Development of a Compound-Specific ELISA for Flufenoxuron and an Improved Class-Specific Assay for Benzoylphenylurea Insect Growth Regulators

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This study describes immunochemical approaches for the compound-specific detection of flufenoxuron and class-specific detection of benzoylphenylurea (BPU) insecticides. With the aim of developing a highly specific immunoassay for flufenoxuron, a hapten was synthesized by introducing a spacer arm at the 2,6-difluoro substituent aromatic ring of a flufenoxuron derivative. An IC_{50} value of 2.4 ppb was obtained for flufenoxuron, with detection of the other four BPUs being more than 4000-fold less sensitive. For the development of class-specific ELISA for five BPUs, a new approach was used for the hapten preparation in which a butanoic acid linkage was introduced into the 3,5-dichloro-substitued aniline ring of chlorfluazuron analogue. Although the resultant ELISA still exhibited slightly differing cross-reactions for these five BPUs, this method had broader specificity than the previously reported polyclonal antibody-based ELISA. Spike and recovery studies for five BPUs in soil and water indicated that both the compound- and class-specific ELISAs were able to quantitatively detect BPU residues in soil and water. This study also provided additional insights into the influence of the immunizing hapten structure on the specificities of the antibodies obtained.

Keywords: *Flufenoxuron; benzoylphenylurea, compound-specific detection; class-specific detection, ELISA*

INTRODUCTION

Flufenoxuron, a benzoylphenylurea (BPU, Figure 1) insect growth regulator, has attractive features from the environmental point of view such as low application rate and less persistence (Gilbert et al., 1992). High-performance liquid chromatography (HPLC) with UV detection or diode array detection is predominantly employed for the determination of flufenoxuron (Hopkins and Lauren, 1990; Huang and Ming, 1993; Tomsej and Hajslova, 1995; Hiemstra et al., 1998) because it is thermally unstable and cannot be analyzed by gas chromatography (GC) directly. The GC methods can be used after derivatization of the parent analyte or its hydrolytic products. However, such methods, normally involving sample extraction followed by a multistep clean up and concentration steps, are time-consuming and laborious. A rapid and reliable immunoassay for this compound is needed to measure flufenoxuron residues in the environment. Wang et al. (1998) earlier developed an ELISA that was able to detect this compound, but the cross-reaction with other BPUs was high. Thus, a highly compound-selective ELISA for flufenoxuron was needed. Such an assay may result in a sensitive and cost-effective means of analyzing environmental samples containing flufenoxuron when compared to the HPLC methods.

The development of an ELISA which enabled the detection of five members of the BPU family, namely, diflubenzuron, chlorfluazuron, flufenoxuron, lufenuron, and teflubenzuron, has been reported (Wang et al., 1998). The immunoassay was based on an antibody raised to a hapten conjugate coupled through a succinyl chain at the 4-position of the aniline ring of diflubenzuron. The assay gave IC_{50} values of 0.6 ppb for diflubenzuron, 5 ppb for teflubenzuron, 10 ppb for flufenoxuron, 31 ppb for lufenuron, and 45 ppb for chlorfluazuron; thus, the detection of the larger BPUs in the group is much less sensitive. The reasons for the better cross-reaction for diflubenzuron were examined in a molecular modeling study, which suggested that the planarity of the molecules, electron-withdrawing groups, and the steric effects of chlorine(s) in the aniline ring may be critical factors affecting antibody binding (Wang et al., 1998). Accordingly, a new approach was undertaken to elucidate a superior mimic of the BPU molecule, in both geometric and electrostatic properties, which would provide maximum determinant exposure for antibody formation.

Antibodies are thought to interact most strongly with the part of the hapten distal from the point of attachment (Landsteiner, 1945). This offers the potential to design two hapten−protein conjugates for either production of a generic assay for detection of a range of analogues or a compound-specific assay with low levels of cross-reactivity with related compounds. In this study, we applied this principle and our previous experience to develop two types of immunoassays, a

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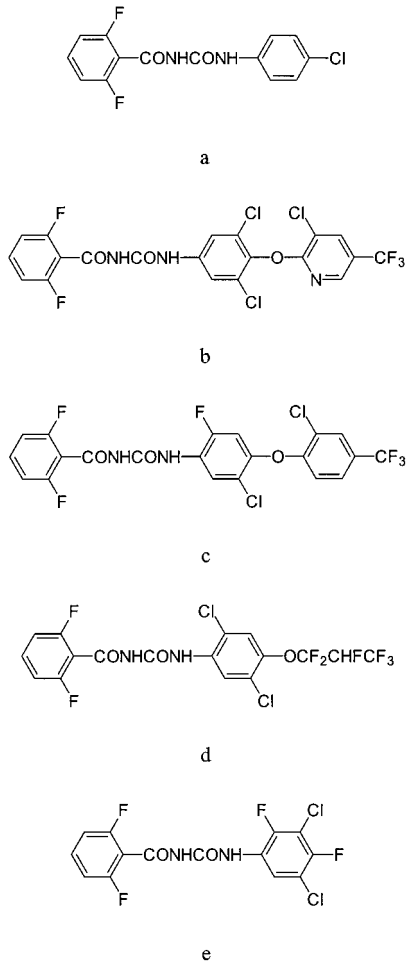

Figure 1. Chemical structures of benzoylphenylureas: (a) diflubenzuron; (b) chlorfluazuron; (c) flufenoxuron; (d) lufenuron; (e) teflubenzuron.

compound-selective assay (detecting flufenoxuron) and a class-specific assay (detecting five BPUs).

MATERIALS AND METHODS

Materials. All organic chemicals used for hapten synthesis were purchased from Aldrich, Milwaukee, WI. Silica gel H used in column chromatography was purchased from Merck, Darmstadt, Germany. Bovine serum albumin (BSA) and horseradish peroxidase (HRP) were purchased from Boehringer-Mannheim, Germany. Keyhole limpet hemocyanin (KLH), Ovalbumin (OA), and dialysis tubing were obtained from Pierce, Rockville, IL. Fish skin gelatin, Tween 20, and Freund's complete and incomplete adjuvants were purchased from Sigma, St. Louis, MO. Protein G sepharose was purchased from Pharmacia, Uppsala, Sweden. Analytical standard diflubenzuron was kindly provided by Uniroyal Chemical Company, Middlebery, CT. Analytical grade chlorfluazuron was kindly supplied by ICI Crop Care, Melbourne, and lufenuron from Ciba-Geigy, Sydney, Australia. Flufenoxuron and teflubenzuron were gifts from the American Cyanamid Company, Princeton, NJ.

Instrumentation. Proton nuclear magnetic resonance (^{1}H NMR) and ^{13}C NMR spectra were obtained on a Varian Gemini-300 instrument (300 MHz). Unless stated otherwise, $CDCl_3$ was used as the solvent with tetramethysilane (TMS) as an internal reference. Mass spectral data refers to chemical ionization using methane as reagent gas on a TSQ46 Finnigan/MAT spectrometer. Melting points were determined on a Gallenkamp melting point apparatus and were uncorrected. Analytical thin-layer chromatography (TLC) was performed on Merck silica gel 60 F_{254} precoated plates with visualization under exposure to either UV light or iodine vapor. Enzyme-linked immunosorbent assays (ELISA) were carried out in Maxisorp polystyrene 96-well microwells (Nunc, Roskilde, Denmark) and read with a Stat Fax 2100 microplate reader in dual-wavelength mode (450−630 nm).

Haptens used in this work were prepared by introduction of alkyl chain spacers, ending in a carboxylic acid, in either the difluoro aromatic ring of flufenoxuron (hapten A) or the aniline ring (hapten B). A terminal carboxylic acid group provided a means of covalently bonding these haptens to various proteins via an activated ester. The purity and structures of the intermediates were supported by melting points, analytical TLC, ^{1}H NMR, ^{13}C NMR, mass spectroscopy, and microanalysis as detailed below.

Syntheses. *2,6-Difluoro-3-iodobenzoic acid (**1**).* In a 250 mL three-neck flask, 15.8 g of 2,6-difluorobenzoic acid (0.1 mol) and 12.7 g of iodine (0.05 mol) were dissolved into 30 mL of acetic acid, and the mixture was heated in an oil bath. After the temperature reached 85−90 °C, a mixture of nitric acid (4.8 mL) and sulfuric acid (10.6 mL) was added gradually from a dropping funnel. The iodine, which sublimed during the experiment, was periodically washed back into the reaction mixture with a small volume of carbon tetrachloride, and the reaction mixture was refluxed for 6 h. The solvent was evaporated under reduced pressure, and the residue was redissolved into 80 mL of ethyl acetate. The iodine color was removed by washing with a solution of $Na_2S_2O_3$, and the ethyl acetate solution was washed with water followed by brine. The aqueous washings were re-extracted with ethyl acetate, and the combined organic fraction was dried with Na_2SO_4. After evaporation of ethyl acetate, 26.5 g of 2,6-difluoro-3-iodobenzoic acid was obtained (93%): mp 134−139 °C; ^{1}H NMR ($CDCl_3$) δ 6.86 (ddd, J = 1.5 Hz, J = 9.0 Hz, J = 9.0 Hz, 1H, aromatic), 7.85−7.92 (m, 1H, aromatic).

*2,6-Difluoro-3-iodobenzamide (**2**).* A solution of 20 g of compound **1** (70.4 mmol) in excess thionyl chloride (100 mL) was refluxed for 1 h, the volatile portion was distilled off, and the residue was cooled on ice. Saturated ammonia solution (28% NH_4OH, ice cooled, 100 mL) was added gradually, and the mixture was stirred vigorously for 10 min. The resulting precipitate was filtered, washed with saturated NH_4OH, and dried to give 19.5 g of crude product. This product was recrystallized from ethanol/water (8:3) to give 16.1 g of 2,6-difluoro-3-iodobenzamide as white crystals (81%): mp 162−165 °C; TLC R_f = 0.54 (ethyl acetate/toluene 1:1); ^{1}H NMR ($CDCl_3$) δ 6.82 (ddd, J = 1.4 Hz, J = 9.0 Hz, J = 9.0 Hz, 2H, aromatic), 7.20 (s, 2H, NH_2), 7.73−7.81 (m, 1H, aromatic).

*2,6-Difluoro-3-[2-(ethyoxycarbonyl)vinyl]benzamide (**3**).* To a stirred solution of 11.32 g of compound **2** (0.04 mol) in 40 mL acetonitrile, 2.8 g of dichloro bis(triphenylphosphine)palladium(II) catalyst, 44 mL of freshly distilled ethyl acrylate (0.4 mol), and 8.1 g of triethylamine (0.08 mol) were added under N_2. The reaction mixture was refluxed overnight. At the end of the reaction, the solvent was removed under reduced pressure and the resulting gum was purified by column/chromatography (ethyl acetate/toluene 1:1) to give 2.25 g of crude product. Recrystallization from ethyl acetate/petroleum ether yielded 1.59 g of compound **3** as plates (16%): mp 122−124 °C; TLC R_f = 0.46 (ethyl acetate/toluene 1:1); ^{1}H NMR ($CDCl_3$) δ 1.34 (t, J = 7.1 Hz, 3H, OCH_2CH_3), 4.28 (q, J = 7.1 Hz, 2H, OCH_2CH_3), 6.00 (s, 2H, NH_2), 6.50 (d, J = 16.2 Hz, 1H, =CHCOO), 7.02 (ddd, J = 1.4 Hz, J = 8.8 Hz, J = 8.8 Hz, 1H, aromatic), 7.58−7.66 (m, 1H, aromatic), 7.75 (d, J = 16.2 Hz, 1H, =CHAr,); MS m/z 256 (M+1, 100%), 238 (6).

*2,6-Difluoro-[2-(ethyoxycarbonyl)ethyl]benzamide (**4**).* A solution of 1.3 g of compound **3** (5.1 mmol) in 130 mL of ethyl acetate was reduced with hydrogen over 0.26 g of platinum oxide catalyst under 1 atm at room temperature. When no more hydrogen was absorbed, the reaction mixture was filtered through Celite and the solvent was evaporated to yield a pale-yellow solid. Recrystallization from ethyl acetate/petroleum ether gave 1.2 g compound **4** as a white fine solid (92%): mp 84−86 °C; TLC R_f = 0.42 (ethyl acetate/toluene 1:1); ^{1}H NMR ($CDCl_3$) δ 1.24 (t, J = 7.1 Hz, 3H, OCH_2CH_3), 2.62 (t, J = 7.5 Hz, 2H, CH_2COO), 2.96 (t, J = 7.5 Hz, 2H, CH_2Ar), 4.12 (q, J

= 7.1 Hz, 2H, OCH_2CH_3), 6.02 (s, 2H, NH_2), 6.90 (ddd, J = 1.4 Hz, J = 8.9 Hz, J = 8.9 Hz, 1H, aromatic), 7.27−7.34 (m, 1H, aromatic). Found: C, 56.05%; H, 5.07%; N, 5.37%. Requires: C, 56.03%; H, 5.09%; N, 5.45%.

*2-Fluoro-4-(2-chloro-4-trifluoromethylphenoxy)aniline (**5**).* A suspension of 1.6 g of flufenoxuron (3.3 mmol) in 50 mL of acetic acid and 50 mL of 6 M HCl was stirred and heated in an oil bath at 120 °C overnight. The solvent was evaporated under vacuum and the product was redissolved in 200 mL of ethyl acetate and then extracted with 6 M HCl (3 × 50 mL). The combined aqueous phase was washed with ethyl acetate (3 × 50 mL) and 6 M NaOH was added to obtain a pH of about 13. The aniline was then re-extracted with ethyl acetate (3 × 100 mL). This organic portion was evaporated to yield 1.02 g of 2-fluoro-4-(2-chloro-4-trifluoromethylphenoxy)aniline as an oil (54%): TLC R_f = 0.73 (toluene/methanol 17:3); ^{1}H NMR ($CDCl_3$/DMSO) δ 3.12 (bs, 2H, NH_2), 6.67−6.82 (bd, J = 8.8 Hz, 1H, aromatic), 7.02 (m, 2H, aromatic), 7.39 (bt, J = 8.5 Hz**,** 1H, aromatic), 7.50 (bdd, J = 2.2 Hz, J = 8.5 Hz, 1H, aromatic), 7.74 (d, J = 2.0 Hz, 1H, aromatic); MS m/z 306 (M+1, 100%), 286 (80), 270 (28), 250 (23), 139 (14).

*1-[4-(2-Chloro-α,α,α-trifluoro-4-tolyloxy)-2-fluorophenyl]-3-[2,6-difluoro-3-(2-ethoxycarbonylethyl)benzoyl]urea (**6**).* Compound **4** was reacted with freshly distilled oxalyl chloride to make 2,6-difluoro-[2-(ethyoxycarbonyl)ethyl]benzoyl isocyanate according to the method of Wie et al. (1982). Next, a solution of the crude isocyanate in dry benzene was added directly to a stirred solution of compound **5** in dry benzene. The mixture was stirred overnight at room temperature with the exclusion of moisture, the solvent was removed by evaporation under reduced pressure, and the residue was recrystallized from acetonitrile to give white crystals of compound **6**: ^{1}H NMR ($CDCl_3$) δ 1.23 (t, J = 7.1 Hz, 3H, OCH_2CH_3), 2.62 (t, J = 7.4 Hz, 2H, CH_2COO), 2.97 (t, J = 7.5 Hz, 2H, CH_2Ar), 4.11 (q, J = 7.1 Hz, 2H, OCH_2CH_3), 6.78 (bd, J = 8.8 Hz, 1H, aromatic), 6.85 (dd, J = 2.4, J = 12.8 Hz, 1H, aromatic), 6.97 (dt, J = 1.3 Hz, J = 8.9 Hz, 1H, aromatic), 7.03 (d, J = 8.7 Hz, 1H, aromatic), 7.42 (dt, J = 6.3 Hz, J = 8.5 Hz, 1H, aromatic), 7.49 (dm, J = 8.7 Hz, 1H, aromatic), 7.76 (d, J = 2.2 Hz, 1H, aromatic), 8.05 (t, J = 8.8 Hz, 1H, aromatic), 9.16 (s, 1H, NH), 10.69 (s, 1H, NH).

*1-[4-(2-Chloro-α,α,α-trifluoro-4-tolyloxy)-2-fluorophenyl]-3-[2,6-difluoro-3-(2-carboxyethyl)benzoyl]urea (**7**, Hapten A).* The hydrolysis reaction of compound **6** (0.6 g) was conducted in the aqueous methanol (1:1) with 2 equiv of KOH. After 7 h, the reaction mixture was extracted with chloroform (3 × 60 mL). The aqueous phase was acidified with 6 M HCl to pH 2 and extracted with chloroform (3 × 60 mL). The combined organic layers were then dried with Na_2SO_4 and concentrated under reduced pressure. The residue was purified using column chromatography on silica gel (ethyl acetate, 10% methanol in ethyl acetate) to give hapten A as a pale-yellow solid (0.254 g): ^{1}H NMR ($CDCl_3$) δ 2.71 (t, J = 7.4 Hz, 2H, CH_2COO), 2.98 (t, J = 7.3 Hz, 2H, CH_2Ar), 6.82 (bd, J = 8.8 Hz, 1H, aromatic), 6.87 (dd, J = 2.6, J = 11.3 Hz, 1H, aromatic), 6.97 (dt, J = 1.1 Hz, J = 8.9 Hz, 1H, aromatic), 7.05 (d, J = 8.5 Hz, 1H, aromatic), 7.39 (dt, J = 6.3 Hz, J = 8.5 Hz, 1H, aromatic), 7.50 (dm, 1H, aromatic), 7.76 (d, J = 2.1 Hz, 1H, aromatic), 8.16 (t, J = 8.7 Hz, 1H, aromatic), 10.34 (s, 1H, NH), 10.81 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 23.3, 33.3 (s, CH_2CH_2), 106.3 (d, C_3), 110.9 (dd, C'_5), 112.5 (t, C'_1), 114.3 (d, C_5), 118.8 (s, C_6), 120.9 (s, C''_2), 122.2 (s, C''_6), 122.4 (d, C_1), 123.7 (dd, C'_3), 124.8 (q, C''_4 and C''_5), 125.3 (q, CF_3), 127.5 (q, C''_3), 132.7 (t, C'_4), 150.0 (s, CONH−Ar), 150.9 (s, C_4), 154.6 (d, C_2), 154.9 (s, C''_1), 155.4 (q, C'_2), 158.7 (q, C'_6), 162.6 (s, Ar−CONH), 173.2 (s, COOH); MS m/z 561 (M+1, 13%), 332 (100), 306 (29), 230 (91), 213 (45).

*Preparation of the Active Ester of 1-[4-(2-Chloro-α,α,α-trifluoro-4-tolyloxy)-2-fluorophenyl]-3-[2,6-difluoro-3-(2-carboxyethyl)benzoyl]urea (**8**).* The *N*-hydroxysuccinimide (NHS) ester of hapten A was formed as follows. The hapten (0.36 g) was combined with NHS (0.081 g) in freshly distilled tetrahydrofuran (THF, 15 mL), and then *N*,*N*-dicyclohexylcarbodiimide (DCC, 0.145 g) was added with stirring. The reaction was allowed to run overnight under N_2. The mixture was filtered, the precipitate was discarded, and the filtrate was evaporated under reduced pressure. The resulting white solid residue was purified using column chromatography by elution with dichloromethane/ethyl acetate 4:1 giving compound **8** (57%) as a single spot on a TLC plate with R_f = 0.67 (dichloromethane/ethyl acetate 4:1). The structure of this active ester was confirmed by a ^{1}H NMR spectrum to be similar to that of compound **7** but with the succinimide protons at δ 2.84 ($CDCl_3$) and mass spectrum m/z 658 (M+1, 9%), 332 (100), 327 (38), 312 (31), 296 (44).

*3,5-Dichloro-4-[3-(ethyoxycarbonyl)propyloxy]aniline (**9**).* Into 30 mL of dry dimethyl sulfoxide (DMSO), a mixture of 7.2 g of 4-amino-2,6-dichlorophenol (40.4 mmol), 7.15 g of ethyl 4-bromobutyrate (36.6 mmol), and 6.1 g of finely ground of potassium carbonate (44.1 mmol) was added. The mixture was stirred under N_2 at room temperature for 7 h. At the end of the reaction, 100 mL of 1 M sodium hydroxide (NaOH) and 150 mL of ether were added. The aqueous layer was washed with ether, and the combined organic layer was washed with 1 M NaOH, water, and brine and then dried with Na_2SO_4. After removing the solvent under reduced pressure, an oily product was obtained in 64% yield: TLC R_f = 0.73 (ethyl acetate/toluene 1:1); ^{1}H NMR ($CDCl_3$) δ 1.27 (t, J = 7.1 Hz, 3H, OCH_2CH_3), 2.12 (5 line m, J = 6.5 Hz, 2H, $CH_2CH_2CH_2$), 2.64 (t, J = 7.4 Hz, 2H, CH_2CH_2COO), 3.63 (bs, 2H, NH_2), 3.95 (t, J = 6.0 Hz, 2H, $OCH_2CH_2CH_2$), 4.16 (q, J = 7.1 Hz, 2H, OCH_2CH_3), 6.60 (s, 2H, aromatic). This product was not purified further before the next reaction.

*1-[3,5-Dichloro-4-[3-(ethyoxycarbonyl)propyloxy]phenyl]-3-(2,6-difluorobenzoyl)urea (**10**).* A solution of 3.1 g of freshly distilled 2,6-difluorobenzoyl isocyanate (17 mmol) in 10 mL dry benzene was added to a stirred solution of 5 g of compound **9** (17 mmol) in 20 mL dry benzene under N_2. The temperature was kept below 40 °C in an ice bath. After 6 h, the resulting crystals were collected, washed with benzene, and dried to yield 7.7 g of a fine white solid. Recrystallization from acetonitrile gave compound **10** as white needles (87%): mp 134−136 °C; TLC R_f = 0.76 (ethyl acetate/toluene 1:1); ^{1}H NMR ($CDCl_3$) δ 1.28 (t, J = 7.1 Hz, 3H, OCH_2CH_3), 2.16 (5 line m, J = 6.5 Hz, 2H, $CH_2CH_2CH_2$), 2.66 (t, J = 7.3 Hz, 2H, CH_2CH_2COO), 4.04 (t, J = 6.0 Hz, 2H, $OCH_2CH_2CH_2$), 4.17 (q, J = 7.1 Hz, 2H, OCH_2CH_3), 7.07 (t, J = 8.3 Hz, 2H, aromatic). 7.48 (s, 2H, aromatic), 7.50−7.60 (m, 1H, aromatic), 9.29 (s, 1H, NH), 10.50 (s, 1H, NH).

*1-[3,5-Dichloro-4-(3-carboxypropyloxy)phenyl]-3-(2,6-difluorobenzoyl)urea (**11**, Hapten B).* A suspension of 3 g of compound **10** (6.3 mmol) and 0.98 g of finely ground potassium hydroxide in 150 mL of water and 75 mL of methanol was stirred at room temperature for 3 h. The methanol was evaporated, and the residue was washed with ethyl acetate (2 × 150 mL). The aqueous layer was acidified to pH 1 with 1 M HCl, then extracted with ethyl acetate (2 × 200 mL). The combined organic layer was washed with water and brine and dried with Na_2SO_4, and the solvent was removed to produce 2.1 g of a white solid. Recrystallized from acetonitrile yielded white needle crystals of hapten B (67%): mp 173−175 °C; TLC R_f = 0.69 (ethyl acetate); ^{1}H NMR ($CDCl_3$/DMSO) δ 2.14 (5 line m, J = 6.5 Hz, 2H, $CH_2CH_2CH_2$), 2.64 (t, J = 7.4 Hz, 2H, CH_2CH_2COO), 4.05 (t, J = 6.0 Hz, 2H, $OCH_2CH_2CH_2$), 7.02 (t, J = 8.2 Hz, 2H, aromatic), 7.43−7.54 (m, 1H, aromatic), 7.58 (s, 2H, aromatic), 10.27 (s, 1H, NH), 10.31 (s, 1H, NH); ^{13}C NMR (DMSO) δ 25.0, 29.9, 72.6 ($CH_2CH_2CH_2$), 112.0 (dd, J = 2.4, 21.8 Hz, C'_3 and C'_5), 113.3 (t, J = 21.1 Hz, C'_1), 120.6 (s, C_2 and C_6), 128.2 (s, C_3 and C_5), 133.2 (t, J = 10.1 Hz, C'_4), 134.5 (s, C_1), 146.7 (s, C_4), 150.0 (s, NHCONH), 158.6 (dd, J = 7.0, 250.1 Hz, C'_2 and C'_6), 161.9 (s, aromatic-CONH), 173.9 (s, COOH). Found: C, 48.35%; H, 2.96%; N, 6.08%. Requires: C, 48.34%; H, 3.16%; N, 6.26%.

*Preparation of Active Ester of 1-[3,5-Dichloro-4-(3-carboxypropyloxy)phenyl]-3-(2,6-difluorobenzoyl)urea (**12**).* The active ester of compound **11** was prepared as for compound **8**. Purification using column chromatography by elution with dichloromethane/ethyl acetate 4:1 gave compound **12** (74%): TLC R_f = 0.45 (dichloromethane/ethyl acetate 4:1); ^{1}H NMR ($CDCl_3$/DMSO) δ 2.14 (5 line m, J = 6.5 Hz, 2H, $CH_2CH_2CH_2$),

2.86 (s, 4H, succimide H), 3.03 (t, J = 7.4 Hz, 2H, CH_2CH_2-COO), 4.08 (t, J = 6.0 Hz, 2H, $OCH_2CH_2CH_2$), 7.02 (t, J = 8.3 Hz, 2H, aromatic), 7.44−7.54 (m, 1H, aromatic), 7.58 (s, 2H, aromatic), 10.23 (s, NH), 10.51 (s, NH); MS m/z 544 (M, 8%), 429(8), 184 (24), 158 (100).

Preparation of Protein and Enzyme Conjugates. The *N*-hydroxysuccinimide (NHS) esters of each hapten were coupled to ovalbumin (OA), keyhole limpet hemocyanin (KLH), and horseradish peroxidase (HRP) in 50 mM potassium phosphate buffer, pH 9.1 (McAdam et al., 1992). The conjugates were then purified using dialysis tubing or desalting in a PD-10 column (Pharmacia, Uppsala). The concentration of the coupled protein was determined using the Coomassie Brilliant Blue G-250 dye-binding assay method (Bradford, 1976). The concentration of coupled enzyme in solution was determined using an extinction coefficient, $E_{403}^{1cm} = 2.25$ for 1 mg/mL solution (McAdam et al., 1992).

Immunization of Rabbits. Female New Zealand white rabbits were immunized by intradermal and intramuscular injections of haptens conjugated to KLH and OA. The immunization protocol was the same as described in Wang et al. (1998). For initial injection, the immunogens (1 mg) were dissolved in 0.5 mL of 0.9% NaCl (saline) and 0.5 mL of Freund's complete adjuvants. For the booster immunization, 0.5 mg of conjugate in 0.5 mL of saline/0.5 mL Freund's incomplete was used. The boosting was performed 2, 4, and 6 weeks after the initial immunization and then at 1 month intervals. Rabbits were bleed from the marginal ear vein 9 days after each monthly immunization. IgG from the antisera were purified by Protein G-Sepharose affinity chromatography (Akerstrom et al., 1985). Two rabbits were immunized with each hapten−protein conjugate, and the results shown were obtained from the fourth bleed of individual rabbits.

Direct Competitive ELISAs. A direct competitive ELISA (using immobilized antibody) was carried out, with all steps performed at room temperature. The microwell plates were coated overnight with purified *anti*-benzoylphenylurea IgG (1 μg per well in 100 μL 50 mM carbonate buffer, pH 9.6). The next day, coated plates were washed three times with PBST washing solution [PBS (50 mM sodium phosphate/150 mM NaCl, pH 7.2) with 0.05% (v/v) Tween 20] and then nonspecific antibody binding blocked with 150 μL of 1% BSA/PBS per well for 1 h. The assay was performed by the addition of 50 μL of pesticide standard in 0.1% (w/v) fish gelatin in PBS solution containing 5% methanol and 50 μL of enzyme conjugate solution diluted in the same diluent to each well and incubation for 1 h. After five applications of washing solution, 150 μL of 3,3′,5,5′-tetramethylbenzidine peroxide based substrate solution was added to each well. Color development was stopped after 30 min by adding 50 μL of 2.5 N H_2SO_4, and absorbances were read in the microplate reader.

Soil and Water Analysis. The cotton field soil and river water samples (Narrabri, Australia) used in this study had no history of application of BPU insecticides. The extent of matrix interference was determined by comparing the standard curves prepared in field water and diluted soil extract with the standard curves prepared in purified water and diluted soil extractant, respectively (90% methanol in water). A series of pesticide spikes of BPUs at 0.1, 0.5, 1, 5, and 10 ppm in soil and 5, 10, 20, 50, and 100 ppb in water were prepared as described earlier (Wang et al., 1998), and recoveries were determined by ELISA. Water samples were analyzed directly without cleanup, and soil samples were extracted with 90% methanol/water and then diluted 1/10 or more with 0.1% FG-PBS before analysis.

RESULTS AND DISCUSSION

Hapten Synthesis. The two synthetic schemes are shown in Figures 2 and 3. With the aim of developing a highly sensitive immunoassay for flufenoxuron, a hapten in which the spacer arm was attached at the 2,6-difluoro-substituted aromatic ring was attempted. This strategy was designed to preserve the flufenoxuron-specific moiety, the 2-fluoro-4-(2-chloro-4-trifluoromethylphenoxy)aniline group, as the potentially major epitope. Antibodies to this hapten would be expected to selectively recognize flufenoxuron. The successful approach involved a multiple-step reaction, starting with the iodination of 2,6-difluorobenzoic acid (Figure 2). Modification of the procedure using an iodine/HNO_3/H_2SO_4 mixture produced 3-iodo-2,6-difluorobenzoic acid (Tronov and Novtskov, 1953). The linker arm was introduced by the palladium-catalyzed arylation reaction (Heck reaction) of the iodo derivative. Moreno-Manas et al. (1996) reported that such coupling reactions with electron-withdrawing substituents in the para position of the aryl iodide led to a side reaction. This may explain the poor yield (16%) of desired product. The required 2-fluoro-4-(2-chloro-4-trifluoromethylphenoxy)aniline was obtained by hydrolysis of the parent compound flufenoxuron, and reaction with 2,6-difluoro-[2-(ethyoxycarbonyl)ethyl]benzoyl isocyanate yielded the 1-[4-(2-chloro-α,α,α-trifluoro-4-tolyloxy)-2-fluorophenyl]-3-(2,6-difluro-3-(2-ethoxycarbonylethyl)benzoyl)urea. Careful base hydrolysis with 2 equiv of KOH gave hapten A.

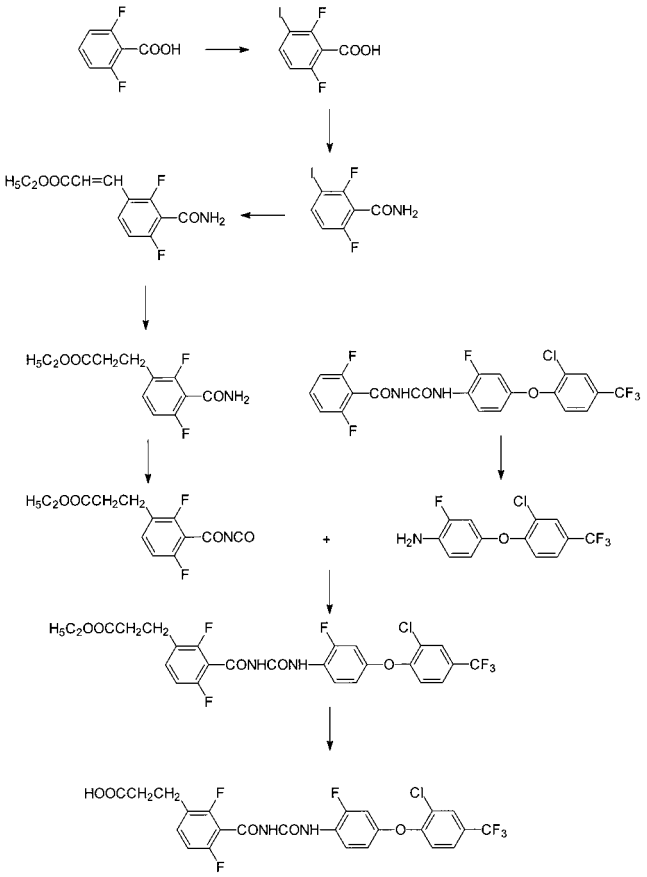

Figure 2. Reaction scheme for the synthesis of hapten A.

In Figure 3, hapten B contains a butanoic acid linkage on the aniline ring. This should allow antibodies to be selective for the common 2,6-difluorobenzoylurea group of the five BPUs. This strategy was designed to provide a class-specific ELISA for these five insecticides. We have previously demonstrated that antibodies developed to a hapten conjugated through a succinyl chain at the 4-position on the aniline of diflubenzuron could detect these five BPUs, but the detection of the BPUs with larger substituents on the aniline ring was less sensitive. A molecular modeling study provided an explanation of the observed results (Wang et al., 1998), sug-

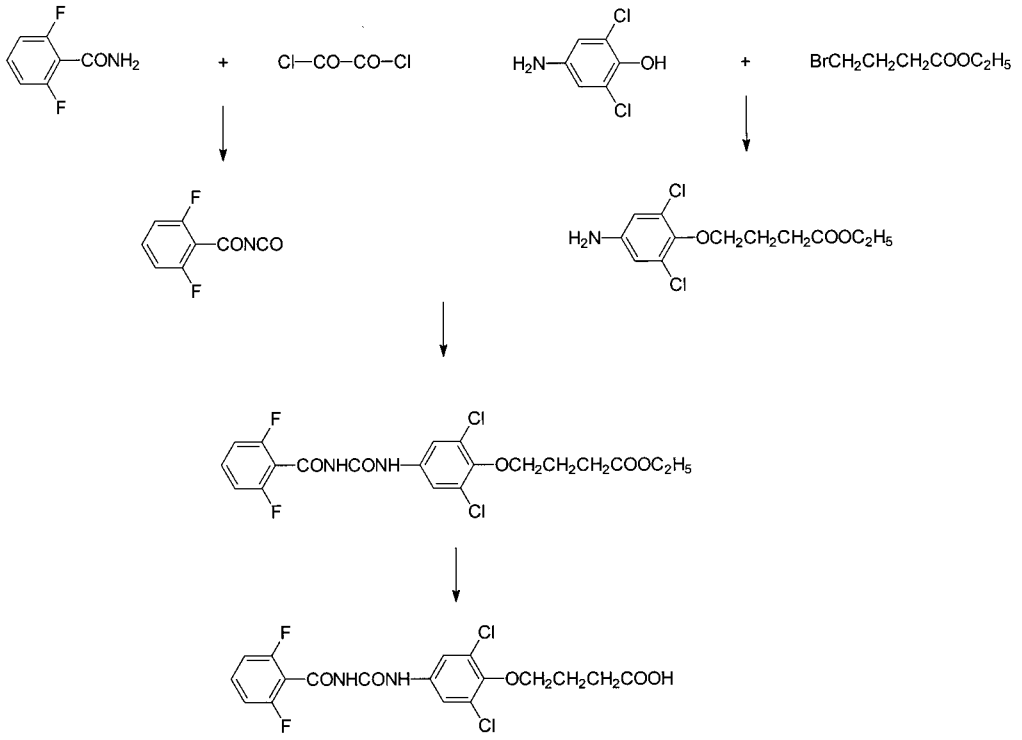

Figure 3. Reaction scheme for the synthesis of hapten B.

gesting that the nonplanarity of an oxygen substituent with the adjacent aniline ring in chlorfluazuron, flufenoxuron, and lufenuron may prevent their recognition by antibodies raised to haptens that had a coplanar group in that position. The electronic characters of the substituent at the 4-position of the aniline ring in the BPU may also have some influence on antibody affinities. The lower reactivity with teflubenzuron compared to diflubenzuron suggests that the chlorine atoms may be necessary for antibody binding. Following that study, investigation was made into introducing a spacer arm at the aniline moiety of the BPUs through an oxygen group at the 4-position, with the inclusion of two chlorine atoms at the 3- and 5-positions. This approach would provide a hapten with the 2,6-difluorobenzoylurea moiety distal to the point of coupling, representing a good mimic of the larger BPU molecules, in both geometric and electronic properties.

The synthetic route to this hapten started with reaction of 4-amino-2,6-dichlophenol and ethyl 4-bromobutyrate to form 3,5-dichloro-4-[3-(ethyoxycarbonyl)-propyloxy]aniline. Addition of 2,6-difluorobenzoyl isocyanate formed compound **11**, which was converted to the final hapten B by base hydrolysis.

Assay Performance. The optimal concentration of enzyme conjugate was determined for each assay as the lowest concentration required to produce an assay color of 0.7−1.2 absorbance units. To select the most suitable combination of antibody- and hapten-labeled enzyme for assaying the target analytes, each antiserum was screened against the panel of six enzyme-labeled haptens (Figure 4) by determining the percentage inhibitions for all five BPUs at 33.3 ppb. Although two antibodies were prepared to each hapten, only results with the antiserum that exhibited superior detection sensitivity for the target compounds are shown (Table 1). Those combinations providing good inhibition and utilizing a low concentration of enzyme conjugate were selected for further characterization (Table 2).

As expected, hapten A produced antibodies and assays with high selectivity for flufenoxuron. The homologous assay was quite sensitive (IC_{50} for flufenoxuron of 2.4 ppb), and detection of the other four BPUs were more than 4000-fold less sensitive. None of the antibodies derived from hapten A recognized conjugates based on hapten B in this paper and heterologous analogues in previous paper (Figure 4). Therefore, although hapten heterology has been demonstrated as a good approach for improving the sensitivity of pesticide immunoassays (Schneider et al., 1994; Schneider and Hammock, 1992; Harrison et al., 1991; Schlaeppi et al., 1994), the heterologous hapten structure and/or the way it is presented to the antibody should not involve great differences with respect to the immunogen hapten structure. Otherwise, haptens could become unrecognizable by the antibody and thus unsuitable to develop competitive immunoassays. The same phenomenon was also observed in our previous study in which antibodies derived from haptens with the spacer arm attached to the aniline ring did not recognize conjugates from the hapten with the spacer arm attached to the central nitrogen atom, and vice versa.

Screening the antisera from hapten B against six hapten-labeled enzymes (Tables 1 and 2) established that combinations of antisera with enzyme conjugates **1** and **2** (Figure 4) provided higher detection sensitivities for the BPU targets. There was not much difference in sensitivities and specificities between these two combinations. However, only results from enzyme conjugate **2** were presented in this paper in order to simplify for comparison with our previous paper (Wang et al., 1998), in which the same enzyme conjugate was used.

The antibodies developed from new hapten B had more equal cross-reactivities to all five BPUs than did the haptens developed in our previous paper, although the detection of the larger BPUs was still less sensitive, compared to the sensitivity to diflubenzuron. If the

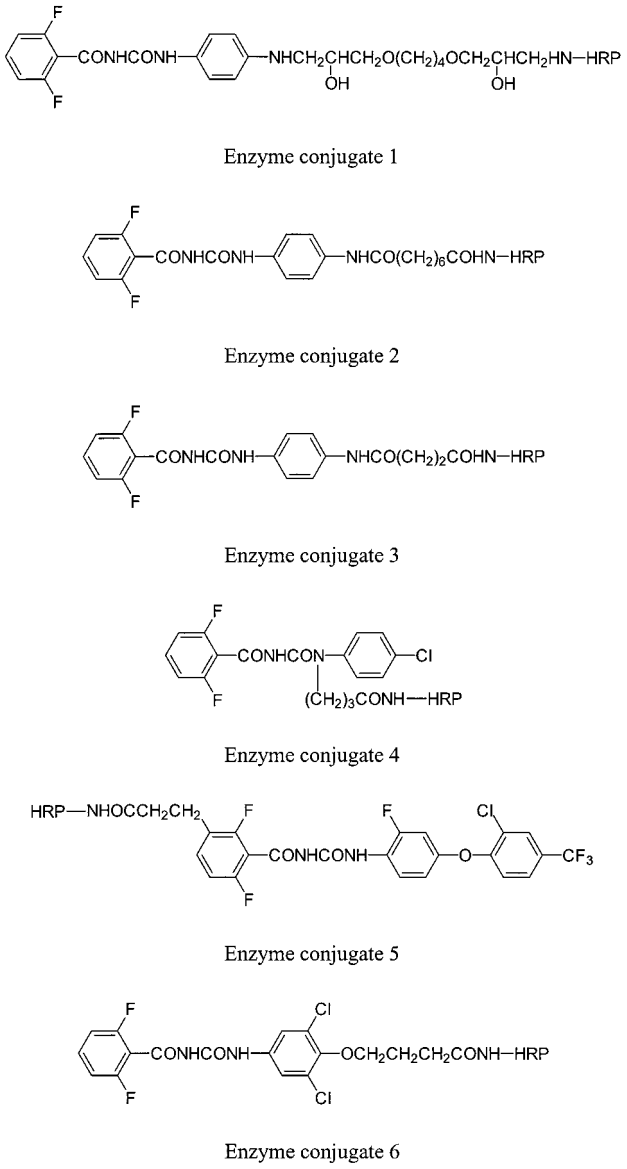

Figure 4. Chemical structures of enzyme conjugates used for assay screening.

sensitivity of each BPU relative to diflubenzuron was calculated using the formula

$$\text{relative sensitivity} = \frac{\text{IC}_{50} \text{ of other BPU}}{\text{IC}_{50} \text{ of diflubenzuron}}$$

then previous studies with the *anti*-hapten II−OA polyclonal antibody showed that the resultant assay was lowered by a factors of 75, 17, 52, and 9 in sensitivity for chlorfluazuron, flufenoxuron, lufenuron, and teflubenzuron, respectively (Wang et al., 1998). In contrast, the current assay using the antibody from hapten A in combination with same enzyme conjugate gave values of 7, 34, 27, and 2 times lower sensitivities compared to diflubenzuron. There are still differences in the extent of cross-reaction in the current assay; however, the relative sensitivities to other BPUs compared to diflubenzuron have been improved, except for flufenoxuron.

A number of aspects of the hapten design for these antibodies may have led to the improvements in cross-reactivity. The assay IC_{50} values for chlorfluazuron and teflubenzuron were 9 and 2 ppb, respectively, which were 5 and 2.5 times more sensitive than the values reported previously using hapten II. This is consistent with a previous prediction (Wang et al., 1998) that chlorine substituents at 3- and 5-positions on the aromatic ring may play a key role in influencing antibody specificity. The relative lower sensitivities to flufenoxuron and lufenuron may be due to the absence of a chlorine atom in the aromatic ring of flufenoxuron and different position (2,5- rather than 3,5-) of chlorine in lufenuron. Because of the important influence of the chlorine substituents on antibody recognition, it may be extremely difficult or impossible to develop an assay with exactly equal cross-reactivity to all five of thses BPUs. The same phenomenon was also observed by Johnson et al. (1998) when they tried to develop a class-specific immunoassay for the detection of organophosphorus pesticides. Although the new hapten with 3,5-dichloro substituents and an ether linkage was different from the previous hapten II, the assay obtained was still more sensitive to diflubenzuron. It may be that diflubenzuron fits into the antibody pocket more exactly since it is smaller. Taking all these factors into account, this new assay can be considered as a class-specific assay for these five BPUs. However, there is still more than a 30-fold IC_{50} difference between these five BPUs, and this may limit the current assay's utility, especially if all five BPUs are expected in the sample.

Interassay precision for both the compound- and class-specific ELISAs was studied by determining the variation in percentage inhibition of antibody binding at the seven BPU concentrations used for preparation of

Table 1. Percent Inhibition at 33.3 ppb for Five Benzoylphenylureas Using Different Immunogen and Enzyme Conjugate Combinations

		immunogens			
HRP conjugate	target	haptenB −OA	haptenB −KLH	haptenA −OA	haptenA −KLH
1	diflubenzuron	52	85	*a*	*a*
	chlorfluazuron	41	71	*a*	*a*
	flufenoxuron	18	52	*a*	*a*
	lufenuron	21	54	*a*	*a*
	teflubenzuron	50	83	*a*	*a*
2	diflubenzuron	54	83	*a*	*a*
	chlorfluazuron	37	68	*a*	*a*
	flufenoxuron	17	44	*a*	*a*
	lufenuron	21	49	*a*	*a*
	teflubenzuron	49	82	*a*	*a*
3	diflubenzuron	47	73	*a*	*a*
	chlorfluazuron	38	56	*a*	*a*
	flufenoxuron	11	21	*a*	*a*
	lufenuron	15	32	*a*	*a*
	teflubenzuron	41	70	*a*	*a*
4	diflubenzuron	*a*	*a*	*a*	*a*
	chlorfluazuron	*a*	*a*	*a*	*a*
	flufenoxuron	*a*	*a*	*a*	*a*
	lufenuron	*a*	*a*	*a*	*a*
	teflubenzuron	*a*	*a*	*a*	*a*
6	diflubenzuron	38	72	*a*	*a*
	chlorfluazuron	18	50	*a*	*a*
	flufenoxuron	2	9	*a*	*a*
	lufenuron	13	9	*a*	*a*
	teflubenzuron	48	69	*a*	*a*
5	diflubenzuron	*a*	*a*	7	0.2
	chlorfluazuron	*a*	*a*	14	6
	flufenoxuron	*a*	*a*	79	80
	lufenuron	*a*	*a*	3	4
	teflubenzuron	*a*	*a*	2	0

[a] Indicates the maxium absorbance in absence of analyte was below 0.7 with enzyme conjugate dilution of 1/500.

Table 2. IC_{50} Values[a] (Parts per Billion, ppb) for Five BPUs Using Different Immunogen and Enzyme Conjugate

immunogens	enzyme conjugate	target				
		diflubenzuron	chlorfluazuron	flufenoxuron	lufenuron	teflubenzuron
hapten A−KLH	**5**	>10 000	>10 000	2.4	>10 000	>10 000
hapten B−KLH	**1**	1.4	7.4	33.6	30.2	1.8
	2	1.3	9.3	44	34.9	2.1
	3	2.9	10.1	67	67	3.1
	6	16	37	300	400	16

[a] Data shown are the geometric mean of 5−20 experiments, with 95% confidence level.

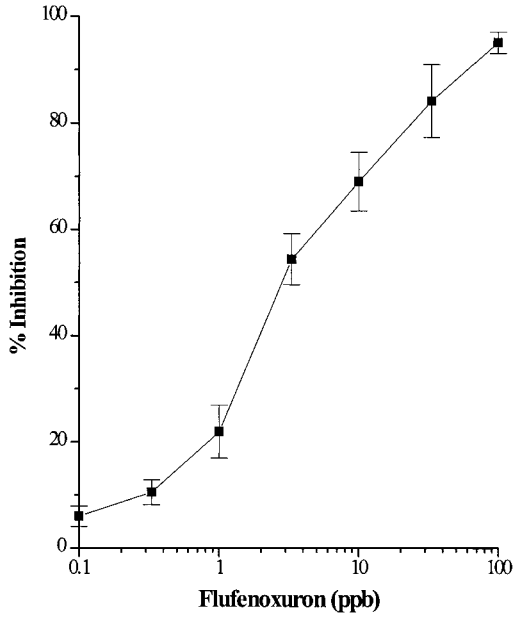

Figure 5. Standard curves for flufenoxuron using antibody to hapten A with hapten A−HRP conjugate.

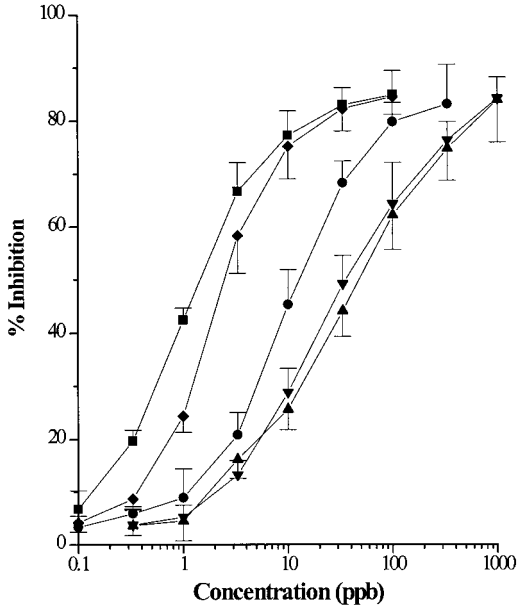

Figure 6. Standard curves for five benzoylphenylureas using antibody to hapten B with hapten I−disuccinimidyl suberate−HRP conjugate: (■) diflubenzuron; (●) chlorfluazuron; (▲) flufenoxuron; (▼) lufenuron; (◆) teflubenzuron.

standard curves. The percent CV values of inhibition at different concentrations for the flufenoxuron assay were as follows: at 100 ppb, 2%; at 33.3 ppb, 8%; at 10 ppb, 8%; at 3.3 ppb, 9%; at 1 ppb, 15%; at 0.33 ppb, 23%; at 0.1 ppb, 28%. The increasing relative error was observed at low flufenoxuron concentration for this assay, as has been discussed by Harrison et al. (1989). The typical standard curve for flufenoxuron (Figure 5), obtained from repeated studies on different days, indicated the limit of detection (LOD) of 0.5 ppb. The definition of LOD used here is the concentration causing 15% inhibition of color development (Lee et al., 1995). The class-specific ELISA also showed similar CV values (data not shown), and the standard curves for five BPUs are shown in Figure 6. The IC_{15} values were 0.2 ppb for diflubenzuron, 1.9 ppb for chlorfluazuron, 2.8 ppb for flufenoxuron, 3.6 ppb for lufenuron, and 0.5 ppb for teflubenzuron.

Assay Specificity. The specificities of both class-specific and flufenoxuron assays were evaluated by performing competitive assays with several structurally related aromatic urea herbicides (chlorobromuron, diuron, fluometuron, monolinuron, metobromuron, metoxuron, neburon, tebuthiuron, 3-(3,4-dichlorophenyl)-1-methylurea, 3,4-dichlorophenylurea), metabolites of diflubenzuron and teflubenzuron (4-chloroaniline, 2,6-difluorobenzamide, 2,6-difluorobenzoic acid, and 2,4-difluoro-3,5-dichlorophenyurea), and some structurally dissimilar compounds that may reasonably be expected to be found in water and soil in the Australian cotton production systems (bifenthrin, deltamethrin, DDE, DDT, endosulfan, and lambda-cyhalothrin). The IC_{50} values for each of these compounds were above 10 000 ppb, indicating that the assays were very specific to the insect growth regulator benzoylphenylureas.

Soil and Water Analysis. Experiments were initially conducted to determine the possible matrix effects from field water and soil extract. Figure 7 shows the results obtained with the flufenoxuron assay, which demonstrated that field water did not interfere with the current assay and thus could be analyzed directly without the need for clean-up procedures. Soil extract (after 1/10 dilution in 0.1% FG-PBS) also did not show significant matrix effects (Figure 8). These two matrixes also did not interfere with the sensitivity and specificity of the class-specific assay (data not shown). Soil and water samples from cotton field were then spiked for recovery study. Good recoveries in water and soil samples illustrate the accuracy of both immunoassays for determination of these BPUs in water and soil environments (Table 3).

CONCLUSIONS

Two competitive immunoassays have been developed for the sensitive analysis of flufenoxuron and the BPU family using two types of immunizing haptens, characterized by the presentation of different parts of the BPU molecular structure. The hapten preserving the characteristic 2-fluoro-4-(2-chloro-4-trifluoromethylphenoxy)-aniline group of flufenoxuron provided a flufenoxuron-specific antibody, with very low cross-reaction with the other four BPUs tested in this study, while the hapten

Table 3. Recoveries of BPUs Spiked at Five Concentrations in Field Soil and Water Samples Using Flufenoxuron-Specific Assay and Class-Specific Assay

		recovery (%)					
		flufenoxuro assay	class-specific assay				
matrix	spike level		diflubenzuron	chlorfluazuron	flufenoxuron	lufenuron	teflubenzuron
soil	0.1 ppm	99	84	90	97	86	91
	0.5 ppm	81	92	102	79	94	82
	1 ppm	92	89	83	109	87	79
	5 ppm	112	86	76	88	94	81
	10 ppm	94	94	89	96	79	92
field water	5 ppb	82	81	94	84	101	95
	10 ppb	83	95	83	82	87	93
	20 ppb	98	88	99	95	98	85
	50 ppb	92	97	76	111	86	87
	100 ppb	109	93	85	99	93	96

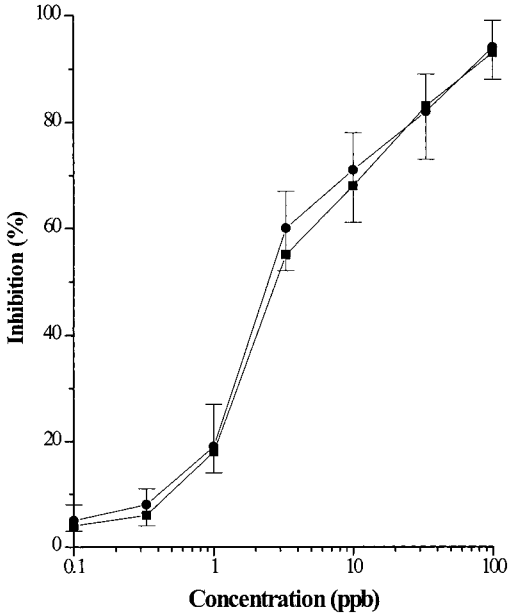

Figure 7. Standard curves for flufenoxuron in purified water (■) and field water (●).

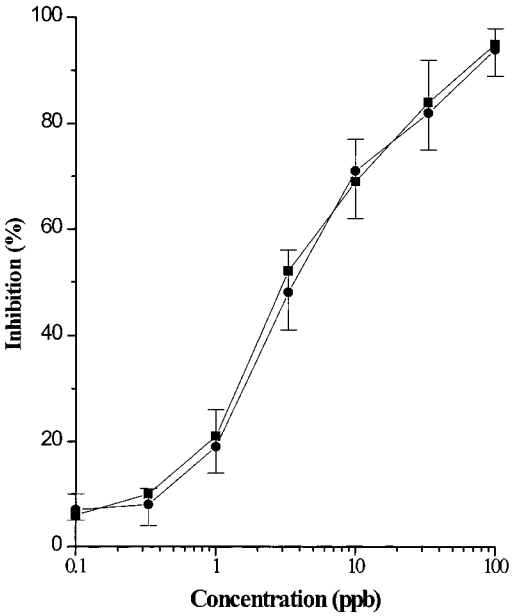

Figure 8. Standard curves for flufenoxuron in methanol (■) and soil extract (●) diluted in 0.1% FG-PBS.

preserving the common characteristic 2,6-difluorobenzoylurea group of five BPUs gave a class-specific antibody for the five BPUs. The work confirms the relevance of hapten design in the production of antibodies for pesticides, in which the position of spacer arm attachment is thought to have profound influence on the selectivity of the assay.

The goal of obtaining an improved class-specific antibody to five BPUs was achieved using hapten B. The antibody obtained improved greatly the cross-reactivities with the chlorfluazuron and teflubenzuron, compared to the previous work (Wang et al., 1998). This result also confirmed the previous prediction that the 3- and 5-position of chlorine substituents on the aniline ring may play an important role in the antibody specificity.

Both immunoassays were able to be applied to study the dissipation of benzoylphenylureas in water and soil. BPU residues in water could be quantitatively analyzed directly without the need for cleanup, and soil samples could be extracted with 90% methanol/water and then diluted 1/10 or more with 0.1% FG-PBS before analysis. No environmental matrix or other agrochemicals tested in this study were found to interfere with the detection of BPUs.

ABBREVIATIONS USED

BPU, benzoylphenylurea; BSA, bovine serum albumin; DMF, *N*,*N*′-dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; 0.1% FG-PBS, phosphate-buffered saline containing 0.1% fish skin gelatin; HRP, horseradish peroxidase; IC_{50}, concentration of analyte giving 50% inhibition of color development; KLH, keyhole limpet hemocyanin; LOD, limit of detection; NMR, nuclear magnetic resonance; OA, ovalbumin; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate); THF, tetrahydrofuran.

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