Development of a Class-Specific Competitive ELISA for the Benzoylphenylurea Insecticides

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A competitive enzyme-linked immunosorbert assay (ELISA) was developed for the quantitative detection of five benzophenylurea insecticides in soil (90% methanol extraction) and water. No significant matrix effects were observed. Conjugates of diflubenzuron derivatives with proteins were prepared by attachment at either the urea nitrogen of diflubenzuron or the 4-position of the aniline ring, wherein bridging groups of various lengths were used between the hapten and the protein carrier. All combinations of antiserum to each immunogen and enzyme tracer were examined, and an assay was developed using an antibody developed to a hapten conjugate coupled through a succinyl chain at the 4-aniline position. The assay gave 50% inhibition of antibody binding (IC_{50}) at 0.6 ppb for diflubenzuron, 5 ppb for teflubenzuron, 10 ppb for flufenoxuron, 31 ppb for lufenuron, and 45 ppb for chlorfluazuron, with detection limits in the range of 0.05−2.3 ppb for these compounds. The basis for the superior cross-reaction of diflubenzuron was examined by molecular modeling, which suggested that the planarity of the molecules, electron-withdrawing groups, and steric effects of chlorines attached to the phenyl ring may be critical factors affecting antibody binding.

Keywords: *Benzoylphenylurea; polyclonal antibody; ELISA; molecular modeling*

INTRODUCTION

Benzoylphenylureas (BPUs) are promising and effective insecticides used for the control of insects attacking a wide range of crops. These compounds are generally recognized as insect growth regulators that interfere with chitin synthesis in target pests, causing death or abortive development (Hajjar and Casida, 1978; Ishaaya, 1990). BPUs are considered to be a fourth generation of insecticides with many attractive properties such as high selectivity, low acute toxicity for mammals, and high biological activity, resulting in low application rates (Metcalf et al., 1975). Five members of this class of compound, diflubenzuron, chlorfluazuron, flufenoxuron, lufenuron, and teflubenzuron (Figure 1), were chosen for their potential use associated with transgenic cotton (Bt cotton) in Australia. The use of Bt cotton is expected to significantly reduce the requirement for endosulfan to control *Helicoverpa* (Fitt, 1994). A single application of BPU could be used on transgenic Bt cottons to prevent survival of resistant larvae. However, in view of previous experience involving serious contamination of produce with chlorfluazuron, an environmental fate study of this group of compounds is essential to minimize their environmental impact.

The most commonly used method for analysis of BPUs is high-performance liquid chromatography (HPLC) (Bogus et al., 1978; Sundaram and Nott, 1989; Hopkins and Laurel, 1990), but this method is expensive and laborious. Rapid monitoring of large numbers of samples for environmental fate studies would benefit from a more cost-effective analytical method. The enzyme-linked immunosorbent assay (ELISA) method offers such a procedure, because it can be very sensitive, often needing little or no sample cleanup and preparation, and applied to numerous samples simultaneously (Gee et al., 1995; Lee et al., 1997; Skerritt, 1995).

The production of antisera that enabled the detection of three members of the BPU family, namely, diflubenzuron, BAY SIR 8514, and penfluron, in immunoassays has been reported (Wie and Hammock, 1982, 1984). The immunoassays were based on antibodies raised to an *N*-carboxypropyl hapten of diflubenzuron and a diflubenzuron phenylacetate derivative. Wie and Hammock (1982, 1984) investigated the sensitivity and specificity of the assays by using different BPU derivatives as immunogens as well as coating antigens and reported that antibodies raised against an *N*-carboxypropyl hapten of diflubenzuron yielded a specific assay for diflubenzuron, while the anti-diflubenzuron phenylacetate antiserum could detect each of these three compounds with IC_{50} values of 4 ppb for diflubenzuron, 7 ppb for penfluron, and 10 ppb for BAY SIR 8514, respectively. They did not test other BPUs with more complicated structures, such as chlorfluazuron and flufenoxuron. The question arises as to whether the extra aromatic ring or extra chloro substituents in the ring would affect the sensitivity and specificity of the assays. The structural similarity of these five BPU derivatives shown in Figure 1 is not as close as for the three BPUs tested in Wie and Hammock's papers (1982, 1984). This study describes the development of a

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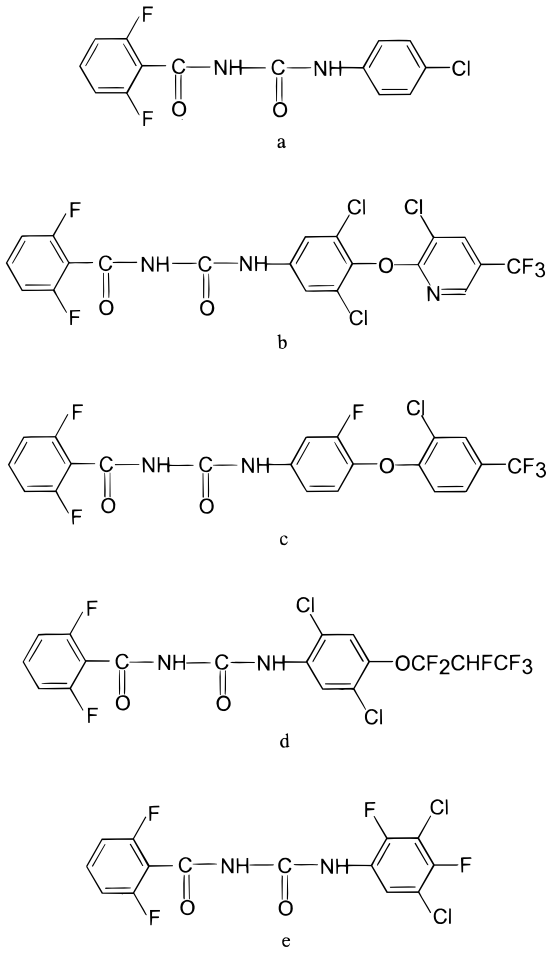

Figure 1. Chemical structures of BPUs: (a) diflubenzuron; (b) chlorfluazuron; (c) flufenoxuron; (d) lufenuron; (e) teflubenzuron.

competitive ELISA for five benzoylphenylureas, namely, diflubenzuron, chlorfluazuron, flufenoxuron, lufenuron, and teflubenzuron, and provides an explanation of observed results using molecular modeling.

MATERIALS AND METHODS

Materials. 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, Rockford, IL. Fish skin gelatin, Tween 20, and Freund's complete and incomplete adjuvants were purchased from Sigma, St. Louis, MO. Protein G agarose was purchased from Pharmacia, Uppsala, Sweden. Maxisorp polystyrene 96-well microwells were from Nunc, Roskilde, Denmark. All organic chemicals used for hapten synthesis were purchased from Aldrich, Milwaukee, WI. Silica gel H was purchased from Merck, Darmstadt, Germany. Analytical standard diflubenzuron was kindly provided by Uniroyal Chemical Co., Middlebery, CT. Analytical 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 Co., Princeton, NJ.

Instrumentation. ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were recorded on a Varian Gemini 300 instrument (300 MHz). Unless stated otherwise, $CDCl_3$ was used as solvent with tetramethysilane (TMS) as internal reference. Mass spectral data refer 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. R_f values refer to thin-layer chromatography (TLC) on Merck silica gel 60 F_{254} precoated plates with visualization under exposure to either UV light or iodine vapor. Immunoassay absorbances were read with a Stat Fax 2100 microplate reader in dual-wavelength mode (450−630 nm).

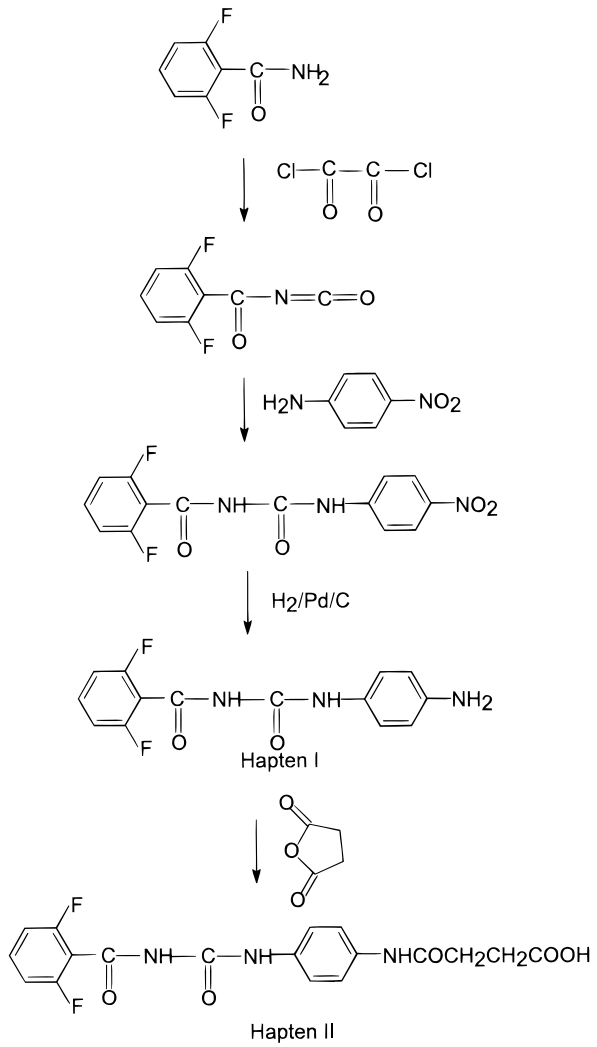

Figure 2. Reaction scheme for the synthesis of haptens I and II.

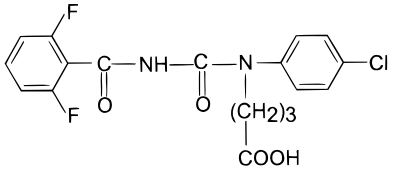

Figure 3. Chemical structure of hapten III.

Hapten Synthesis. Three haptens were synthesized for the production of BPU antibodies and used in the enzyme conjugate. Hapten I was prepared according to a method similar to that used for the preparation of arylureas by Wellingas et al. (1973). Hapten II was prepared by N-acylating the aniline amino group of hapten I with succinic anhydride to provide a four-carbon hemisuccinate spacer arm (Figure 2). Hapten III (Figure 3) was prepared using the same method as described by Wie et al. (1982).

2,6-Difluorobenzoyl Isocyanate. To a stirred suspension of 11.8 g (75 mmol) of 2,6-diflurobenzamide in 65 mL of dry 1,2-dichloroethane was added 9.5 mL of freshly distilled oxalyl chloride. After the initial exothermic reaction subsided, the reaction mixture was refluxed under N_2 overnight. The solvent was then removed by distillation at reduced pressure, and the remaining syrup was distilled under vacuum to give 11.6 g of 2,6-difluorobenzoyl isocyanate as an oil in 87% yield:

bp 54 °C at 0.05 mmHg (bp[lit] 62−63 °C at 0.4 mmHg, Wie et al., 1982); ^{1}H NMR ($CDCl_3$) δ 7.01 (t, J = 8.3 Hz, 2H), 7.48−7.58 (m, 1H).

1-(4-Nitrophenyl)-3-(2,6-difluorobenzoyl)urea. A solution of 4.96 g (27.1 mmol) of 2,6-difluorobenzoyl isocyanate in 15 mL of dry benzene was added to a stirred solution of 3.36 g (24.3 mmol) of 4-nitroaniline in 50 mL of dry benzene. The mixture was stirred overnight at room temperature, the solvent was removed by evaporation under reduced pressure, and the residue was recrystallized from ethyl acetate to give yellow crystals in 88% yield: mp 256−258 °C (mp[lit] 260 °C, Yu and Kuhr, 1976); TLC R_f 0.49 (ethyl acetate/toluene 1:2); ^{1}H NMR ($CDCl_3$) δ 7.02 (t, J = 8.3 Hz, 2H, aromatic), 7.44−7.55 (m, 1H, aromatic), 7.78 (d, J = 9.2 Hz, 2H, aromatic), 8.23 (d, J = 9.2 Hz, 2H, aromatic), 10.95 (s, 1H, NH), 11.14 (s, 1H, NH).

1-(4-Aminophenyl)-3-(2,6-difluorobenzoyl)urea (Hapten I). A solution of 5 g of 1-(4-nitrophenyl)-3-(2,6-difluorobenzoyl)urea (15.6 mmol) in 85 mL of 1-methyl-2-pyrrolidone was reduced with hydrogen over 10% palladium on activated carbon (0.5 g) under 1 atm at room temperature. No more hydrogen was absorbed after overnight treatment. The reaction mixture was then filtered through Celite, and 1-(4-nitrophenyl)-3-(2,6-difluorobenzoyl)urea was precipitated as a yellow powder by adding water. The pure product was obtained by recrystallization from ethyl acetate (3.9 g, 86%): mp 210−212 °C (dec); found: C, 57.81%, H, 3.92%, N, 13.81%; requires: C, 57.73%, H, 3.81%, N, 14.43%; TLC R_f 0.74 (ethyl acetate); ^{1}H NMR (acetone-d_6) δ 6.67 (d, J = 8.8 Hz, 2H, aniline), 7.17 (t, J = 8.3 Hz, 2H, aromatic), 7.31 (d, J = 8.8 Hz, 2H, aniline), 7.59−7.69 (m, 1H, aromatic); ^{13}C NMR (acetone-d_6) δ 112.82 (dd, J = 3.1, 22.5 Hz, C_3' and C_5'), 114.51 (t, J = 21.6 Hz, C_1'), 122.47 (s, C_2 and C_6 or C_3 and C_5), 122.60 (s, C_3 and C_5 or C_2 and C_6), 128.13, (s, C_1 or C_4), 134.02 (t, J = 10.1 Hz, C_4'), 146.24 (s, C_4 or C_1), 150.59, (s, NHCONH), 160.34 (dd, J = 6.8, 250.9 Hz, C_2' and C_6'), 163.09 (s, aromatic CONH); mass spectrum, *m/e* 292 (M + 1, 88%), 158 (100), 141 (6), 135 (71), 107 (5).

Hemisuccinate of Hapten I (Hapten II). Seven hundred milligrams of 1-(4-aminophenyl)-3-(2,6-difluorobenzoyl)urea (2.4 mmol) and 720 mg of succinic anhydride (7.2 mmol) in pyridine (25 mL) were refluxed for 3 h. The pyridine was evaporated under reduced pressure, and the resulting black oil was stirred with 30 mL of ethyl acetate. The precipitated solid was recrystallized from ethyl acetate several times to give hapten II as white needles (197 mg, 21%): mp 278−280 °C (dec); found: C, 55.38%, H, 3.81%, N, 10.53%; requires: C, 55.25%, H, 3.86%, N, 10.47%); TLC R_f 0.73 (ethyl acetate/methanol/acetic acid 10:1:0.1); ^{1}H NMR (methanol-d_4) δ 2.65 (s, 4H, CH_2CH_2), 7.12 (t, J = 8.2 Hz, 2H, aromatic), 7.49 (d, J = 9.2 Hz, 2H, aniline), 7.55 (d, J = 9.2 Hz, 2H, aniline), 7.48−7.59 (m, 1H, aromatic); ^{13}C NMR (methanol-d_4) δ 30.37, 32.35 (s, CH_2CH_2), 113.03 (dd, J = 3.1, 22.4 Hz, C_3' and C_5'), 114.64 (t, J = 20.5 Hz, C_1'), 121.75 (s, C_2 and C_6 or C_3 and C_5), 122.08 (s, C_3 and C_5 or C_2 and C_6), 134.35 (t, J = 10.3 Hz, C_4'), 134.38 (s, C_1 or C_4), 136.61 (s, C_4 or C_1), 152.17 (s, NHCONH), 160.84 (dd, J = 6.5, 251.8 Hz, C_2' and C_6'), 164.15 (s, aromatic CONH), 172.71 (s, NH*C*OCH$_2$), 176.27 (s, COOH); mass spectrum, *m/e* 392 (M + 1, 68%), 374 (23), 292 (32), 184 (100), 158 (62), 141 (52).

Preparation of Active Ester of Acid Derivative. One hundred and thirty-one milligrams of hapten II (0.33 mmol) and 42.3 mg of *N*-hydroxysuccinimide (NHS; 36.7 mmol) were dissolved in 4 mL of freshly distilled tetrahydrofuran (THF). To this solution was added 75.8 mg of *N,N*-dicyclohexylcarbodiimide (DCC; 36.7 mmol) with stirring. The reaction was stirred at room temperature under N_2 for 3.5 h. The precipitate was filtered off and washed with THF, and the combined THF filtrates were evaporated under reduced pressure. The resulting white solid residue was purified by flash chromatography (ethyl acetate/acetone 2:1) to yield a white solid. The structure was confirmed by its ^{1}H NMR spectrum with succinimide protons at δ 2.78 ($CDCl_3$) and mass spectrum *m/e* 489 (M + 1, 4%), 391 (7), 374 (100), 348 (8), 331 (55), 315 (14).

The preparation of hapten III with handle attachment at the middle nitrogen was carried out according to the method of Wie et al. (1982). Briefly, 4-chloroaniline was derivatized by ethyl 4-bromobutyrate, followed by reaction with 2,6-difluorobenzoyl isocyanate. The resultant ethyl ester was hydrolyzed to provide hapten III. The active ester synthesis of this hapten was similar to that above for hapten II.

Preparation of Immunogens. Three haptens were conjugated to ovalbumin (OA) and keyhole limpet hemocyanin (KLH). For hapten I, the diazo conjugate method (Sheth and Sporns, 1991) was used with some modifications. Briefly, hapten I (15.5 mg) dissolved in dry dimethylformamide (DMF) was added to 2 mL of 3.5 N HCl, followed by 0.8 mL of 1% sodium nitrite. After 10 min of stirring at 0 °C, 39.2 mg of ammonium sulfate was added, and the reaction was stirred for another 10 min at 0 °C. The diazonium salt was then reacted with proteins (OA and KLH) in PBS buffer. The pH of the reaction was adjusted to 9, and the reaction mixture was kept stirring at 4 °C overnight and then dialyzed extensively in phosphate-buffered saline (PBS, 50 mM sodium phosphate−0.9% NaCl, pH 7.2) containing 0.01% sodium azide and stored at 4 °C. For linking hapten I to OA through two homolinkers 1,4-butanediol diglycidyl ether (BDE) and disuccinimidyl suberate (DSS), the methods were adapted from previously reports (Lommen et al., 1995; Paek et al., 1993). For BDE, 50 mg of BDE was added to 20 mg of protein in 1 mL of 50 mM sodium carbonate buffer, pH 9.6, and the solution was mixed overnight at room temperature. The solution was then desalted on a PD-10 column (Pharmacia, Uppsala, Sweden), and 10 mg of hapten I in dry dimethyl sulfoxide (DMSO) was added. After mixing overnight at room temperature, the mixture was dialyzed against PBS extensively. For DSS, the hapten solution (10 mg/mL in dry DMSO) was added to the cross-linker solution (20 mg/mL in dry DMSO) such that there was a 2 molar excess of cross-linker. This mixture was stirred for 30 min at room temperature. The mixture was then added to 10 mg of protein in 2 mL of 10 mM phosphate buffer at pH 7, and a 20 molar excess of hapten was used. After incubation for 2 h at room temperature, the mixture was dialyzed against PBS as described above. The conjugation method for the NHS activated ester of haptens II and III was adapted from that of McAdam et al. (1992). The active ester dissolved in dry DMF was slowly added to a precooled buffer solution (50 mM K_2HPO_4, pH 9.1) containing the protein, and the reaction solution was gently mixed by hand and then left at 4 °C overnight. The next day the conjugates were transferred to dialysis tubing and dialyzed extensively. The concentration of the coupled protein was determined using the Coomassie Brilliant Blue G-250 dye-binding assay method (Bradford, 1976).

Preparation of Enzyme Tracers. HRP was used for the preparation of enzyme conjugates. The methods were similar to those described for the conjugation of hapten to the protein, except that the diazo method was not chosen for conjugating hapten I to the enzyme. 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).

Antibody Production. Female New Zealand white rabbits were immunized by intradermal and intramuscular injections of haptens conjugated to KLH and OA. The initial immunizing dose consisted of 1 mg of hapten−protein conjugate in 0.5 mL of 0.9% NaCl (saline) and 0.5 mL of Freund's complete emulsion. Subsequent booster injections (0.5 mg of conjugate in 0.5 mL of saline−0.5 mL of Freund's incomplete emulsion) were performed 2, 4, and 6 weeks later and then at monthly intervals. Blood was collected from the marginal ear vein 9 days after each monthly injection. Antiserum was purified using affinity chromatography on protein G−Sepharose (Akerstrom et al., 1985). The concentration of the purified antibody was determined by Beer−Lambert's law using the extinction coefficient of 1.35 for 1 mg/mL rabbit IgG and then stored at 4 °C after 0.1% (w/v) sodium azide was added. 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. The microwell plates were coated with purified anti-BPU IgG at 1 μg per well in 100 μL

of 50 mM carbonate buffer, pH 9.6, overnight at room temperature. Plates were then washed three times with PBST washing solution [PBS with 0.05% (v/v) Tween 20)], and unbound active sites were blocked with 150 μL of 1% BSA/PBS per well for 1 h. After the plate was blotted dry, 50 μL of pesticide standard in 0.1% (w/v) fish gelatin in PBS solution containing 5% methanol followed by 50 μL of enzyme conjugate solution diluted in the same diluent was added to each well and incubated for 1 h. Following washing (five times) with washing solution, 150 μL of substrate solution (1.25 mM 3,3′,5,5′-tetramethylbenzidine−1.6 mM hydrogen peroxide in acetate buffer, pH 5.0) was added to each well. The reaction was stopped after 30 min at room temperature by adding 50 μL of 2.5 N H_2SO_4, and absorbances were recorded in the microplate reader.

Soil and Water Analysis. The soil used in this study was collected from a cotton field located near Narrabri, in the northwest of New South Wales, Australia. This soil was a self-mulching, gray cracking clay (vertisol) and had no applications of BPU insecticides previously. For a spiking study, 10 g of air-dried soil in a glass jar was spiked with a known concentration of one of the five BPUs dissolved in methanol. The soil was mixed thoroughly and then allowed to stand at room temperature for 48 h to allow the methanol to evaporate and BPUs to equilibrate with the soil colloids. After incubation, soil was shaken with 20 mL of 90% methanol for 3 h on an orbital shaker and then allowed to stand overnight. The supernatant was then diluted 1:10 with 0.1% FG-PBS buffer. Runoff water from an irrigation on a cotton field (pH 7.4) with no history of BPU application was used to evaluate potential matrix interference. The field water samples were fortified with several concentrations of individual BPUs and analyzed by ELISA without any further treatment.

Molecular Modeling. Minimum energy conformations of the five BPUs and hapten II were performed using Spartan 5.0 software (Wavefunction, Irvine, CA) running on an O_2 SiliconGraphics workstation under Irix 6.3. The stereochemical conformation of the urea structure, common to all compounds, was based on the crystal structure of diflubenzuron (Cruse, 1978). In deriving least energy molecular models, molecules were initially set up as approximately planar, and minimization was performed using an AM1 semiempirical method; the results were displayed with electron probability density set at 0.002 electron/Å^3. Electron density on the surface was illustrated in seven steps. PowerFit software (MicroSimulations, Mahwah, NJ) was used to compare hapten II with each target compound. The fitting was carried out using the automatic atom-based rigid fitting process. It involved superimposing substituent atoms that may be exposed for interaction with antibody: the hydrogens and substituent fluorines or other atoms on the two phenyl rings and two hydrogens and two oxygens from the urea moiety.

RESULTS AND DISCUSSION

Hapten Selection and Conjugation. Since antibodies are thought to best recognize the part of the hapten that is most distant from the conjugate linkage, a linking group opposite from the most characteristic groups of the molecule was desirable (Burrin and Newman, 1991). In the case of BPU insecticides, we attempted to exploit this principle to produce polyclonal antibodies designed to detect all five members of this group of pesticides. Compounds a−e in Figure 1 are each 1-(2,6-difluorobenzoyl)-3-(substituted phenyl)ureas, containing a common aromatic ring substituted with fluoro atoms in the 2- and 6-positions. This provided an opportunity to make a derivative in which the common benzoylurea group is preserved by coupling onto the distal aniline. This approach could theoretically provide a broad-specificity assay for all five of these BPUs. It is known from previous work (Wie and Hammock, 1984) that a hapten connecting the protein through an acetic acid replacing the chloro of diflubenzuron resulted in assays which could recognize BAY SIR 8514 and penfluron with comparable sensitivities. We decided to adopt a related strategy that included an amide linkage for reasons of simplicity and to produce longer spacer arms.

Usually the optimal linking group is a straight chain of about four to six atoms (Franek, 1987; Hill et al., 1993; Newsome et al., 1993; Yeung and Newsome, 1995). Hapten I was relatively easily made using 4-nitroaniline, followed by reduction (Figure 2). This hapten provides a short linkage to the protein by using the diazonium method and 10- and 8-carbon spacer arms by linking through BDE and DSS, respectively. The acylation of the amino hapten with succinic anhydride can also provided a 4-carbon succinyl spacer with a terminal carboxylic group for coupling to the protein. Whereas N-acylation of hapten I with succinic anhydride produced hapten II with a 4-carbon spacer arm, all attempts at making an active ester in DMF failed. The active ester may have undergone intramolecular acylation, as TLC and ^{1}H NMR evidence suggested that the cyclic succinimide derivative rather than active ester had been formed. DMF is usually used as solvent to make active esters of carboxylic acid haptens. This phenomenon has also been observed by other researchers (Tatake et al., 1991; Roseman et al., 1992). Fortunately, this problem was resolved by using the less polar THF.

The active ester of hapten II was used for linking to the protein and the peroxide enzyme. However, coupling with diazonium salts often leads to a considerable loss of enzyme activity (McAdam et al., 1992) becasue histidine/tyrosine residues are present near or in the active site of most enzymes (Averamaes et al., 1978). The diazonium method was therefore not chosen for conjugating hapten I to enzyme HRP; instead, two bifunctional linkers were used to provide enzyme conjugates.

Assay Performance. Enzyme conjugate dilution factors were determined in preliminary experiments as those yielding an absorbance of 0.7−1.2 units under the conditions described under Materials and Methods. All antibodies were checked with each enzyme conjugate to determine the sensitivity of individual antibody−enzyme conjugate combinations for the target compounds using the lowest conjugate dilution. The resulting IC_{50} (calculated as the concentration of BPU giving 50% inhibition of color development for each combination) is shown in Table 1.

In most cases, using the same enzyme conjugate, antibodies to the hapten II−protein conjugate provided higher detection sensitivities for the target compounds than antibodies from hapten I−protein antigens. However, even higher antibody sensitivities for the target compounds were not achieved by extending the hapten further out from the protein by using the DSS and BDE cross linkers. This result suggests the presence of the appropriate spacer could enhance the production of high-affinity antibodies. Antibodies raised to hapten−protein conjugates linked through the 4-aniline position provided much higher cross-reactivity toward the targets than the antibodies to the antigens linked through the middle nitrogen of the hapten. One possible explanation would be the greater change of dihedral angles of urea with the planes of the adjacent rings due to the coupling through the middle nitrogen group, as deter-

Table 1. IC_{50} Values (Parts per Billion) for Five BPUs Using Different Immunogen and Enzyme Conjugate Combinations

		immunogens							
HRP conjugate	target	hapten I−OA	hapten I−KLH	hapten I−DSS−OA	hapten I−BDE−OA	hapten II−OA	hapten II−KLH	hapten III−OA	hapten III−KLH
hapten I−BDE−HRP	diflubenzuron	14 ± 2.1	5 ± 0.4	180 ± 40	165 ± 55	4.1 ± 0.3	2.1 ± 0.2	−[a]	−
	chlorfluazuron	>1000	>1000	>1000	920 ± 110	120 ± 15	43 ± 13	−	−
	flufenoxuron	>1000	>1000	>1000	>1000	49 ± 6.5	43 ± 9.8	−	−
	lufenuron	>1000	>1000	>1000	850 ± 90	110 ± 20	69 ± 11	−	−
	teflubenzuron	95 ± 17	67 ± 8.7	830 ± 85	710 ± 95	27 ± 4.5	7.5 ± 2.1	−	−
hapten I−DSS−HRP	diflubenzuron	8.9 ± 1.5	2.0 ± 0.3	130 ± 31	150 ± 45	0.6 ± 0.2	1.4 ± 0.4	−	−
	chlorfluazuron	>1000	>1000	>1000	>1000	45 ± 12	41 ± 12	−	−
	flufenoxuron	>1000	110 ± 19	>1000	>1000	10 ± 2.8	19 ± 4.7	−	−
	lufenuron	510 ± 45	610 ± 65	>1000	>1000	31 ± 5	70 ± 14	−	−
	teflubenzuron	90 ± 11	39 ± 8	940 ± 105	960 ± 90	5.1 ± 1.5	7.1 ± 1.1	−	−
hapten II−HRP	diflubenzuron	72 ± 23	3.2 ± 0.7	170 ± 45	190 ± 35	3.1 ± 0.9	2.4 ± 0.8	−	−
	chlorfluazuron	>1000	50 ± 7.3	780 ± 75	>1000	51 ± 8.7	>1000	−	−
	flufenoxuron	>1000	25 ± 5.5	670 ± 70	>1000	12 ± 2.3	520 ± 25	−	−
	lufenuron	>1000	56 ± 16	>1000	>1000	28 ± 2.4	>1000	−	−
	teflubenzuron	110 ± 35	66 ± 23	560 ± 75	>1000	8.0 ± 1.3	66 ± 12	−	−
hapten III−HRP	diflubenzuron	41 ± 9.2	27 ± 2.4	150 ± 25	170 ± 35	17 ± 2	15 ± 3.1	640 ± 35	250 ± 25
	chlorfluazuron	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
	flufenoxuron	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
	lufenuron	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
	teflubenzuron	210 ± 40	160 ± 45	>1000	>1000	85 ± 15	71 ± 11	>1000	>1000

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

mined by molecular modeling studies. The low color development for combinations of antibodies raised to hapten III and enzyme tracers derived from the other two haptens demonstrated that the haptens I and II used for enzyme tracers may not have the necessary structural attributes for recognition by the antibodies raised to hapten III.

It was proposed that antibodies against such haptens could provide a broad-specificity assay for all five BPUs, but, from the results listed in Table 1, detection of the larger BPUs was less sensitive. Generally, the most sensitive assay for all five BPUs was obtained when using hapten II−OA for immunization and hapten I−DSS−HRP as enzyme tracer. Therefore, this combination was chosen for more detailed work, and all following results presented in this paper are based on this combination. Standard curves for all five BPUs with this combination are shown in Figure 4. The limit of detection (LOD) of an assay was reported to be calculated as the compound concentration causing 10% inhibition of color development (Midgely et al., 1969). Using this criterion, the LODs for the five BPUs were 0.05 ppb for diflubenzuron, 2.3 ppb for chlorfluazuron, 0.7 ppb for flufenoxuron, 1.2 ppb for lufenuron, and 0.2 ppb for teflubenzuron.

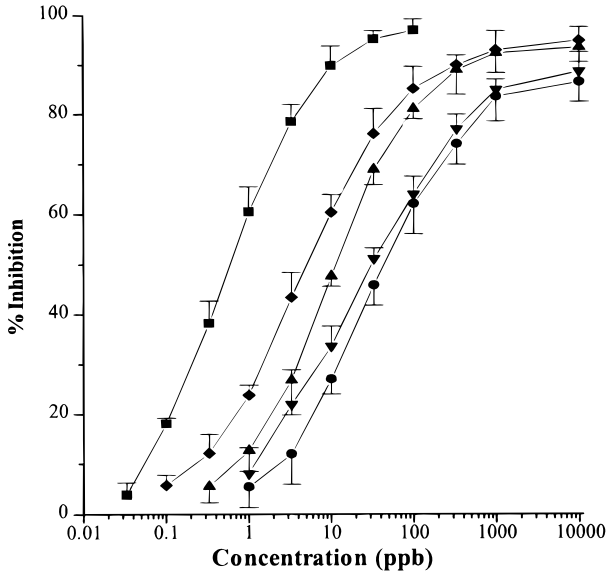

Figure 4. Standard curves for five BPUs using antibody to hapten II with hapten I−DSS−HRP conjugate: (■) diflubenzuron; (●) chlorfluazuron; (▲) flufenoxuron; (▼) lufenuron; (◆) teflubenzuron.

Assay Optimization. To test for solvent effects on the assay, methanol, acetone, acetonitrile, and DMSO were added to the assay buffer at four levels: serially diluted (a 2000 ppb standard was serially diluted to appropriate concentrations in borosilicate glass tubes), and 1, 5, and 10% final concentrations. Solvent effects were determined by comparing the standard curve prepared in the 0.1% FG-PBS-containing solvents at the above concentrations. Figure 5 shows that methanol (at 10%) did not alter the sensitivity of assay for the BPUs. For diflubenzuron, there was little change in the detection sensitivity among the four methanol concentrations. However, the detection sensitivities of the other four BPUs were significantly reduced, and slopes of the sample curves were changed at methanol concentrations <5%, perhaps due to the lower water solubilities of these four compounds. Similar results were also observed for the other three solvents. This result showed the necessity of using a water-miscible solvent in BPU ELISAs. The four solvents had limited effects on the sensitivity of the ELISA at 5% final concentration (Table 2). However, acetone and DMSO caused less color development. On the basis of these results and the fact that methanol has been reported to be a suitable solvent for extraction of diflubenzuron residues in cotton-growing soil (Bull and Ivie, 1978), methanol was chosen as the extraction solvent and was reduced to a concentration between 5 and 10% by dilution in the 0.1% FG-PBS buffer solution.

Because proteins (such as *Teleostean* fish skin gelatin) and detergents such as Tween 20 are commonly used in ELISA to reduce nonspecific interactions, their influence on assay performance was also examined. Four different enzyme conjugate diluents (PBS, PBS/T, 0.1% FG/PBS, and 0.5% FG/PBS) were tested for their effects. Assay sensitivities were reduced by the addition of

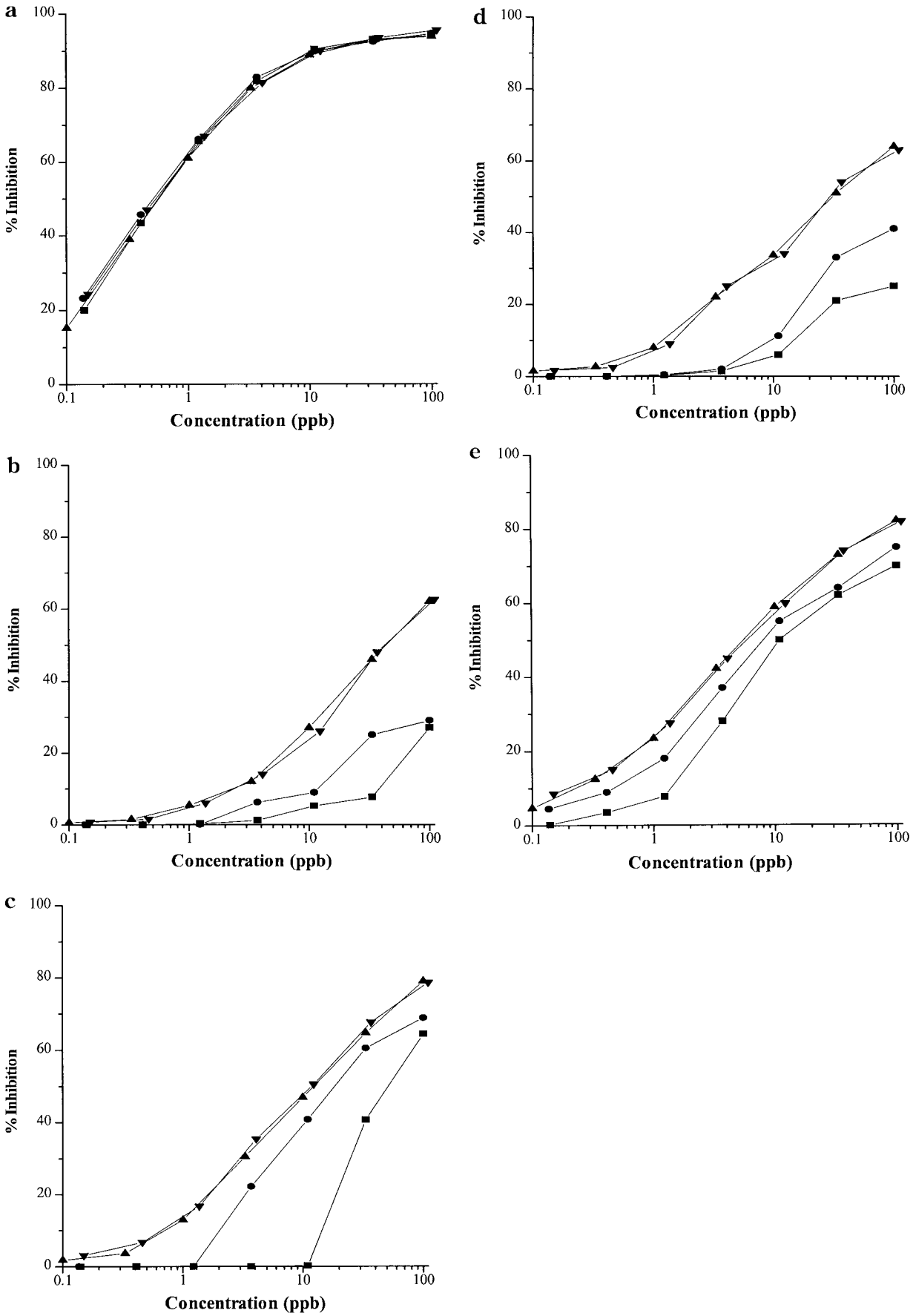

Figure 5. Effect of methanol on BPU ELISA standard curves: (a) diflubenzuron; (b) chlorfluazuron; (c) flufenoxuron; (d) lufenuron; (e) teflubenzuron. Methanol concentrations are (■) serially diluted and (●) 1%, (▲) 5% and (▼) 10% final.

Tween 20. There was little difference in assay sensitivities among the other three diluents, but 0.5% fish gelatin decreased color development and PBS alone caused a higher standard deviation between assays,

Table 2. Effect of Different Organic Solvents (5%) on the Performance of BPU ELISA

solvent	target	absorbance[a]	IC_{50} (ppb)
methanol	diflubenzuron	0.95	0.6
	chlorfluazuron	0.94	45
	flufenoxuron	0.96	10
	lufenuron	0.98	31
	teflubenzuron	0.94	5.1
acetone	diflubenzuron	0.74	0.8
	chlorfluazuron	0.78	51
	flufenoxuron	0.75	12
	lufenuron	0.69	35
	teflubenzuron	0.77	7.4
acetonitrile	diflubenzuron	0.88	0.8
	chlorfluazuron	0.89	50
	flufenoxuron	0.84	13
	lufenuron	0.86	37
	teflubenzuron	0.87	6.5
DMSO	diflubenzuron	0.53	0.9
	chlorfluazuron	0.52	53
	flufenoxuron	0.49	13
	lufenuron	0.55	36
	teflubenzuron	0.51	7.5

[a]Absorbance in the absence of analyte.

perhaps due to the adsorption of conjugates to walls of tubes and pipets.

Assay Specificity. Assay specificity was evaluated using 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 (4-chloroaniline, 2,6-difluorobenzamide, 2,6-difluorobenzoic acid), and some structurally dissimilar compounds that may reasonably be expected to be found in the Australian cotton production systems (bifenthrin, deltamethrin, DDE, DDT, endosulfan, and λ-cyhalothrin). The IC_{50} values for each of these compounds were >10 000 ppb, suggesting that the assay was very specific to the insect growth regulator BPUs. No cross-reaction with the structurally related aromatic urea herbicides was noted, which indicated that both the aromatic rings and the urea moiety in the target structure were critical for antibody binding.

Soil and Water Analysis. To study possible matrix interference from soil extracts and field water samples, standard curves were prepared in soil extracts, field water samples, and 0.1% FG-PBS buffers as control. Figure 6 shows the result obtained using diflubenzuron. The superimposition of the standard curves suggested that there were no significant matrix effects from field water and soil extract. There also were no noticeable differences between the corresponding standard curves in buffer, field water, and soil extract for the other four BPUs (data not shown). Spiking matrix samples with several amounts of analyte is a common practice to evaluate the reliability of the analytical assay. Hence, soil and water samples from cotton field were spiked at five concentrations of each of the five BPUs (Table 3). Good recoveries were obtained for soil and water samples.

Molecular Modeling. Modeling studies were performed to understand more clearly the specificities and hapten−conjugate preferences we observed. Figure 7 shows models of five BPUs and hapten II displayed as electrostatic potential mapped onto the electron density isosurface. The X-ray structure of crystalline diflubenzuron showed considerable nonplanarity of the urea group and the 2,6-difluorobenzene and 4-chlorobenzene

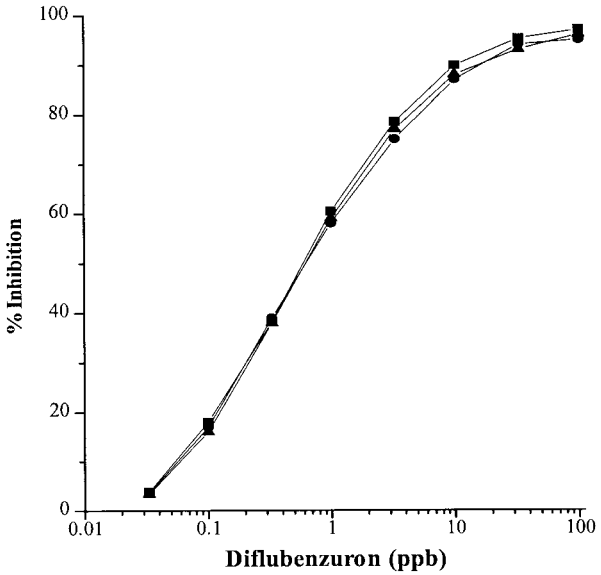

Figure 6. Standard curves for diflubenzuron in field water (■), soil extract (●), and 0.1% FG-PBS (▲).

Table 3. Recoveries for Soil and Water Spiked Studies

		recovery (%)				
matrix	spike level	diflubenz-uron	chlorflu-azuron	flufen-oxuron	lufen-uron	teflubenz-uron
soil extract	0.1 ppm	89	81	90	79	86
	0.5 ppm	87	79	83	84	84
	1 ppm	91	79	86	81	85
	5 ppm	93	87	84	89	85
	10 ppm	105	91	89	83	89
field water	5 ppb	86	86	104	82	102
	10 ppb	95	101	95	79	102
	20 ppb	110	95	105	91	95
	50 ppb	90	86	102	84	94
	100 ppb	110	87	89	92	93

groups (44.8 and −12.9°, respectively between the planes of the adjacent six-membered rings and the relevant nitrogen to carbonyl carbon bond); computer minimization changed these angles to about 50° and −25 to −35° (except flufenoxuron is 10°), respectively, for each of the five BPUs and hapten II.

The molecular fits of the five BPUs to hapten II are shown in Figure 8. The target compounds and hapten II superimposed well, especially with regard to the common phenylbenzoylurea groups. However, the ELISA results showed that the antibody developed against hapten II detected diflubenzuron more sensitively than the other four BPUs. On closer examination of the molecular models for the compounds shown in Figure 7, it was found, in the compounds with an oxygen-linked group on the aniline ring, chlorfluazuron, flufenoxuron, and lufenuron, that the oxygen held the alkyl or aryl group almost completely out of plane. In contrast, halogen substituents in diflubenzuron and teflubenzuron, as well as the nitrogen of the amide in the hapten II, were in the same plane as the adjacent aromatic ring. The relatively lower affinities of the antibody for chlorfluazuron, flufenoxuron, and lufenuron may thus be explained by this difference in geometry; that is, the nonplanar oxygen-linked group sterically hindered the recognition of these compounds by the antibodies.

Because the amide group in the 4-position of aniline ring in hapten II is a weakly electron-withdrawing group, antibody raised against this hapten may be more likely to bind compounds containing an electron-withdrawing group in the same position. Diflubenzuron

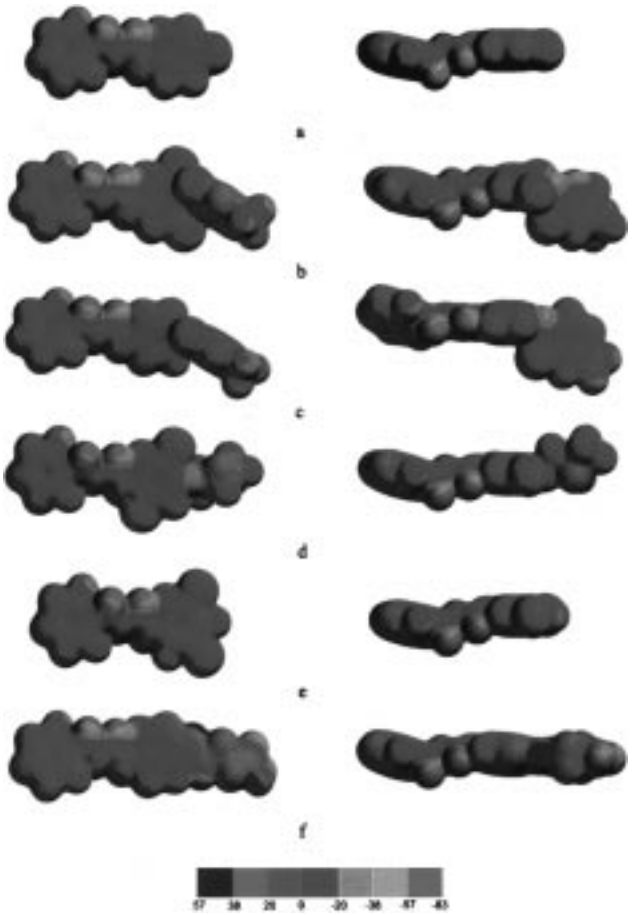

Figure 7. Molecular models of two views of the minimum energy conformation displayed with electrostatic potential isosurfaces for (a) diflubenzuron, (b) chlorfluazuron, (c) flufenoxuron, (d) lufenuron, (e) teflubenzuron, and (f) hapten II.

and teflubenzuron have 4-chloro and 4-fluoro groups. Alternatively, the oxygen in the other three BPUs is a electron-donating atom, shown by lighter shading of this atom in the electrostatic potential isosurfaces (Figure 7), and such strong negative portion may be less well recognized by such an antibody. This electronic effect may explain, or at least contribute to, the observed results. The relatively low antibody binding to teflubenzuron compared with diflubenzuron is possibly due to the two chlorines substituted in the aniline ring. The large size of the chlorines may sterically hinder the association of antibody with the target compound.

Conclusions. An ELISA method has been developed and optimized for the detection of each of the five chosen BPUs. Although the present assay is of relatively low sensitivity for the four larger BPUs compared to diflubenzuron, the IC_{50} values of this assay for all five targets are <50 ppb and the limits of detection are <3 ppb, which are sensitive enough to detect BPUs in soil at 0.1 ppm, enabling the assays to be used in a study of the dissipation of the BPUs in the soil environment. Cross-reaction and recovery studies also revealed that neither the environmental matrices nor other cotton agrochemicals would interfere with the detection of benzoylphenylureas.

Molecular modeling has produced 3-D models of the hapten and five BPUs, allowing an interpretation of why antibodies produced to diflubenzuron derivatives had lower cross-reaction for the other four BPUs. The nonplanarity of an oxygen substituent with the adjacent

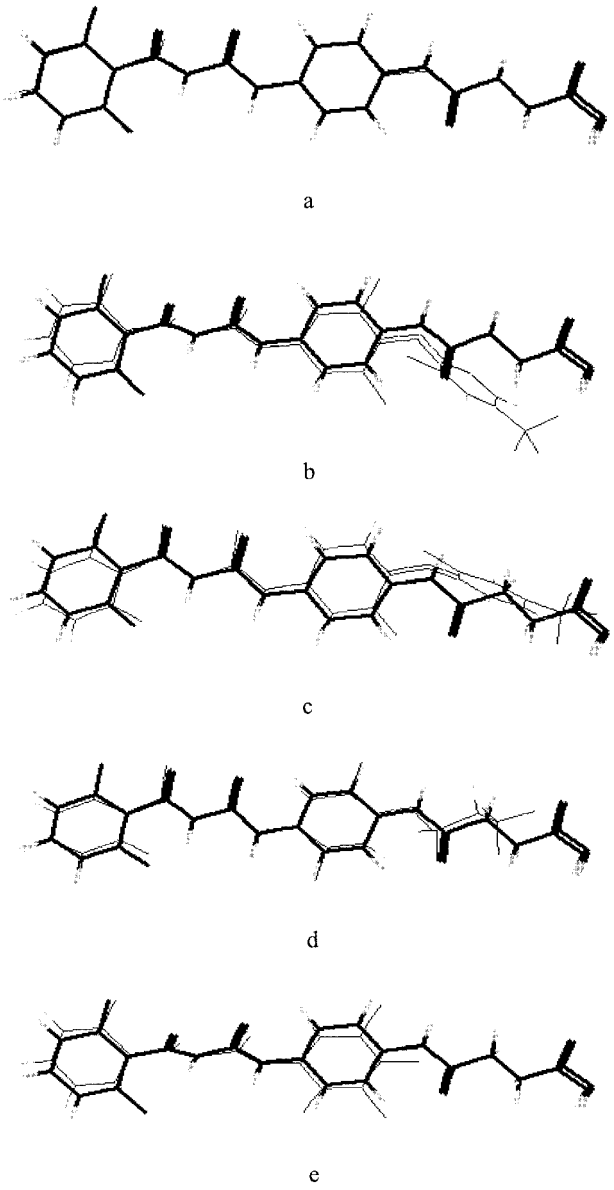

Figure 8. Molecular fits of hapten II (thick line) and BPUs (thin line): (a) diflubenzuron; (b) chlorfluazuron; (c) flufenoxuron; (d) lufenuron; (e) teflubenzuron.

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 characterics at the 4-position of the aniline ring in the BPU also may have some influence on antibody affinities. The lower reactivity with teflubenzuron compared to diflubenzuron indicates that the chlorine atoms may be necessary for antibody binding. The ELISA results showed that both aromatic rings and the urea moiety must be involved in antibody binding and that any change in the aniline ring which results in major variation of the electrostatic potential isosurface may have a significant effect on the binding activity.

ABBREVIATIONS USED

BDE, 1,4-butanediol diglycidyl ether; BPU, benzoylphenylurea; BSA, bovine serum albumin; DMF, *N*,*N*′-dimethylformamide; DMSO, dimethyl sulfoxide; DSS, disuccinimidyl suberate; ELISA, enzyme-linked immunosorbent assay; 0.1% FG-PBS, phosphate-buff-

ered 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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