earlier, to synthesize a TCP hapten with carboxylic groups for its use in immunogens and plate-coating antigens. However, we encountered some difficulties in practical application when synthesizing the reactive hapten of TCP as reported in previous studies (Manclús and Montoya, 1995; Lee et al., 2003).

TCP derivatives that can be used as immunizing hapten have different alkyl chain spacers, ending in a carboxylic acid, in two sites of the pyridyl ring. Among these TCP derivatives, 3-(3,5-Dichloro-6-hydroxy-2-pyridyl) thiopropanoic acid with a shorter methylene spacer (two carbons) has been reported to produce high affinity anti-TCP polyclonal antibodies (pAbs) and exert the most immunogenic properties (Manclús and Montoya, 1995 ; Chuang et al., 2004).

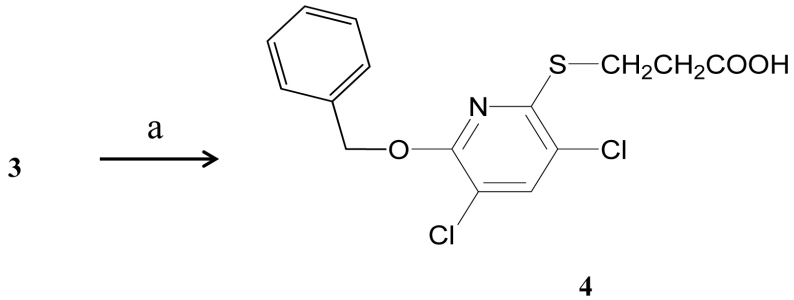
In this study, we have tried to identify a urinary biomarker that is useful to assess chlorpyrifos exposure in farmers. Herein, we describe an effective synthesis of this TCP hapten, which consists of a spacer arm attachment through a thioester linkage to the 6-positon of the pyridyl ring (Fig. 1).

Chlorpyrifos was obtained from Sigma-Alrich Company Ltd. (St. Louis, MO, USA). All chemicals were of analytical grade. Nuclear magnetic resonance (NMR) spectra were obtained with a Bruker AVANCE 500 spectrometer (500 MHz) (Bruker, Germany). Chemical shift values are given relative to internal tertramethylsilane. Liquid chromatography–mass spectrometry (LC-MS) was carried out by coupling a Waters 2695 Alliance HT system (Waters, USA) equipped with a Waters 2996 photodiode array detector and a C-18 column.

First, TCP was prepared by spacer attachment through hydrolysis of thiophosphate ester from chlorpyrifos (*O,O*-diethyl O-[3,5,6-trichloro-2-pyridinyl] phosphorothioate) as follows (Scheme 1). Potassium hydroxide (KOH, 7.2 g) was added to a mixture of



Scheme 2. (a) Benzyl bromide, (b) anhydrous DMF and 60% NaH stirred at room temperature for 18 hrs.



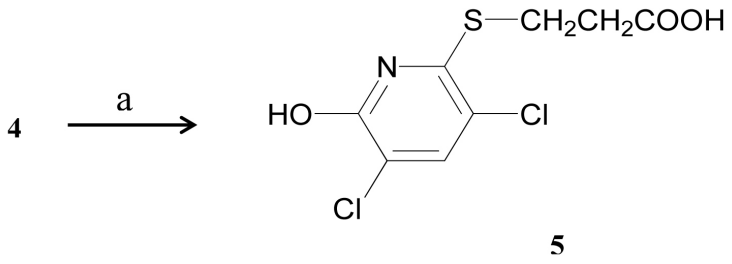
Scheme 3. (a) 3-mercaptopropanoic acid refluxed for 18 hours.

chlorpyrifos **1** (3.18 g, 30 mmol) in anhydrous ethanol (EtOH, 150 mL). The reaction mixture was reflexed for 1 hr and then cooled to room temperature before the solvent was removed by the evaporator. After adding distilled water (150 mL) to the crude residue, the resulting mixture was acidified to pH 3 with 2 N HCl and then was stirred for 1 h. The solid mixture was filtered, washed with distilled water (100 mL), and vacuum dried to obtain 1.4 g of TCP **2** as pale brown solid: ^{1}H-NMR ($CDCl_3$): δ 7.82 (1H, s). LC-MS calculated for $C_5H_2Cl_3NO$ (3,5,6-trichloro-2-pyridinol) [M+]: 198.43, found: 198.43.

Next, to the solution of TCP **2** (992 mg, 5 mmol) dissolved in anhydrous dimethylformamide (DMF, 100 mL), 60% sodium hydride (NaH, 380 mg) and benzyl bromide (1.025 g, 6 mmol) were added in the order. The reaction mixture was stirred at room temperature for 18 hrs under the nitrogen atmosphere. After stopping the reaction by adding a small amount of deionised water to the mixture, extraction was conducted with deionised water (200 mL) and ethyl acetate (EtAc; 200 mL, twice). EtAc layer was washed three times with brine (200 mL) and deionised water (200 mL, tree times), dried over $MgSO_4$, filtered, and evaporated. The residue was purified by column chromatography (EtAc/hexane, 1:20) to give 1.05 g of 2,3,5-trichloro-6-benzyloxy pyridine **3**: 1H NMR ($CDCl_3$): δ 7.75 (1H, s), 7.5-7.38 (5H, m, aromatic proton of benzyl group), 5.45 (s, 2H, CH_2 of benzyl group). LC-MS calculated for $C_{12}H_8Cl_3NO$ (2,3,5-trichloro-6-benzyloxy pyridine) [M+]: 288.55, found: 288.55.

The ionizable hydroxyl groups in the 6th position of the ring in the TCP structure (compound **2**) can be used as the spacer attachment point to offset different parts of molecules. Therefore, a protection reaction of alcoholic hydroxyl groups was necessary to prevent unwanted chemical reaction solely at the 6th position of the ring, as can be seen in Scheme 2. Electronegative nitrogen supplied to TCP structure stabilized the intermediate anion and nucleophilic substitution took place preferentially at the 6th position.

Third, to the solution of 2,3,5-trichloro-6-benzyloxy pyridine **3** (96 mg, 3.3 mmol) dissolved in anhydrous EtOH (660 mg, 12 mmol), KOH (660 mg, 12 mmol) and 3-Mercaptopropanoic acid (426 mg, 4 mmol) were added in the order. After the reaction mixture was refluxed for 18 h and cooled, EtOH layer was evaporated. After the residue was extracted with EtAc twice, the extracts were washed with brine (100 mL) and distilled water (100 mL), dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography (methyl cellulose(MC): methanol



Scheme 4. (a) H, Pd-C catalyst stirred for 6 hrs under 1 bar of hydrogen gas.

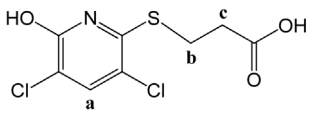
(MeOH), 30:1) to give 240 mg of (2-carboxyethyl) thio-3,5-dichlro-6-benzyloxy pyridine **4** : ^{1}H-NMR ($CdCl_3$): δ 7.75 (1H, s), 7.5-7.38 (5H, m, Aromatic proton of benzyl group), 5.45 (s, 2H, CH_2 of benzyl group), 3.38 (t, 2H, -SCH_2-), 2.77 (t, 2H, -SCH_2CH_2-). LC-MS calculated for $C_{12}H13Cl_2NO_3S$ ((2-carboxyethyl) thio-3,5-dichlro-6-benzyloxy pyridine) [M+1]: 358.21, found: 358.21. The chlorine in the 2nd position of the pyridyl ring was substituted by a 3-mercaptopropanoic acid spacer arm in this step, as can be seen in Scheme 3.

In final step, to a solution of (2-carboxyethyl) thio-3,5-dichlro-6-benzyloxy pyridine 4 (190 mg, 0.53 mmol) in anhydrous EtOH, 10% Pd-C catalyst (30 mg) was added for deprotection of alcohol groups, and the mixture was stirred at room temperature for 6 h under 3 bar of hydrogen gas. After filtering through celite to remove Pd, the EtOH layer was evaporated, extracted with EtAc (50 mL, twice), washed with brine (50 mL) and deionised water (50 mL), dried over $MgSO_4$, and concentrated. The residue was purified by column chromatography (MC: MeOH, 20: 1), and recrystallized from MC/Hex system to give 50 mg of 3-(3,5-Dichloro-6-hydroxy-2-pyridyl) thiopropanoic acid **5** (TCP hapten) as a pale brown solid. Phenyl group attached to the 6th position in pyridyl ring was finally removed for producing TCP hepten.

Complete NMR data of TCP hapten **5** are given in Table 1. Synthetic intermediates were analyzed by LCMS and confirmed by ^{1}H NMR ($CDCl_3$): ^{1}H-NMR ($CdCl_3$): δ 7.75 (1H, s), 3.38 (t, 2H, -SCH_2-), 2.77 (t, 2H, -SCH_2CH_2-). LCMS calculated for $C_8H_7Cl_2NO_3S$ (3-(3,5-Dichloro-6-hydroxy-2-pyridyl)thiopropanoic acid) [M+1]: 268.12, found: 268.12.

Previous study reported that this hapten, 3-(3,5-Dichloro-6-hydroxy-2-pyridyl) thiopropanoic acid, with a shorter methylene spacer (two carbons) showed high affinity anti-TCP MAbs and the most immunogenic properties among other TCP derivatives (Manclús and Montoya, 1995). It was reported in earlier studies

Table 1. NMR data of TCP hapten (δ, ppm).

Residue		^{1}H
	a	7.75 (s, 1H)
	b	3.38 (t, 2H)
	c	2.77 (t, 2H)

that, hapten was synthesized by direct substitution of chlorine in the 6th position of the pyridyl ring of chlorpyrifos by a 3-mercaptopropanoic acid spacer arm and then thiophosphate ester was hydrolysed (Manclús et al., 1994, Manclús and Montoya, 1995). However, our trial encountered some difficulties in synthesis of this hapten due to differences in reactivity of chlorine in different positions of the pyridyl ring.

The synthetic method of TCP hapten developed in this study consisted of four steps. It was successfully applied in the laboratory for the synthesis of hapten for chlorpyrifos. Further study is needed to validate the affinity and specificity of the monoclonal antibody produced against TCP using TCP hapten synthesized in this study. Henceforth, this TCP hapten would be used in developing immunoassay studies for the detection and quantitation of TCP in urine samples, especially when assessing farmers exposed to chlorpyrifos.

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