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Synthesis of Hapten and Development of Immunoassay Based on Monoclonal Antibody for the Detection of Dufulin in Agricultural Samples

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ABSTRACT: An indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) was developed for the quantitative detection of the antiviral agent against tobacco mosaic virus (TMV). Hapten 6-[2-((methyllbenzothiazol-2-yl)-1-(2-ethoxy)-*O*, *O*-diethyl-α-aminophosphonate)acetamido)] hexanoic acid (DHS) was prepared from commercial chemicals and incorporated into the spacer arm through a carbon−carbon single bond. The prepared hapten was then coupled to carrier proteins keyhole limpet hemocyanin (KLH) to be used as an immunogen for monoclonal antibody (mAb) production together with ELISA development. This assay was further optimized by the assessment of the dependence of assay parameters on organic solvents, pH, and ionic strength. The IC_{50} values of the optimized assay for Dufulin and the calculated limit of detection in phosphate-buffered saline (PBS) were 9.6 ± 0.59 and 0.3 ± 0.05 ng/mL, respectively. Using the optimized assays Dufulin residues in soil and tobacco samples were determined with recovery values ranging from 81.5 to 95.3%, intra-assay variation ranging from 2.88 to 6.10%, and interassay variation ranging from 6.11 to 9.42%.

KEYWORDS: *Dufulin, ELISA, immunoassay, hapten synthesis*

INTRODUCTION

Phosphonates belong to an important class of compounds with diverse biological activities. Some of them have potential to act as enzyme inhibitors, antibiotics, and anticancer agents. They are extensively used as insecticides and herbicides because of their wide range of antiviral and antifungal properties.[1−5] Selected phosphonates also play significant roles in hapten design for antibody generation.[6]

Dufulin, an aminophosphonate compound, is a plant antiviral agent with a novel molecular structure (Figure 1) and good

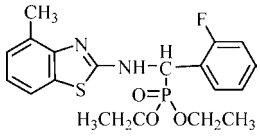

Figure 1. Structure of Dufulin.

antiviral activities against tobacco mosaic virus (TMV), cucumber mosaic virus, and potato virus Y.[7−10] Some studies have shown Dufulin is an immunoactivator of systemic acquired resistance (SAR) that confers antiviral activity to cells via regulation of the SA signaling pathway.[11] In recent years, Dufulin has been used widely to prevent and control tobacco and rice viral diseases and is the first antiplant viral agent to meet the environmentally friendly criteria defined in China.[12,13] It was granted registration as a new chemical entity with high anti-TMV activity by the Ministry of Agriculture of China.

However, limited methods have been reported for the analytical evaluation of Dufulin and α-aminophosphonates. Some liquid chromatographic techniques with diode array detection (DAD) have been used for the quantitative analysis and separation or semipreparation of α-aminophosphonates.[14−17] Chromatographic analyses provided sensitive and specific techniques. However, they usually required highly skilled personnel and expensive equipment. Moreover, these laborious sample pretreatment procedures involved numerous extraction steps that were time-consuming and were unsuitable for routine analysis of a large number of samples or on-site determinations. Compared with instrumental methods, immunoassays (IAs) are portable and cost-effective with adequate sensitivity, high selectivity, and simple sample extraction processes. Therefore, immunochemical techniques have become popular and increasingly considered as alternative or complementary methods for residue analysis.

Recently, monoclonal antibodies (mAbs) have been generated and a variety of immunoanalytical methodologies based on the ELISA technique have been developed for the determination of pesticide residues. To our knowledge, no antibody against Dufulin has been published so far and no immunoassay is available for this antivirus agent.

In this paper, we described a novel synthesis of a functionalized derivative of Dufulin and its conjugation to carrier proteins. Using this reagent, a high-affinity mAb against Dufulin was raised for the first time, and the specificity of this antibody was evaluated and the indirect competitive (ic)-ELISA was characterized. The application of the optimization IAs to the analysis of Dufulin residues in soil and tobacco samples was discussed.

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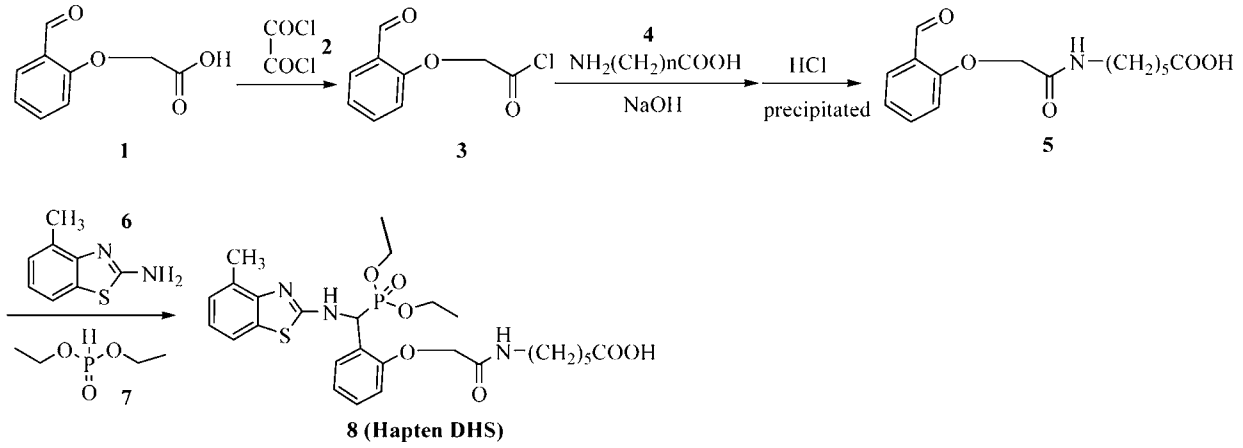

Figure 2. Synthetic route for the preparation of hapten.

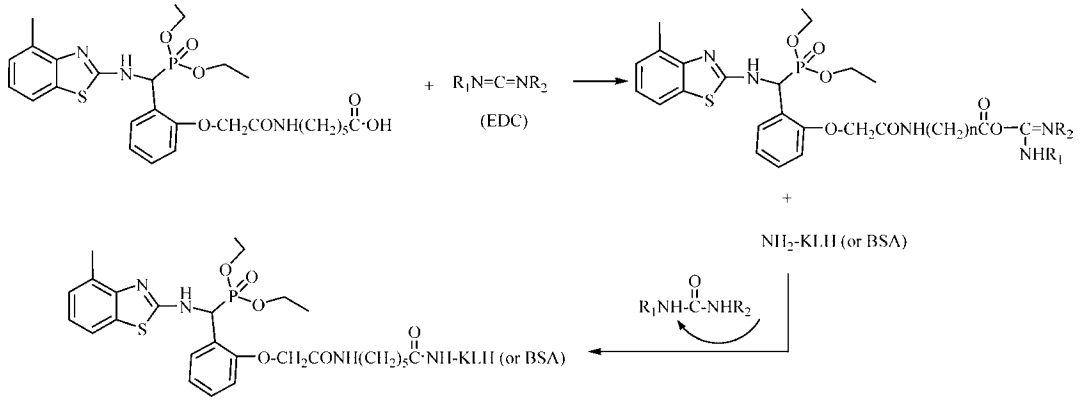

Figure 3. Synthetic route of antigen.

MATERIALS AND METHODS

Reagents and Chemicals. The analytical standard of 2-formylphenoxyacetic (CAS Registry No. 6280-80-4, MW 180.04) was obtained from Sigma-Aldrich. 6-Aminocaproic acid, 2-amino-4-methylbenzothiazole, and diethyl phosphite were purchased from Aladdin (Shanghai, China). All reagents and solvents used for the synthesis of haptens and for the activaction of their functions were supplied by commercial sources.

For conjugate and antibody purification, Sephadex G-25 Hitrap Desalting columns and Hitrap G HP columns from GE Healthcare (Sweden) were used, respectively. Horseradish peroxidase-labeled goat anti-mouse IgG (IgG–HRP) was obtained from Wuhan Boster Biotech Co. Ltd. (Wuhan, China). Keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), and Freund's complete and incomplete adjuvants were purchased from Sigma (St. Louis, MO, USA). *o*-Phenylenediamine (OPD) and other chemical reagents were purchased from Shanghai Chemical Reagents Co. (Shanghai, China). Hypoxanthine, aminopterin, and thymidine (HAT), hypoxanthine and thymidine (HT), Dulbecco's modified Eagle medium (DMEM), and polyethylene glycol (PEG 1450) were all obtained from Sigma-Aldrich (USA). Costar flat-bottom high-binding polystyrene ELISA plates were procured from Corning (New York, USA). Balb/c mice and myeloma cells SP2/0 were purchased from Youke Biotechnology Co. Ltd. (China), and an Immunopure Monoclonal Antibody Isotyping Kit I was prepared by Pierce (Rockford, IL, USA).

Instruments. Nuclear magnetic resonance (NMR) spectra were obtained on a JEOL ECX-500 (Japan) instrument operating at 500 MHz in the solvent DMSO-d_6. Chemical shifts were given relative to tetramethylsilane (TMS). Ultraviolet–visible (UV–vis) spectra were recorded on a spectrophotometer (Persee, China). Plate washing was carried out in an ELx405 microplate washer from BioTek Instruments (Winooski, VT, USA), and well absorbences were measured on a 680 plate reader (Bio-Rad, Hercules, CA, USA). Chromatographic experiments were performed on a high-performance liquid chromatograph–diode array detector (HPLC-DAD) Agilent 1100 instrument (USA).

Buffers and Solutions. The following buffers and solutions were used: 10 mmol/L phosphate-buffered saline (PBS, pH 7.4), PBS with 0.05% Tween-20 (PBST), coating buffer (50 mmol/L sodium carbonate buffer, pH 9.6), OPD, substrate solution (3.7 mmol/L *o*-phenylenediamine and 30% hydrogen peroxide in acetate buffer, pH 5.0), and stopping solution (2 mol/L H_2SO_4).

Hapten Synthesis. Hapten was synthesized following the route shown in Figure 2. A detailed description of each synthetic step as well as the complete spectroscopic and spectrometric characterization data of the main intermediate (**5**) and the hapten (**8**) are presented below.

*Synthesis of 2-Formylphenoxyacetic-6-aminocaproic Acid (**5**).* A mixture of 2-formylphenoxyacetic acid (**1**, 7.2 g, 40 mmol), oxalyl chloride (**2**, 10.08 g, 80 mmol), and DMF (100 μL) in dry dichloromethane was stirred for 14 h under nitrogen. The intermediate (**3**) was obtained after the removal of dichloromethane and oxalyl chloride. 6-Aminocaproic acid (**4**, 60 mmol) was dissolved in dichloromethane and sodium hydroxide (20 mL, 0.6 mol/L), and a solution of intermediate (**3**) in dichloromethane was added dropwise to the solution of 6-aminocaproic acid. The mixture was stirred on an ice bath for 0.5 h. The water layer was separated from the organic layer using a separation funnel and then transferred slowly into a hydrochloric acid solution. A white crude crystal was separated out from the solution and washed with water to yield pure 2-formylphenoxyacetic-6-aminocaproic acid (**5**, 72% yield): mp 85–87 °C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 12.02 (s, 1H, COOH), 10.46 (s, 1H, CHO), 8.17 (s, 1H, CONH), 7.73 (d, 1H, J = 7.5 Hz, Ph–H), 7.64 (t, 1H, J = 7.2 Hz, Ar–H), 7.12 (m, 2H, 2Ar–H), 4.66 (s, 2H, $ArOCH_2$–H), 3.15 (m, 2H, CH_2), 2.18 (t, 2H, J = 6.5 Hz, CH_2), 1.49

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(m, 4H, $2CH_2$), 1.20 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 190.2, 174.6, 166.8, 160.0, 136.4, 128.2, 124.7, 121.4, 113.7, 67.6, 38.7, 33.9, 29.3, 26.4, 24.8; IR (KBr, cm^{-1}) ν 761, 1244, 1645, 1720, 2941, 3408; ESI-MS, m/z 294.2 $[M + H]^+$, 316.2 $[M + Na]^+$, 332.1 $[M + K]^+$.

Synthesis of 6-[2-((Methylbenzothiazol-2-yl)-1-(2-ethoxy)-O,O-diethyl-α-aminophosphonate)acetamido)] Hexanoic Acid (8, Hapten DHS). *p*-Toluenesulfonic (0.5 mL) was added to a mixture of 2-formylphenoxyacetic-6-aminocaproic acid (**5**, 1.78 g, 10 mmol) and 2-amino-4-methylbenzothiazole (**6**, 1.968 g) in methylbenzene. The reaction mixture was refluxed for 6 h at 120 °C. Then, diethyl phosphate (**7**) was added and refluxed for 8 h again. The reactions were monitored by TLC and purified by column chromatography to afford a white crystal (**8**, 2.77 g, 48% yield): mp 148−151 °C; ^1H NMR (DMSO-d_6, 500 MHz) δ 12.02 (s, 1H, COOH), 8.33 (t, 1H, J = 6.5 Hz, CHN−H), 7.52−6.33 (m, 7H, Ph−H), 4.74 (s, 2H, CH_2), 3.02 (t, 2H, J = 4.2 Hz, CH_2), 2.20 (t, 2H, J = 4.4 Hz, CH_2); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 174.9, 167.9, 165.9, 155.0, 150.6, 130.7, 129.1, 129.0, 128.0, 126.8, 121.8, 121.5, 119.0, 117.4, 111.7, 67.4, 63.5, 63.1, 60.2, 38.4, 34.0, 29.0, 26.1, 24.6, 16.8, 16.7, 16.5; IR (KBr, cm^{-1}) ν 732, 1027, 1256, 1541, 1665, 2975, 3371; ESI-MS, m/z 578.2 $[M + H]^+$, 600.2 $[M + Na]^+$, 616.2 $[M + K]^+$.

Hapten−Carrier Protein Conjugation. For immunization purposes, hapten DHS was added to KLH using the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) as shown in Figure 3.[18] Conjugation reactions were set up as follows: hapten (50 mg) was dissolved in 1 mL of dimethyl sulfoxide (DMSO), and then DHS/DMSO (20 μL) was added dropwise to KLH (10 mg/mL, 0.1 mL) in conjugate buffer (0.2 M HEPES, pH 7.2); EDC (2 mg) was then added to the reaction and mixed immediately by gently pipetting the solution. Conjugations were allowed to proceed at room temperature for 2 h. A Sephadex G-25 with gel filtration was used to remove autocoupling products. The resulting solution was then dialyzed against 0.01 mol/L PBS at 4 °C for 24 h with three changes of PBS and stored frozen at −20 °C. Meanwhile, DHS−BSA conjugate was prepared in the same way and used as a coating conjugate for antibody titer detection.

Preparation of Monoclonal Antibodies. The immunization of BALB/c female mice was carried out according to the methods described in the literature.[19,20]

Immunization. Five female BALB/c mice (6 weeks old) were injected subcutaneously at multiple points with a DHS−KLH immunogen (60 μg in 0.2 mL of PBS, mixed with an equal volume of FCA to form an emulsion). Booster injections were given at 2 week intervals with the same amount of immunogen emulsified with incomplete Freund's adjuvant. After the fourth booster immunization, tail bleeding was carried out, and antiserum was determined before fusion. The mouse exhibiting the highest titer was sacrificed after the last immunization, and the spleens were removed for hybridoma production.[21]

Cell Fusion and Hybridoma Selection. SP2/0 murine myeloma cells were cultured in high-glucose DMEM supplemented with 20% fetal bovine serum and 1% penicillin−streptomycin. The splenocytes from the immunized mice were mixed with the myeloma cells at 4:1 ratio using 1.0 mL of PEG1450 as the fusing agent. The fused cells were plated in 96-well plates at a density of 1.5×10^5 cells/well per 100 μL of s-DMEM with 15% FBS. After 24 h of plating, 100 μL of HAT selection medium (s-DMEM supplemented with 100 μM hypoxanthine, 0.4 μM aminopterine, and 16 μM thymidine) with 20% FBS was added to each well. Half of the medium in the wells was replaced by fresh HAT medium every third day. When most of the nonfused cells disappeared, HAT was substituted by HT medium without aminopterin. The cultures in the plates were incubated at 37 °C with 5% CO_2. After about 10 days, hybridoma culture supernatants were screened for the presence of antibodies that recognized Dufulin by an ic-ELISA. Hybridoma supernatants from the cells that gave an absorbency of >2.0 were transferred to 24-well microculture plates in HT medium. Supernatants from 24-well culture plates were tested, and only those that exhibited inhibition by deltamethrin at 5.0 μg/mL were transferred to a cell culture bottle; supernatants from the cell culture bottle were tested again. Selected positive hybridoma cell lines were subsequently subcloned by limited dilution technique, and stable antibody-producing clones were expanded.

Production and Characterization of Monoclonal Antibody. *Large Amount Production and Purification of Monoclonal Antibodies.* A large amount of monoclonal antibody was produced in vitro.[22] Ascites fluid was produced in female BALB/c mice, which were preinjected with 0.5 mL of paraffin and then intraperitoneally injected with $(1-2) \times 10^6$ hybridoma cells 1 week later. Two weeks after the injection, the ascites fluid was harvested. Immunoglobulins (Ig) were purified from late stationary phase culture supernatants by ammonium sulfate precipitation and protein G affinity chromatography.

Affinity and Isotype. The affinity and specificity of the produced antibodies were again confirmed by competitive ELISA. The relative affinity of the monoclonal antibodies for Dufulin was measured by determining the 50% inhibition of control values (IC_{50}). The isotype of the purified monoclonal antibody was identified by a commercially available Immunopure Monoclonal Antibody Isotyping Kit I (Pierce). The purified antibodies were stored at −70 °C.

Indirect Competitive ELISAs. *ic-ELISA Protocol.* All incubations were carried out at 37 °C, and the plates were washed three times with PBST after each incubation, unless specified descriptions are given. For competition assays, the antibody and coating antigen concentrations were optimized by checkerboard titration. Standards were prepared with 5% methanol−PBS by serial dilutions from a stock solution in methanol. The ELISA was run as follows. The 96-well plate was coated with 100 μL of coating antigen (DHS−BSA) overnight at 4 °C and then washed three times. The excess binding sites were blocked with 300 μL/well of blocking buffer, followed by incubation for 2 h at 37 °C. After the plate was washed as described above, mAb was added (50 μL/well), followed by addition of buffer with or without competitor (Dufulin, 50 μL/well), and the plate was incubated for 1.5 h. After washing, goat anti-mouse IgG−HRP (100 μL/well) was added, followed by incubation for 0.5 h at 37 °C. Unbound goat anti-mouse IgG−HRP was removed by washing, and substrate solution was added (100 μL/well). After the plates were incubated for another 15 min at 37 °C, the chromogenic reaction was inhibited by adding stopping solution (50 μL/well), and absorbances were measured at 490 nm with a microplate reader (model 680; Bio-Rad). Absorbances were corrected by blank reading with preimmune serum used as a negative control. The result was expressed as percentage inhibition (B/B_0), where B is the absorbance of the well containing analyte and B_0 is the absorbance of the well without analyte. The inhibition curve was plotted as B/B_0 versus the logarithm of Dufulin concentration.

Competitive curves were obtained by plotting the relative absorbance of B/B_0 against the logarithm of the analyte concentration. Sigmoid competitive curves were fitted to a four-parameter logistic equation and the IC_{50} value was calculated.[23,24] The limit of detection (LOD) of the assay was defined as the concentration of analyte that provided a 10% reduction of the A_{max} (IC_{10}). The dynamic range of the assay was established between the values of IC_{80} and IC_{20}, and they were considered as the upper and lower limits of quantification (LOQ), respectively.

Assay Optimization and Characterization. Taking into account the low water solubility of Dufulin (0.04 g/L, 22 °C), we used organic solvent to improve the analyte solubility in assay medium. For the test, Dufulin standard solutions were prepared in various concentrations of methanol, acetone, DMF, or acetonitrile (10, 20, 40, and 60% in PBS, which become 5, 10, 20, and 30%, respectively, after combination with an equal volume of diluted antiserum). The effect of ionic strength of assay solution on ELSA performance was also researched using different concentrations of phosphonate ion in 5% methanol-PBS to dissolve Dufulin (40, 60, 100, 200, and 400 mmol/L NaCl, which become 20, 30, 50, 100, and 200 mmol/L, respectively, after combination with an equal volume of diluted antiser). The influence of pH (5.0, 6.0, 7.4, 9.6 and 10) of the assay solution was studied.

Antibody Specificity Determination. Cross-reactivity (CR) was tested to determine the specificity of the antibody. Some commercial

organophosphate pesticides (phoxim, parathion, fenthion, chlorpyrifos), original materials (2-amino-4-methylbenzothiazole, 2-fluorobenzaldehyde), and intermediate (2-fluorinephenyl-4-methylbenzothiazolimide) of Dufulin were selected and evaluated. Standard solutions of testing compounds were analyzed by the ELISA procedures. CR was expressed as the ratio of IC_{50} values for Dufulin to that for competitors and described by the following formula: CR (%) = ($IC_{50\ Dufulin}$/$IC_{50\ competitor}$) × 100.

Preparation of Spiked Samples. The procedure was the same as for the matrix effect study except that different volumes of standard solution were spiked to sample (spiked concentration of 0.01, 0.1, and 1.0 mg/kg) before the extraction; standards and samples were run in triplicate wells. Determination of spiked samples was performed by interpolating the mean absorbance values in the standard curve running on the same plate.

HPLC-DAD Analysis of Dufulin. To evaluate the accuracy of the ELISA, a high-performance liquid chromatography (HPLC) method was also performed. An 1100 (Agilent, USA) HPLC with DAD detector was used for instrumental analysis of Dufulin using an Eclipse XDB-C18 (4.6 × 250 mm, 5 μm) obtained from Agilent. The λ_{max} is 254 nm and mobile phase (CH_3OH/H_2O = 7:3, v/v) with 1.0 mL/min.

Statistical Analysis. All statistical analysis was performed with SPSS version 19. Data were analyzed by one-way ANOVA. Mean separations were performed using the least significant difference method. Each experiment was replicated three times, and all experiments yielded similar results. Measurements from all of the replicates were combined, and treatment effects were analyzed.

RESULTS AND DISCUSSION

Hapten Synthesis and Conjugate Preparation. Proper hapten design was essential for the antibody production and ELISA development. Dufulin, like most pesticides, is a small and simple organic molecule. It is nonimmunogenic by itself and lacks a functional group for coupling to proteins. Therefore, the primary goal of this study was to synthesize a hapten to yield a specific immunoassay for Dufulin. At the same time, a suitable hapten and its artificial antigen for immunization should preserve the structure of the target compound as much as possible. Furthermore, the analyzed hapten bore a spacer (carboxyl, amino, or hydroxyl group) to couple with the carrier protein. Both the spacer length and connection position at the hapten molecule were important to the production of a successful antibody. With respect to the spacer arm, it was generally accepted that medium-sized aliphatic linkers (three to six carbon atoms) are appropriate for an optimum exposure of the conjugated hapten to the immune system.[25]

It can be seen from the structure of Dufulin that three parts can be distinguished as the benzene ring and its substituents, the benzothiazole ring, and phosphonate. As shown in Figure 2, the preparation of hapten DHS (**8**) was completed by using readily available 2-formylphenoxyacetic acid (**1**) and oxalyl chloride (**2**) in a satisfactory way by treatment of **1** with **2** in dichloromethane at reflux with 2 drops of DMF. After removal of dichloromethane and oxalyl chloride, pure 2-formylphenoxyacetic acyl chloride (**3**) was obtained. Then the aliphatic spacer arm with six carbon atoms was reacted with the acyl chloride group in the presence of 6-aminocaproic acid dissolved in dichloromethane and sodium hydroxide, which provided a six carbon spacer arm with a terminal carboxylic group for coupling to the carrier protein. After the addition of hydrochloric acid solution into the water layer, an intermediate (**5**) was obtained with good yield (72%), and no recrystallization or column chromatography was required. Finally, hapten DHS (**8**) was prepared by reaction of 2-amino-4-methylbenzothiazole (**6**) with *p*-toluenesulfonic acid (**7**) by using the modified procedure developed earlier.[26]

As described by Kurtz et al.,[27] there were varieties of carrier proteins for immunogens and coating antigens, and they recommended the use of KLH as a first choice for immunization because of its good potent immunogen. After being purified by column chromatography, the carboxyl group of the DHS was covalently coupled with the amino group of the carrier protein. Hapten ratios for the KLH conjugates could not be measured due to the large size of KLH, so conditions were standardized using BSA as a model carrier protein. The prepared DHS−KLH conjugate product was examined by UV spectroscopy to confirm the coupling reaction. If conjugation occurred, the UV spectrum of the conjugate was different from that of the free protein (Figure 4). Therefore, the curve diagram of the conjugate that combined hapten and KLH presented successful conjugation.

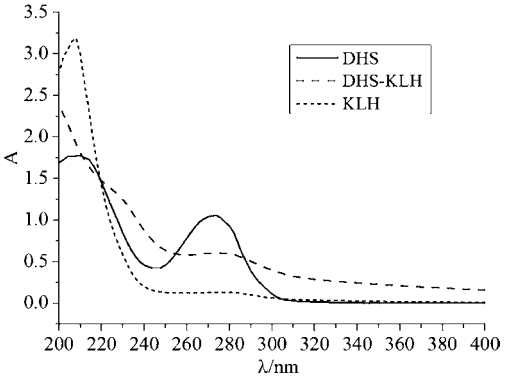


Figure 4. UV−vis sepectrum for the artificial antigen of DHS−KLH.

Production of Hybridomas and Cloning. Among the antisera collected from the mice previously injected, the one with the highest antibody titer was chosen for cell fusion. The supernatants from hybridoma cells were detected by ic-ELISA for the presence of antibodies against Dufulin. In all of the wells tested, 35 wells gave an absorbency of >2.0 and then were transferred to 24-well microculture plates in HT medium. Among the 35 wells, 24 wells exhibiting inhibition by Dufulin at 5.0 μg/mL were transferred to a cell culture bottle. Three positive wells that exhibited high inhibition in the presence of 5.0 μg/mL Dufulin were chosen to be cloned by limiting dilution. Final clones were obtained from wells that had been seeded with 1 cell/well. Finally, after three clonings, we selected a stable hybridoma, 3A2, which was used to produce ascites. After purification by Protein G column, IC_{50} values were obtained and used to evaluate the sensitivity of the obtained antibody, and the mAb was of IgG1 isotype by enzyme immunoassay using an ImmunoPure Monoclonal Antibody Isotyping Kit I.

Analytical Parameters of ELISA. Dufulin is considered to be a hydrophobic compound because of its lower solubility in water. Organic solvents are often added to the running buffer to improve the solubility of the target analyte. The influence of most relevant organic solvents over selected immunoassay parameters was studied, as described in Table 1. Methanol, acetonitrile, acetone, and DMF were evaluated at concentrations ranging from 5 to 30%. In acetonitrile, acetone, and DMF trials, a clear decrease in maximum signal was observed

Table 1. Influence of Organic Solvent of the Assay Solution on ic-ELISA

variable		A_{max}[a]	IC_{50} (ng/mL)	A_{neg}[b]
methanol (%)	5	1.093	26.4	0.008
	10	1.129	28.2	0.012
	20	1.075	35.5	0.035
	30[c]	1.115		
acetone (%)	5	1.097	32.6	0.053
	10	1.102	40.3	0.078
	20[c]			
	30[c]			
DMF (%)	5	1.165	29.5	0.128
	10	1.132	38.2	0.084
	20	1.096	26.9	0.049
	30[c]			
acetonitrile (%)	5	1.075	30.8	0.064
	10	1.098	47.6	0.075
	20[c]			
	30[c]			

[a]Maximum absorbance (A_{max}). [b]Blank control (A_{neg}). Data are the means of triplicates. [c]Data fitting was impossible due to poor color development.

when increasing amounts of the organic solvents were added to the assay buffer. In the methanol trial, the lowest IC_{50} values were found at 5% methanol (26.4 ng/mL). In addition, a very high concentration of the organic solvents could affect and retard the interaction between antibody and antigen and may denature protein reagents such as antibodies. Methanol was selected as the most suitable organic solvent for the ELISA, and on the basis of the IC_{50} values for the standard curves, 5% methanol as the final concentration was used in the following experiments.

With regard to the effect of the ionic strength, immunoassay performance was investigated in medium at a concentration of NaCl ranging from 20 to 100 mmol/L. As shown in Table 2, it was observed that in the absence of NaCl the IC_{50} values ranged from 29.6 to 64.8 ng/mL. Table 2 presents the effect of the ion strength on ELISA characteristics at the competition step. Increasing the concentration of NaCl caused an initial improvement followed by a decline in assay sensitivity. The optimum concentration selected was 50 mmol/L, which showed the lowest IC_{50} value.

Table 2. Influence of Ionic Strength and pH of the Assay Solution on ic-ELISA

variable		A_{max}[a]	IC_{50} (ng/mL)	A_{neg}[b]
NaCl (mmol/L)	20	1.212	43.2	0.005
	30	1.209	37.5	0.016
	50	1.178	29.6	0.065
	100	1.103	31.5	0.041
	200	1.115	64.8	0.092
pH	5.0	0.975	32.3	0.102
	6.0	1.032	29.5	0.084
	7.4	1.027	27.8	0.027
	8.0	1.063	30.5	0.015
	9.6[c]			

[a]Maximum absorbance (A_{max}). [b]Blank control (A_{neg}). Data are the means of triplicates. [c]Data fitting was impossible due to poor color development.

Table 2 also presents the effect of the pH of the assay solution on ELISA. pH 7.4 was selected as the optimum for the assay.

The antibody and coating antigen concentrations (dilution of 1:320000 and 1.25 μg/mL, respectively) were optimized by checkerboard titration. Ninety-six-well plates were coated with 100 μL/well of a coating antigen in carbonate−bicarbonate buffer (50 mmol/L, pH 9.6) by overnight incubation at 4 °C. The plates were washed three times with PBST (10 mmol/L PBS containing 0.05% Tween 20, pH 7.4) and were blocked by incubation with 5% skim milk in PBS (300 μL/well) for 2 h. After another washing step, 50 μL/well of standard (serial dilutions of the analyte) or sample in 5% methanol−PBST (pH 7.4, 50 mmol/L, containing 0.05% Tween 20) was added, followed by 50 μL/well of a previously determined antiserum dilution. After incubation for 1 h, the plates were washed and 100 μL/well of a diluted (1/5000) goat anti-mouse IgG−HRP was added. The mixture was allowed to incubate for 1 h, and after another washing step, 100 μL/well of OPD solution was added. The reaction was stopped after an appropriate time by adding 50 μL of 2 mol/L H_2SO_4, and the absorbance was read at 490 nm. Competition curves were obtained by plotting absorbance against the logarithm of analyte concentration. Sigmoidal curves were fitted to a four-parameter logistic equation, from which IC_{50} values were obtained.

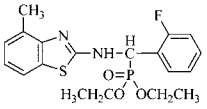
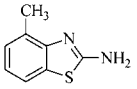
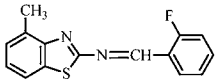
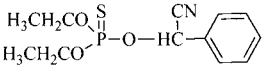
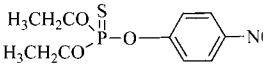
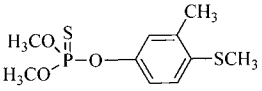
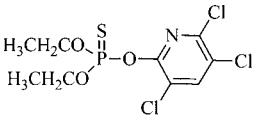
Cross-Reactivity (CR) Assay. A competitive standard curve for Dufulin was obtained using the optimized conditions. The curve was plotted with $\%(B/B_0)$ on the y-axis and log$C$ on the x-axis: $y = -26.56 \lg C + 76.10$, $R^2 = 0.9942$. The Dufulin assay had an IC_{50} of 9.6 ± 0.59 ng/mL, LOD (IC_{10}) was 0.3 ± 0.05 ng/mL, and the linear range was 0.3−308.1 ng/mL.

The specificity of the antibody for Dufulin was determined by testing some commercial organophosphate pesticides, original materials, and intermediate compounds of Dufulin, and the results are summarized in Table 3. It can be seen that most tested compounds showed low cross-reactivity (<0.01%). Only original material 2-amino-4-methylbenzothiazole and intermediate showed cross-reactivity with CR values of 1.52 and 11.1%, indicating that the presence of the benzothiazole ring enabled the antibody to recognize its own specific antigen, which confirms the suitability of the hapten structure for this particular chemical.

Analysis of Spiked Samples. Sample pretreatment procedures were performed as follows. Ten grams of sample was added to 30 mL of petroleum ether/ethyl acetate (50:50, v/v) as extracting solvent, and the mixture was blended by homogenization for 1 min. Then 5 g of anhydrous sodium sulfate was added, followed by 1 min of oscillation. After centrifugation (5 min, 5000 rpm), 3 mL of supernatant was transferred and evaporated at 40 °C under a nitrogen stream. The dry residue was dissolved into different volumes of PBST (containing 5% methanol). Finally, 50 μL portions were used for the selected ELISA test to investigate the matrix interference on the standard curve.

To study the matrix effect, tobacco and soil were chosen as samples and diluted several times with PBS, and then a Dufulin standard curve was prepared by mixing each diluted sample and the mAb in PBS for ELISA. Figure 5 shows the average interferences produced by 1/1, 1/2, 1/4, 1/10, and 1/20 diluted tobacco and soil samples over the developed ELISAs. As

Table 3. Cross-Reactivity of the mAb with Dufulin and Related Compounds

Analyte	Structure	IC_{50} (ng/mL)	CR (%)
Dufulin		9.616	100
2-Amino-4-methylbenzothiazole		630.9	1.52
2-Fluorobenzaldehyde		N/A[a]	<0.01
2-Florinephenyl-4-methylbenzothiazol-imide		86.3	11.1
Phoxim		N/A	<0.01
Parathion		N/A	<0.01
Fenthion		N/A	<0.01
Chlorpyrifos		N/A	<0.01

[a]Unavailable.

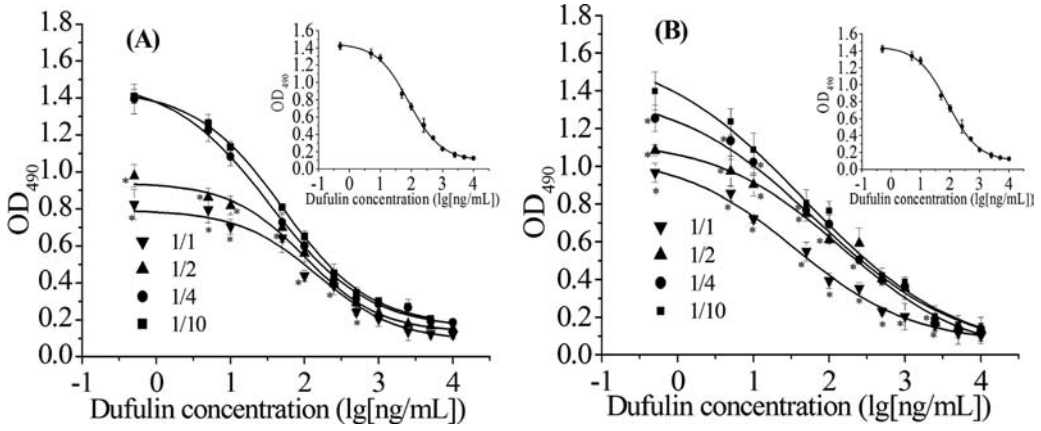


Figure 5. Effect of sample matrix: (A) soil; (B) tobacco (embedded diagram is curve of dilution with 0.05 mol/L PBST). ∗, $P < 0.05$.

Table 4. Recovery and Relative Standard Deviation of Dufulin from Spiked Samples by ELISA

		intra-assay				interassay			
sample	level added (μg/kg)	*n*	measured (μg/kg)	recovery (%)	RSD (%)	*n*	measured (μg/kg)	recovery (%)	RSD (%)
soil	100	6	92.54 ± 5.64	92.5	6.10	12	95.27 ± 6.95	95.3	7.30
	10	6	8.69 ± 0.25	86.9	2.88	12	8.15 ± 0.74	81.5	9.08
tobacco	100	6	87.34 ± 3.21	87.3	3.68	12	82.14 ± 5.02	82.1	6.11
	10	6	8.89 ± 0.52	88.9	5.85	12	9.02 ± 0.85	90.2	9.42

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can be seen, matrix effects were completely eliminated if a 1/4 dilution of soil and a 1/10 dilution of tobacco were carried out.

Fortified standards in assay buffer were checked by the optimal ELISA. To evaluate the accuracy and reproducibility of the developed ELISA, soil and tobacco samples were collected and spiked with known amounts of Dufulin. Each sample was spiked with Dufulin at concentrations of 10 and 100 μg/kg and extracted ultrasonically with a petroleum ether/ethyl acetate (50:50, v/v) for 30 min. The extract was diluted 1/4 (for soil) and 1/10 (for tobacco) times in PBS and then analyzed by ic-ELISA. With these dilutions, the matrix effect was eliminated and rendered negligible.

To study the application of the developed ELISA method in tobacco and soil, the measured recoveries and intra-assay and interassay variations of Dufulin from the spiked samples were determined. As shown in Table 4, the recoveries of Dufulin ranged from 81.5 to 95.3%, and the intra-assay variation (<6.10%) and interassay variation (<9.42%) were also acceptable. It was indicated that the developed ELISA method was especially suitable for the analysis of Dufulin in tobacco and soil.

Comparison of ELISA and HPLC Analyses. Although previous studies have demonstrated that ELISA offered a comparatively simpler and more rapid method than the traditional instrumental techniques, the confirmatory study is still required for legal and statutory purposes because of the possibility of false-positive results of ELISA. Comparison studies were performed on tobacco and soil samples by HPLC. The extraction procedure for HPLC analysis was more complicated, with a LOD of 39 ng/mL, lower sensitivity than that of ELISA method. Figure 6 shows a typical chromatogram

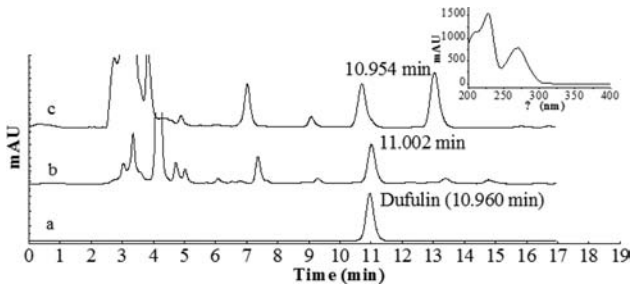

Figure 6. HPLC chromatogram at 270 nm for (a) Dufulin standard at 5.0 μg/mL, (b) soil, and (c) tobacco samples spiked with Dufulin at 0.5 mg/kg.

acquired by HPLC analysis of soil and tobacco samples spiked at 0.5 mg/kg. Soil and tobacco matrices did not present interference compounds in the chromatographic area of interest, thus allowing an optimal determination of Dufulin.

We also weighed the analytical characteristics of the proposed ELISA with those of the HPLC methods. For the HPLC, 2−3 h was the pretreatment period for the sample. Because the pretreatment for the ELISA analysis completed only extraction and dilution of samples, about 40 min was required per sample. The ELISA analysis has practically no burden to the environment or health hazard to analysts because of the reduction of approximately 95% on consumption of organic solvents. Moreover, the ELISA lowered by approximately 90% the required time for acquirement of analytical results when 40 samples were simultaneously handled as an example. Therefore, the proposed ELISA fulfilled the analytical requirements for screening pesticide residues.

In conclusion, a high-affinity monoclonal antibody against Dufulin was developed by means of immunized mice as a promising tool for analyzing and monitoring Dufulin in soil and tobacco samples. In this study, the derivatization of the benzene ring of Dufulin with carbon chain was demonstrated as an appropriate synthetic strategy for the production of mAb specific to this antivirus agent. Following an optimization process, an immunoassay was proposed with an IC_{50} value for Dufulin in a buffer of 9.6 ± 0.59 ng/mL. Finally, the performance of the ic-ELISA for the analysis of Dufulin in environmental samples was assessed in a preliminary study using fortified soil and tobacco. Most probably, the chemical properties of this pesticide and the complexity of the samples account for the interferences that were encountered. Despite these matrix effects, Dufulin could be accurately determined in these two types of samples at relevant analytical levels. Further studies will be performed for additional validation of this ic-ELISA.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ic-ELISA, indirect competitive enzyme-linked immunosorbent assay; DHS, 6-[2-((methyllbenzothiazol-2-yl)-1-(2-ethoxy)-*O*,*O*-diethyl-α-aminophosphonate)acetamido)] hexanoic acid; Ig, immunoglobulins; IA, immunoassay; mAb, monoclonal antibody; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; IC_{50}, concentration of competitor giving 50% inhibition of maximum signal; LOD, limit of detection; IgG−HRP, immunoglobulin G horseradish peroxidase conjugate; HAT, hypoxanthine, aminopterin, and thymidine; HT, hypoxanthine and thymidine; DMEM, Dulbecco's modified Eagle medium; PEG, polyethylene glycol; s-DMEM, DMEM including 20% fetal bovine serum; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 0.05% Tween 20; OPD, *o*-phenylenediamine; CR, cross-reactivity; HPLC-DAD, high-performance liquid chromatography−diodearray detector

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