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Development of an Enzyme-Linked Immunosorbent Assay for the Insecticide Thiamethoxam

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An enzyme-linked immunosorbent assay (ELISA) was developed for the neonicotinoid insecticide thiamethoxam, 3-(2-chlorothiazol-5-ylmethyl)-5-methyl-4-nitroimino-1,3,5-oxadiazinane. Three antisera were raised from rabbits immunized with the hapten−KLH conjugate. On the basis of the computational analysis of hapten candidates, the hapten with a spacer arm on the thiazolyl ring of thiamethoxam was synthesized to elicit thiamethoxam-specific antisera. The hapten was 3-[2-(2-carboxyethylthio)-5-ylmethyl]-5-methyl-4-nitroimino-1,3,5-oxadiazinane. Antisera were characterized with indirect competitive ELISA. Cross-reactivity and effects of organic solvents, pH, and ionic strengths were evaluated. The antiserum was specific for thiamethoxam and tolerant of up to 5% acetonitrile and 5% acetone. Various ionic strengths and pH values in the tested ranges had negligible effect on the assay performance. Under the optimized conditions, the half-maximal inhibition concentration (IC_{50}) and the limit of detection were approximately 9.0 and 0.1 μg/L of thiamethoxam, respectively. ELISA analysis of stream and tap water samples showed an excellent correlation with the fortification levels.

KEYWORDS: ELISA; immunoassay; thiamethoxam; neonicotinoid insecticide; hapten design

INTRODUCTION

Thiamethoxam, 3-(2-chlorothiazol-5-ylmethyl)-5-methyl-4-nitroimino-1,3,5-oxadiazinane, belongs to a relatively new class of insecticides known as neonicotinoids. Thiamethoxam, imidacloprid, and related neonicotinoids have several advantages over pyrethroid, organophosphate, and carbamate insecticides and are rapidly replacing them worldwide.

Pyrethroids interact mainly with presynaptic sodium channels in vertebrates as well as insects (*1*−*3*). Organophosphate and carbamates act by inhibiting acetylcholine esterases and thus are toxic to vertebrates, insects, and other invertebrates (*4*). By contrast, neonicotinoids act similarly to nicotinic acid as agonists of the postsynaptic nicotinic acetylcholine receptors (nAChR). Neonicotinoids are 100-fold or more selective for insect nAChRs than for vertebrate nAChRs (*5*, *6*). Several research groups worldwide have published evidence related to the submolecular basis for this selectivity, from the standpoint of the receptor subunit composition and properties, as well as the steric and charge distribution characteristics of the neonicotinoids (*6*−*18*).

The U.S. EPA promulgated rules for thiamethoxam use beginning in 2000 (*19*−*22*). During 2001, thiamethoxam was approved for various uses by the Massachusetts Department of Food and Agriculture, the Canadian Pest Management Regulatory Agency, and the Australian National Registration Authority for Agricultural and Veterinary Chemicals. Abundant details of the chemistry, metabolism, bioavailability, human toxicology, and ecotoxicology, as well as approved uses, analytical procedures, and potential risks, are presented in refs *23*−*25*. The major concerns are wind drift and leaching of thiamethoxam into surface water and ground water (*23*), toxicities to a few vulnerable aquatic species such as mysid shrimp and rainbow trout embryos (*25*), toxicity to honey bees, parasitic wasps, and other beneficial insects (*25*), and the development of resistance by target insects (*24*). In addition, thiamethoxam in the formulation known as Actara25-WG may lose activity or become phytotoxic when mixed with certain fungicides such as mancozeb, metalaxyl, and folpet (*26*). Despite these drawbacks, thiamethoxam, imidacloprid, and other neonicotinoids are expected to replace organophosphates as the most widely used insecticides worldwide (*23*).

Thiamethoxam and related neonicotinoids also have low acute dermal and inhalation toxicities and in normal use do not cause allergic reactions or skin or eye irritation in humans and animals.

Because of their high affinity for insect nAChRs (*9*), neonicotinoids are very potent, and application of 5−8 oz/acre is effective for some soil and foliar applications.

Currently, two generations of neonicotinoid insecticides are in use. These are the chlorothiazolyl derivatives, such as imidacloprid, and the thianicotinyl derivatives, exemplified by thiamethoxam. High-performance liquid chromatography (HPLC) and HPLC−mass spectrometry (HPLC-MS) are currently preferred for determination of neonicotinoids in environmental samples (*23*). The thermolability and high polarity of neonicotinoids make them difficult to analyze with gas chromatography (GC) or GC-MS (*27*−*29*). However, the water solubility, asymmetric structure, and potential for leaching into surface water and ground water make ELISA potentially a good analytical

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method. To date, our laboratory and one other have developed haptens and ELISAs based on rabbit sera, and Watanabe et al. derived another hapten and monoclonal mouse antibodies for ELISA of the most heavily used chlorothiazolyl neonicotinoid insecticides, imidacloprid and acetamiprid (*30*−*32*). In this paper we describe the design of a hapten and development of serum-based direct and indirect competition ELISAs for thiamethoxam, the most used thianicotinyl insecticide in this class. Hapten design was facilitated by computational modeling of the energy-minimized structures and their charge distributions.

MATERIALS AND METHODS

Reagents. All reagents were of analytical grade unless specified otherwise. Reference standards of clothianidin (99.9%), acetamiprid (99.5%), and dinotefuran (99.7%) were kindly provided by the National Institute of Agricultural Science and Technology, South Korea. Primary stock solutions were prepared by dissolving each reference standard in methanol at a concentration of 0.5 mg/mL. Chemicals purchased from Sigma (St. Louis, MO) were goat anti-rabbit IgG−horseradish peroxidase (IgG-HRP), keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), HRP, phosphate−citrate buffer capsules with sodium perborate, carbonate−bicarbonate buffer capsules, and *o*-phenylenediamine (OPD). TiterMax Gold adjuvant was obtained from CytRx Corp. (Norcross, GA). The ELISAs were carried out in 96-well polystyrene microplates (MaxiSorp F96, Nalge Nunc International, Roskilde, Denmark). Thiamethoxam, the thiamethoxam hapten, and the hapten conjugates were synthesized in this laboratory. The antiserum was purified with a HiTrap Protein G HP column (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. Concentration of antibody in the final preparation was determined with the Bio-Rad Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). The purified IgG in phosphate-buffered saline (PBS, 12 mM phosphate, 137 mM NaCl, and 2.7 mM KCl, pH 7.5) was stored at −20 °C.

Spectroscopy and Chromatography. ^{1}H NMR spectra were obtained on a Nicolet NT-300 MHz for solutions in $CDCl_3$ and are described as multiplicity, coupling constant (*J*) in hertz (Hz), number of protons, and assignment. Chemical shifts (δ, ppm) are relative to internal tetramethylsilane (TMS). Low-resolution mass spectra were recorded with a Hewlett-Packard 5958 GC-MS system with an electron impact (EI, 70 eV) ionization and are given as $[M]^+$.

Molecular Modeling. Global energy minimum structures were searched by preliminary conformational analysis with all of the rotatable bonds, and refined energy minimization was done by PM5 force field. To evaluate the structural similarity, root-mean-square (RMS) deviations were calculated by superimposing other chemicals on thiamethoxam. Atomic properties, including superdelocalizability, partial charge, and electron density, were calculated with the same force field. Electron density isosurfaces colored by electrostatic potential were also calculated to evaluate the electron distribution over the molecules. All of the calculations were done by CAChe Worksystem Pro (version 5.0, Fujitsu, Japan). Partial dipole moment, molecular dimension, and hydrogen bonding properties were calculated with Molecular Modeling Pro (version 5.0.8, ChemSW Inc.). Atomic polarizability and partial π and σ charges were calculated with PETRA (*33*).

Safety Precaution. Hapten syntheses were done in a chemical fume hood with charcoal filters. All synthetic byproducts and wastes and solutions of thiamethoxam analytes were disposed of as hazardous materials. Goggles, spill-resistant gowns, and chemical-impermeable nitrile gloves were worn.

Synthesis of Hapten (Figure 1). In general, the final hapten, thiamethoxam, and intermediates were synthesized according to established methods (*30*, *34*−*37*).

*Preparation of 3-Methyl-4-nitroimino-1,3,5-oxadiazinane (**I**).* A solution of *N*-methyl-*N*′-nitroguanidinene (6.0 g) and 37% aqueous formaldehyde solution (37.5 mL) was added to 37.5 mL of 90% formic acid and heated to 80 °C for 16 h. After the solution had cooled to 0 °C, an aqueous sodium hydroxide solution (300 g/L, ∼70 mL) was added to adjust the solution to pH 8.0. The resulting mixture was extracted with CH_2Cl_2. The organic phase was washed with saturated sodium chloride, dried by anhydrous sodium sulfate, and evaporated to dryness. The solid residue was recrystallized from ethyl acetate and ethyl ether to get a white solid: 5.5 g, yield 67%; mp 141−143 °C; GC-MS $C_4H_8N_4O_3$, 160 (M^+).

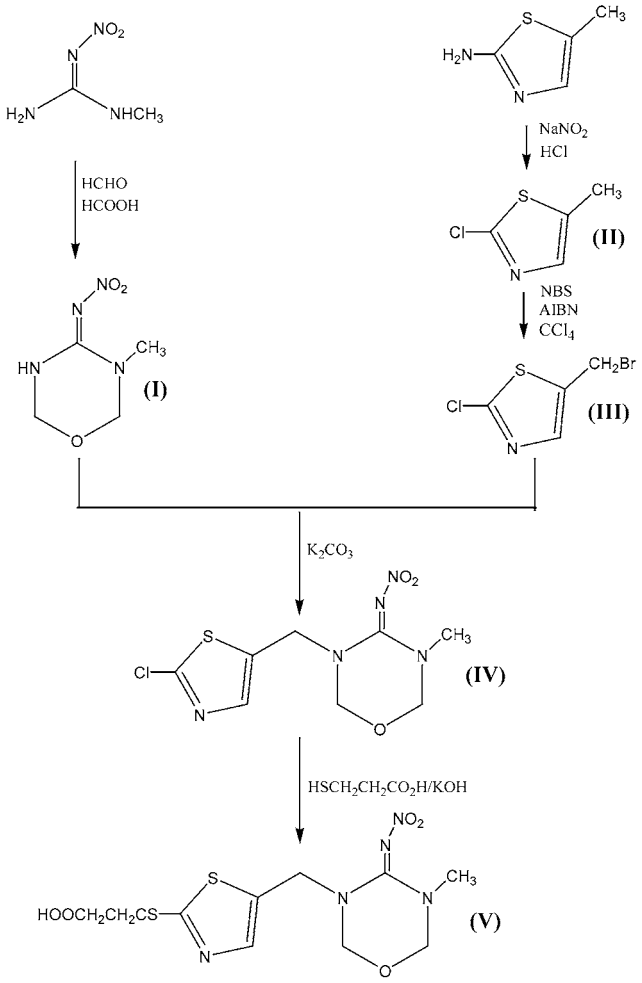

Figure 1. Synthetic scheme of thiamethoxam hapten.

*Preparation of 2-Chloro-5-methylthiazole (**II**).* A solution of 2-amino-5-methylthiazole (10 g) in 40 mL of concentrated HCl and 20 mL of water was cooled to 0 °C. Aqueous sodium nitrite (7 g in 15 mL of water) was added dropwise to the reaction mixture. After the mixture had been maintained at 0 °C for 3 h, the solution was heated to 80 °C for 3 h and then cooled to room temperature. The reaction mixture was extracted with chloroform (400 mL × 3), and the extracts were combined and dried with anhydrous sodium sulfate. The solvent was removed under reduced pressure, and the residue was purified with a silica gel column and eluted with CH_2Cl_2: GC-MS $C_4H_4ClNSH^+$, 134 (MH^+).

*Preparation of 2-Chloro-5-bromomethylthiazole (**III**).* In a 50-mL round-bottom flask, a solution of **II** (8.0 g), *N*-bromosuccinimide (NBS) (12.8 g), and benzoyl peroxide (AIBN) (28.8 mg) in CCl_4 (200 mL) was heated to 80 °C for 36 h, cooled to room temperature, and filtered. The filtrate was washed to neutral with water, 5% sodium bicarbonate, and water and dried by anhydrous sodium sulfate. After solvent removal, the residue was purified by flash chromatography (ethyl acetate/hexane = 1:9): yield 85%; GC-MS $C_4H_3BrClNSH^+$, 213 (MH^+).

*Preparation of Thiamethoxam (**IV**).* A solution of **I** (3.0 g), **III** (5.0 g), and potassium carbonate (6.25 g) in DMF (20 mL) was heated to 30 °C overnight and then to 50 °C for 16 h. After the reaction mixture had been filtered through Celite, DMF was removed at reduced pressure. The residue was purified by flash chromatography (CH_2Cl_2/CH_3OH = 95:5). The purified product has mp 140−142 °C; LC-MS $C_8H_{10}ClO_3SH^+$, 292 (MH^+); ^{1}H NMR (DMSO-d_6) δ 2.832 (3H, s, CH_3), 4.746

(2H, s, CH_2), 4.968 (2H, s, CH_2), 5.039 (2H, s, CH_2), and 7.639 (1H, s, CH═).

Preparation of 3-[2-(2-Carboxyethylthio)-5-ylmethyl]-5-methyl-4-nitroimino-1,3,5-oxadiazinane (V). In a 50-mL round-bottom flask, a solution of thiamethoxam (0.5 g), 3-mercaptopropionic acid (0.2 g), and 85% potassium hydroxide (powder, 0.2 g) in DMSO (15 mL) was heated to 75 °C for 3 days. The solution was poured into water (100 mL), adjusted to pH 3 with 1 N HCl, and then extracted with ethyl acetate (30 mL × 3). The organic phase was combined, washed with brine solution, and dried with anhydrous Na_2SO_4. After the solvent was removed at reduced pressure, the residue was dissolved in methanol. The product was separated by preparative thin-layer chromatography (CH_2Cl_2/CH_3OH = 9:1). The yield was 55.6%: LC-MS $C_{11}H_{15}N_5O_5S_2H^+$, 362 (MH^+); ^1H NMR (DMSO-d_6) δ 2.756 (2H, t, CH_2, J = 7.0 Hz), 2.980 (3H, s, CH_3), 3.406 (2H, t, CH_2, J = 7.0 Hz), 4.806 (2H, s, CH_2), 4.976 (2H, s, CH_2), 5.032 (2H, s, CH_2), and 7.616 (1H, s, CH═).

Preparation of Protein—Hapten Conjugates. *Hapten−KLH and −BSA Conjugation.* The hapten (*V*) was conjugated to KLH and BSA as previously described (*30*) with slight modification. To 0.05 mmol of the hapten was added 500 μL of DMF solution containing 0.1 mmol each of *N*-hydroxysuccinimide (NHS) and 1-[3-(Dimethylamino)propyl]-3-ethylcarbodimide hydrochloride (DEC). The mixture was stirred at room temperature for 4 h and then centrifuged to remove precipitated urea. The clear supernatant was slowly added, a few microliters per 20 min intervals, to KLH solution (30 mg of KLH in 10 mL of a 0.1 M borate buffer, pH 9.0) or BSA solution (150 mg of BSA in 10 mL of a 0.1 M borate buffer, pH 9.0). The reaction mixture was stirred overnight at 4 °C and then dialyzed against PBS (1.5 L) for 3 days at 4 °C with two buffer changes per day.

Preparation of Enzyme Tracer. The hapten−HRP conjugate was prepared as above with the following exception. Hapten (6 μmol) was activated with NHS (30 μmol) and DEC (60 μmol) in 260 μL of DMF, and the reaction mixture was added to HRP (2 mg) in 3 mL of 0.13 M $NaHCO_3$ buffer. After the dialysis against PBS, the crude hapten−HRP conjugate was diluted with an equal volume of glycerol and stored at −20 °C.

Immunization. The hapten−KLH conjugate was used to immunize rabbits. A biochemically defined adjuvant, TiterMax Gold, was selected due to its ability to produce high titers of antisera (*38*). Three New Zealand white rabbits (2−4 kg) were immunized with hapten−KLH emulsified in TiterMax Gold as previously described (*30*). One received 60 μg and the others received 100 μg of emulsion in 0.4 mL. Two booster injections were given at 4-week intervals. Injections were made intradermally and subcutaneously at multiple sites on the animals' backs. The titers of the antisera were monitored by ELISA using checkerboard titration. Seven days after the last injection, the rabbits were bled, and the antisera were collected and stored at −80 °C. Inhibition of antibody binding by free thiamethoxam was monitored with an indirect competitive ELISA (icELISA) (*30*).

Direct Competitive ELISA (dcELISA). A microtiter plate was coated with rabbit anti-thiamethoxam−KLH antibody (100 μL of 2.0 μg antibody/well in 0.1 M carbonate−bicarbonate buffer, pH 9.6) overnight at 4 °C. The following day, the plate was washed four times with PBS containing 0.05% Tween 20 (PBST) and then blocked with 1% BSA in PBS (200 μL/well) for 1 h at room temperature. After the plate was washed (five times) with PBST, a solution of 50 μL of the analytes or standards diluted in PBST and 50 μL of thiamethoxam−HRP conjugate (0.16 ng/μL in PBST) was added. After 40 min of incubation at 37 °C and another five washings, the substrate solution (100 μL per well, containing 1.0 mg/mL of OPD in 0.05 M citrate−phosphate and 0.03% sodium perborate, pH 5.0) was added. The reaction was stopped with sulfuric acid (4 N, 50 μL/well) after 15 min at room temperature, and absorbance at 490 nm was read with a Vmax microplate reader (Molecular Devices, Sunnyvale, CA). Inhibition curves were fit to a four-parameter logistic equation with Softmax software v 2.35 (Molecular Devices).

Fortification of Thiamethoxam in Water. Water samples were from a tap in the laboratory and were collected from Manoa stream in Honolulu, HI. Tap and stream water samples were fortified with thiamethoxam up to 100 ng/mL. The optimized dcELISA was used to determine thiamethoxam concentrations in the fortified water samples. The samples were buffered with 4-fold concentrated PBST.

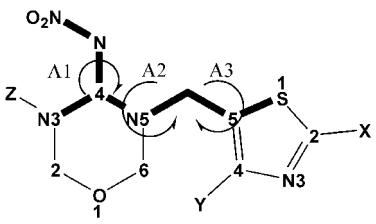

Figure 2. Structures and dihedral angles of thiamethoxam and hapten candidates.

RESULTS AND DISCUSSION

Hapten Design and Synthesis. There are several possible linker positions (X, Y, and Z) in thiamethoxam for hapten preparation (**Figure 2**). To obtain compound-specific antibodies, a good option is to place a linker on a remote part of the molecule and to maximally reserve the parent molecular property (*39*, *40*). The hapten design was aided with computational analysis of hapten candidates. Various chemical and physical properties of thiamethoxam and its analogues were predicted with computational modeling to identify a suitable linker position (**Figures 2** and **3**; **Table 1**). The computational analysis suggested that a thioalkyl spacer on the 2-position of the thiazolyl ring alters the molecular properties much less than one on the 4-position of the thiazolyl ring or the 5-position of the oxadiazinane ring (**Figures 2** and **3**; **Table 1**). In addition, replacing the *N*-methyl group with a spacer (proposed hapten **C**) may not produce a suitable hapten because clothianidin and thiamethoxam share the same thiazole structure, located at the end of the molecule remote from the spacer. The proposed hapten **B** may not be an adequate hapten either because a large steric hindrance of the linker at the 4-position of thiazole significantly distorts the geometry from that of thiamethoxam (**Figure 3**; **Table 1**). These considerations suggest that the proposed haptens **B** and **C** would not elicit thiamethoxam-specific antibodies. The computational modeling indicated that hapten **A** has almost the same geometric overlap and electrostatic properties as thiamethoxam (**Figure 3**; **Table 1**) and is a suitable hapten candidate. Therefore, it was synthesized and used to induce antibodies.

Thiamethoxam (**IV**) and three intermediates (**I**−**III**, **Figure 1**) were synthesized according to established methods (*34*−*37*). Thiamethoxam was prepared by reaction of compounds **I** and **III** as shown in **Figure 1**. Introduction of the spacer to the selected site of thiamethoxam was carried out under a mild alkaline condition because conversion of ═N−NO_2 to ═O at a strong alkaline condition was observed in the previous work (*30*). The molecular ratio of hapten to protein was estimated by UV absorbance and was 10 and 43 for hapten−BSA and hapten−KLH, respectively.

Immunization and Characterization of Antisera. Table 2 shows the titers and IC_{50} values of the three antisera for free thiamethoxam. The titer of antiserum−**II** (Ab-**II**) from rabbit II was 5- and 10-fold higher than those from rabbits I (Ab-**I**) and III (Ab-**III**), respectively. However, the antiserum-**I** (Ab-**I**) from rabbit I had the lowest IC_{50} (278 ng/mL) in an icELISA (**Table 2**). Protein G affinity-purified Ab-**I** was stored at −20 °C and used throughout this work. When compared with the titers of imidacloprid antisera obtained with the same procedure (*30*), the titers of these thiamethoxam antisera were low, which may be due to the lower ratio of thiamethoxam hapten to KLH and differences in the hapten structures and individual animals.

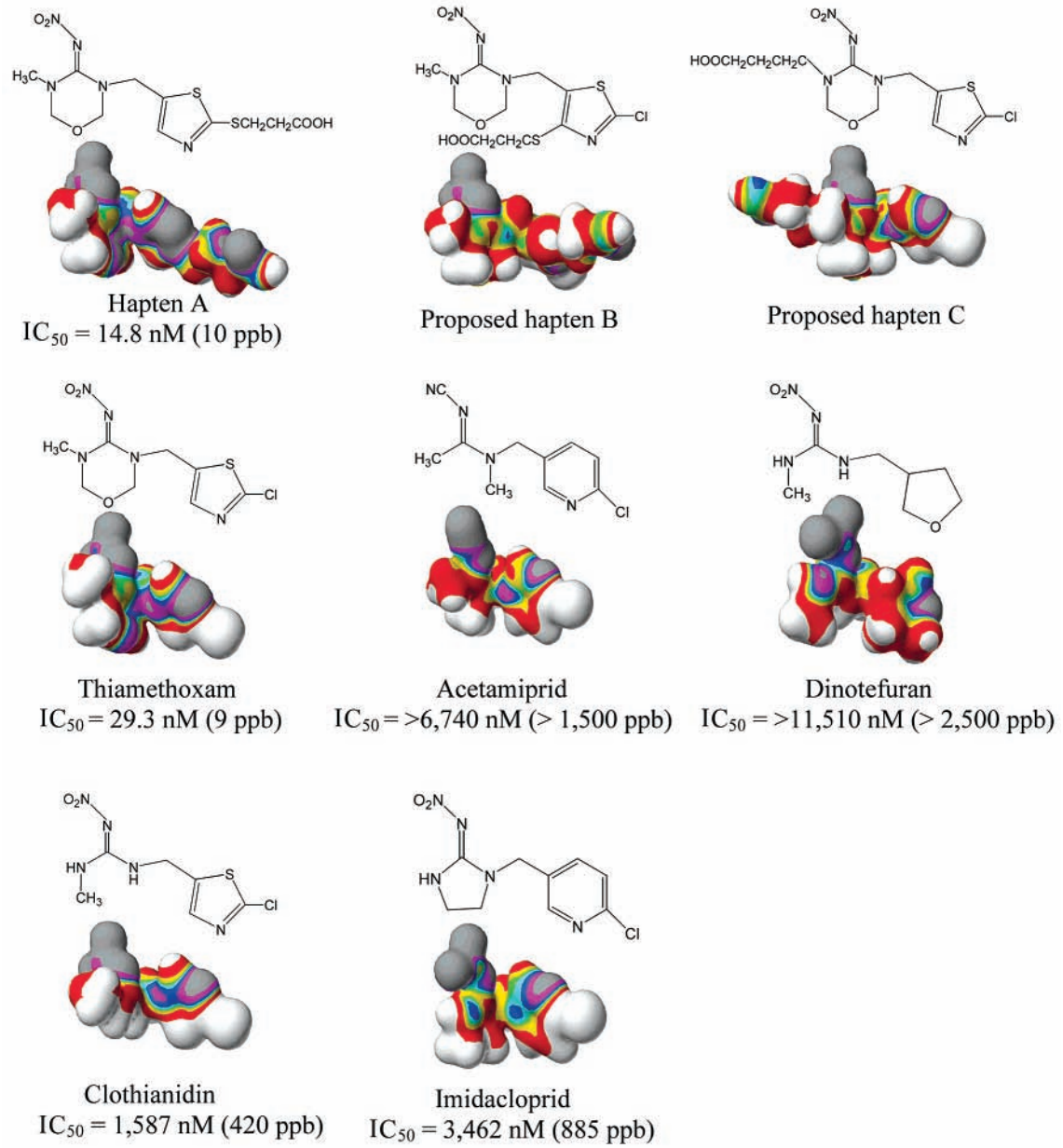

Figure 3. Electron density isosurfaces colored by electrostatic potential: white, >0.09; 0.03 < red < 0.09; 0.01 < yellow < 0.03; 0.00 < pale green < 0.01; –0.01 < pale blue < 0.00; –0.03 < blue < –0.01; –0.06 < pink < –0.03; gray < –0.06. The structures of haptens B and C were proposed but not synthesized.

Table 1. RMS Errors of the Superimposition on Thiamethoxam and Molecular Dimensions of Global Energy Minimum Structures

		dihedral angle (deg)			molecular dimension (Å)		
compound	RMS deviation	A1	A2	A3	length	width	depth
acetamiprid	0.1092	–1.2	70.1	–111.1	11.35	8.77	7.62
clothianidin	0.0982	–61.8	74.3	–125.8	10.80	8.78	7.67
dinotefuran R	0.2813	–3.2	–169.9	173.2	11.20	8.82	6.16
dinotefuran S	0.2796	–3.4	115.2	–71.7	11.10	8.82	6.35
imidacloprid	0.1127	–0.2	69.0	–90.0	10.68	9.18	7.98
thiamethoxam	0.0000	–119.3	82.9	–72.9	12.17	9.01	7.91
hapten **A** (synthesized)	0.0242	–119.7	82.3	–73.9	12.16	9.03	7.92
hapten **B** (proposed)	0.2868	–89.1	155.2	–79.8	12.35	9.12	7.82
hapten **C** (proposed)	0.0562	–105.2	84.4	–73.8	12.15	9.11	7.53

Competitive Inhibition. Figure 4 shows representative standard curves for thiamethoxam obtained with dcELISA and icELISA. The calibration ranges of the dcELISA and icELISA curves were approximately 0.1−200 and 10−5000 μg/L, respectively. The IC_{50} values of dcELISA and icELISA were approximately 8 and 160 μg/L, respectively, indicating that use of dcELISA and purification of antibody improved the sensitivity by 20-fold compared with that of an icELISA. A similar improvement with the dcELISA format was observed with the imidacloprid polyclonal antibody (*40*). A dcELISA format using

Table 2. Titer and Apparent Binding Affinity of Antisera

antiserum	titer[a]	IC_{50}[b] (ng/mL)
I	8000	280
II	40000	>1500
III	4000	>1200

[a] The titer of antiserum is defined as the antiserum dilution that produced an absorbance of 1 at 490 nm, 20 min after the addition of the substrate. [b] IC_{50} values represent the concentration of thiamethoxam in ng/mL that produced 50% inhibition of antiserum binding to the hapten conjugate using icELISA. The wells were coated with 100 μL of conjugate of 25 ng/mL.

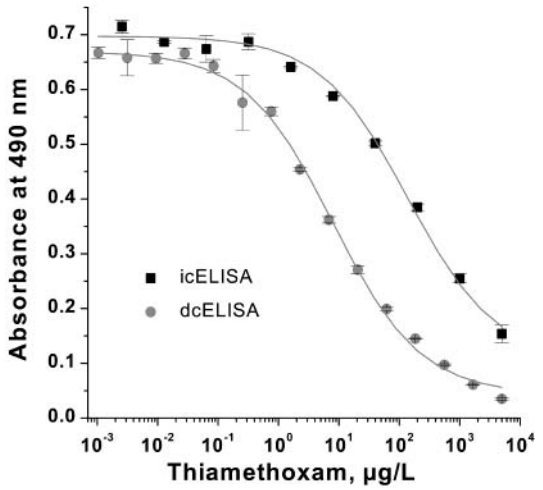

Figure 4. Standard inhibition curves of thiamethoxam in dcELISA and icELISA formats. Plates were coated with 100 μL of 2.0 μg of purified Ab-**I** for dcELISA or 2.5 ng of hapten–BSA per well for icELISA. Each value represents the mean of four replicates.

Table 3. Cross-Reactivity of Ab-**I** to Thiamethoxam and Its Structural Analogues[a]

compound	IC_{50} (nM, ng/mL)	cross-reactivity (%)
thiamethoxam	29.3 (9.0)	100
thiamethoxam hapten	14.8 (10)	198
clothianidin	1587 (420)	1.8
imidacloprid	3462 (885)	0.8
dinotefuran	>11 510 (>2500)	<0.25
acetamiprid	>6740 (>1500)	<0.4

[a] The plate was coated with 2.0 μg of affinity-purified antibody (100 μL/well). [b] Numbers in parentheses are IC_{50} values in ng/mL.

the purified Ab-**I** was employed throughout this study because it significantly improved the assay sensitivity.

Cross-Reactivity. The specificity of Ab-**I** was estimated by performing dcELISA with four neonicotinoid insecticides as competitors, which were imidacloprid, acetamiprid, dinotefuran, and clothianidin. Ab-**I** was specific for thiamethoxam and showed <2% cross-reactivity for imidacloprid, chlothianidin, and acetamiprid (**Table 3**). No response in the assay was observed up to 12 μM dinotefuran. These results support the hapten design suggested by the computational analysis.

The relationship between the antibody specificity and competitor structures was examined by computational modeling. The largest structural difference was found in dihedral angles (**Figure 2**; **Table 1**). The nitro group in thiamethoxam appears to be distorted into a nearly perpendicular position (dihedral angle A1) to the thiadiazinane ring, which coincides well with crystallographic data of structurally related *N*-methylimidacloprid (*41*). Computational modeling suggested that the steric volume of the pyridine of acetamiprid and imidacloprid (55.29 cm^2/mol) is similar to that of the thiazole of thiamethoxam and clothianidin (**Figure 2**). In addition, there appeared to be similar electrostatic properties between the pyridine and thiazole rings. Large IC_{50} differences for thiamethoxam, clothianidin, and imidacloprid indicate that the conservation of the nitroiminothiadiazinane moiety in the thiamethoxam hapten distal to the linker was important for the production of thiamethoxam-specific antibodies. Clothianidin and thiamethoxam have a chlorothiazole moiety; however, the large structural differences in their nitroimino groups probably result in low binding affinity of clothianidin to the antibody. The electrostatic properties of imidacloprid are more similar to thiamethoxam than clothianidin, but large differences exist in dihedral angle A1 among imidacloprid, clothianidin, and thiamethoxam (**Table 1**). The large IC_{50} value for imidacloprid strongly suggests that the geometry of the nitroimino group is an important factor for the selectivity between chemicals (**Figure 3**; **Table 1**). The *R*- and *S*-enantiomers of dinotefuran share the same nitroimino structure with clothianidin, but dihedral angle A1 and the molecular shapes are completely different. Acetamiprid shows good geometric overlap with thiamethoxam, but the large electrostatic difference permits no cross-reactivity.

Chemical Effects on Assay Performance. Assay performance is often affected by ionic strength, pH, and solvent concentration of samples. The pH of aqueous samples may interfere with ELISAs due to the disturbance of weak molecular interactions in antibody−antigen binding caused by pH derived alteration (e.g., hydrolysis, conformational change, ionization) of either an analyte or molecules (antibody or enzyme tracer) participating in the interaction (*42*−*44*). Salt concentrations in real world samples can often affect assays (*40*, *44*). The effects of these parameters on the assay were evaluated.

Solvent Effects. The effects of DMSO, MeOH, acetone, and acetonitrile were studied because they are water-miscible and are commonly used in sample extractions. Serial dilutions of thiamethoxam standard in PBST containing 0, 5, 10, 20, or 40% of organic solvent were mixed with an equal volume of antibody solution diluted in PBST for dcELISA. In general, maximum absorbance (A_{max}) gradually decreased as the concentration of solvent increased. Acetonitrile and acetone showed less effect on the inhibition curves (shape and position) than MeOH and DMSO (**Figure 5**). Little change in IC_{50} values was observed in up to 5% of acetonitrile and 5% of acetone. However, IC_{50} values increased gradually as concentrations of MeOH and DMSO increased.

Effect of pH and Ionic Strength. To evaluate the influence of pH and ionic strength on assay performance, thiamethoxam was diluted in PBST of different pH values (4.0−9.0) with the same ionic strength or different ionic strengths (0.15−1.5 M) at pH 7.5 and enzyme tracer in PBST, pH 7.5. A_{max} values decreased at pH <6; however, there were negligible shifts of the inhibition curves in the pH range of 4−9 (data not shown). All subsequent assays were performed at pH 7.0. There were no significant changes in IC_{50} values and A_{max} in the tested range of ionic strength (data not shown).

Thiamethoxam-Fortified Water Samples. Thiamethoxam in water samples was analyzed with the dcELISA. The concentrations of thiamethoxam by ELISA correlated very well with the fortification values with a slope of 0.94 and a correlation coefficient of 0.99 for the tap water samples and a slope of 0.94 and a correlation coefficient of 0.99 for the

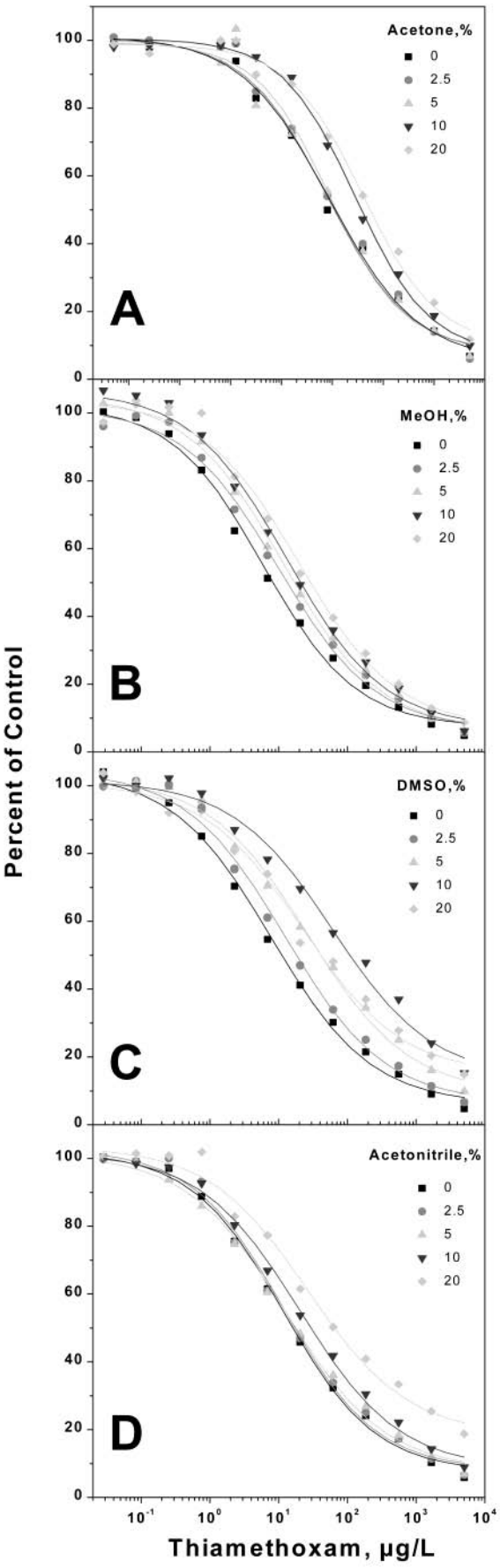

Figure 5. Effects of acetone (A), methanol (MeOH) (B), DMSO (C), and acetonitrile (D) on the assay. Values refer to solvent v/v concentration in the competitive assay solution. Each value represents the mean of four replicates.

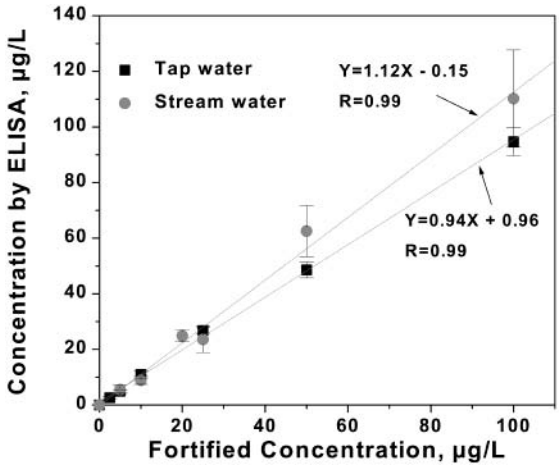

Figure 6. Correlation between thiamethoxam concentrations determined by ELISA and those fortified in tap water and stream water samples. The plate was coated with 2.0 μg of Ab-I in 100 μL/well. The concentration of enzyme tracer was 0.16 ng/μL in PBST, pH 7.0. Each value represents the mean of four replicates.

stream water (**Figure 6**). The results showed that the ELISA can accurately measure the concentration of thiamethoxam in water.

Summary and Conclusions. Thiamethoxam-specific polyclonal antibodies were obtained from rabbits immunized with a hapten−KLH conjugate of hapten having a thioalkyl spacer at the thiazolyl ring of thiamethoxam. The hapten design was aided by computational analysis of RMS deviations, dihedral angles, and molecular dimensions of candidate haptens. The experimental results were consistent with our prediction that the substitution of the chlorine atom with a thioalkyl group is a suitable position to derivatize in as well to obtain thiamethoxam-specific antibodies. All of the molecules that have a flat dihedral angle (A1) showed little cross-reactivity. In addition, the nitro group configuration and the presence of the oxadiazinane group may play a key role in the antigen−antibody recognition.

An ELISA was developed for the detection of thiamethoxam. The use of the protein G-purified IgG and the dcELISA format significantly improved sensitivity and analysis time for the thiamethoxam ELISA. The assay was specific for thiamethoxam with an IC_{50} of 29 nM and had <2% cross-reactivity with structural analogues of thiamethoxam. The good recovery of thiamethoxam in water samples showed that the assay can be used for the determination of thiamethoxam residues in water, but further work is needed to validate this assay with other matrices.

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